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UNITED STATES DISTRICT COURT

SOUTHERN DISTRICT OF CALIFORNIA

SAN DIEGO DIVISION

FATE THERAPEUTICS, INC. and WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH,

Plaintiffs,

v.

SHORELINE BIOSCIENCES, INC. and DAN S. KAUFMAN,

Defendants.

CASE NO. '22CV676 RBM MSB

COMPLAINT FOR PATENT INFRINGEMENT

JURY TRIAL DEMANDED

Plaintiffs Fate Therapeutics, Inc. (“Fate Therapeutics”) and Whitehead Institute for Biomedical Research (“Whitehead Institute”) bring this Complaint for monetary and declaratory relief against Defendants Shoreline Biosciences, Inc. (“Shoreline”) and Dan S. Kaufman, M.D., Ph.D. to address Defendants’ infringement of Plaintiffs’ patented technology.

NATURE OF THE ACTION

1. This action arises under the patent laws of the United States, 35 U.S.C. § 1 *et seq.*, based on Defendants infringement of United States Patent Nos. 8,071,369 (“the ’369 Patent”), 8,932,856 (“the ’856 Patent”), 8,951,797 (“the ’797 Patent”), 8,940,536 (“the ’536 Patent”), 9,169,490 (“the ’490 Patent”), 10,457,917 (“the ’917 Patent”) (collectively, “the Asserted Patents”). True and correct copies of the Asserted Patents are attached hereto as **Exhibits A, B, C, D, E, and F**, respectively.

2. This action concerns Plaintiffs’ groundbreaking research tool—a proprietary, human induced pluripotent stem cell (“iPSC”) platform that enables creation of genetically engineered, clonal master cell lines. Using this foundational technology, researchers can determine the fate of clonal master iPSC lines to produce, for example, well-defined and uniformly composed immunotherapy cells that can be used for a variety of cell therapies.

3. Fate Therapeutics’ natural killer immunotherapy cells derived from induced pluripotent stem cells are highly efficacious in the treatment of cancer patients. For example, Fate Therapeutics’ FT596 natural killer immunotherapy cells have shown dramatic results in the treatment of B-Cell lymphoma. **Exhibit G**, December 13, 2021 Fate Therapeutics Press Release.

4. This proprietary iPSC platform belongs to Plaintiffs, as do the Asserted Patents covering the exclusive right to use this platform.

5. Shoreline, through at least the actions of its founder and former Scientific Advisor to Fate Therapeutics, Dr. Kaufman, as well as Dr. Kaufman, individually, infringe the Asserted Patents.

6. On information and belief, while under exclusivity obligations to Fate Therapeutics as its Scientific Advisor, Dr. Kaufman secretly founded, participated in, advised and served as a director for his own rival company, Shoreline. On information and belief, to generate investor interest quickly and compete against Fate Therapeutics, Shoreline and Dr. Kaufman used and continue to use Plaintiffs' iPSC platform to generate induced pluripotent cells that are subsequently differentiated for use in cancer immunotherapies. Indeed, Dr. Kaufman founded Shoreline to develop and use competing "off-the-shelf" allogeneic natural killer immunotherapy cells derived from induced pluripotent stem cells.

7. On information and belief, in violation of his exclusivity agreement with Fate Therapeutics, and while still serving as Fate Therapeutics' Scientific Advisor, Dr. Kaufman helped his rival company raise investment funds and pursue strategic partnerships. On information and belief, Dr. Kaufman concealed and misled Fate Therapeutics as to his activities for and participation in Shoreline despite a contractual duty to disclose them.

8. When Shoreline's existence came to light, Fate Therapeutics informed Dr. Kaufman that he was in breach of his exclusivity obligations and demanded that he "immediately terminate his relationship" with Shoreline. On information and belief, Fate Therapeutics' efforts did not deter Dr. Kaufman from breaking his promises to Fate Therapeutics; they caused Dr. Kaufman to escalate. On information and belief, instead of terminating his relationship with Shoreline, Dr. Kaufman helped Shoreline raise over $43 million in investor funds, including from Kite Pharma, Inc. On information and belief, Dr. Kaufman also helped Shoreline pursue strategic partnerships to develop "off-the-shelf" allogeneic natural killer immunotherapy cells derived from induced pluripotent stem cells with BeiGene, Ltd. and Kite Pharma, Inc. valued at over $4 billion.

9. Industry news, republished on the Shoreline website, explains the significance of Dr. Kaufman to the Kite transaction: "Kite…selected Shoreline as its strategic partner for a strategic expansion into allogeneic iPSC therapies based around NK cells, in [sic] due

to the expertise of the laboratory of Dan S. Kaufman, MD, PhD, a UCSD investigator and Shoreline co-founder, who serves as the company's Chief Scientific Officer." **Exhibit H**, November 8, 2021 GeneEdge article [republished on the Shoreline website www.shorelinebio.com].

10. Only through the unauthorized and infringing use of Plaintiffs' breakthrough iPSC platform were Defendants able to develop "off-the-shelf" allogeneic natural killer immunotherapy cells derived from induced pluripotent stem cells within months of creation, earning them over $4 billion in funding to date. All of this was a benefit that Shoreline enjoyed from its and Dr. Kaufman's choice to use Plaintiffs' iPSC platform.

11. This action follows because Defendants made the deliberate decision to infringe Plaintiffs' valuable intellectual property and infringe its patents to gain, *inter alia*, a commercial head start.

THE PARTIES

12. Plaintiff Fate Therapeutics, Inc. is a corporation organized and existing under the laws of Delaware, with its principal place of business at 12278 Scripps Summit Drive, San Diego, CA 92131.

13. Fate Therapeutics is a clinical-stage biopharmaceutical company dedicated to the development of first-in-class cellular immunotherapies for patients with cancer. Fate Therapeutics' mission statement includes the tenet that better cell therapies start with better cells. To produce better cell therapies, Fate Therapeutics' proprietary product platform is uniquely designed to overcome numerous limitations associated with the production of cell therapies using patient- or donor-sourced cells, which is logistically complex, expensive, and subject to variability that can affect clinical safety and efficacy.

14. Fate Therapeutics engineers human iPSCs in a one-time genetic modification event and selects a single engineered iPSC for maintenance as a clonal master iPSC line. Clonal master iPSC lines are a renewable source for manufacturing cell therapy products that are well-defined and uniform in composition, can be mass produced at significant scale

in a cost-effective manner, and can be delivered "off-the-shelf" for patient treatment.

15. Fate Therapeutics' cell therapy product candidate pipeline is comprised of immuno-oncology programs, including off-the-shelf NK- and T-cell product candidates, that synergize with well-established cancer therapies and target tumors.

16. Fate Therapeutics has an exclusive license to the Asserted Patents, including the right to sue for infringement.

17. Plaintiff Whitehead Institute for Biomedical Research is a world-renowned non-profit research institution dedicated to improving human health through basic biomedical research. Whitehead Institute is a Delaware corporation, with a principal office at 455 Main Street, Cambridge, MA 02142.

18. Whitehead Institute is the owner and assignee of the Asserted Patents and exclusively licensed the Asserted Patents to Fate Therapeutics.

19. On information and belief, Defendant Shoreline Biosciences, Inc. is a corporation organized and existing under the laws of Delaware, with its principal place of business at 11408 Sorrento Valley Road, San Diego, CA 92121.

20. On information and belief, Defendant Dr. Kaufman is a co-founder, director, and participant in Shoreline. On information and belief, Dr. Kaufman secretly founded, participated in, advised and served as a director for Shoreline while serving as a Scientific Advisor to Fate Therapeutics.

21. On information and belief, Dr. Kaufman resides in this District.

22. In his and Shoreline's acts of infringement, Dr. Kaufman was the agent, servant, co-conspirator, or employee of Shoreline, and the acts and omissions herein alleged were done or caused by them, acting individually, in concert, and/or through or by their alleged capacity, within the scope of their authority. Each of the Defendants aided and abetted and rendered substantial assistance in the accomplishment of the acts complained of herein. In taking the actions, as particularized herein, to aid and abet and substantially assist in the commission of the misconduct complained of, each Defendant acted with an

awareness of his, her or its primary wrongdoing and realized that his, her or its conduct would substantially assist in the accomplishment of that misconduct and was aware of his, her or its overall contribution to, and furtherance of the conspiracy, common enterprise, and common course of conduct. Defendants' acts of aiding and abetting included, inter alia, all the acts each Defendant is alleged to have committed, individually or in concert, in furtherance of the conspiracy, common enterprise, and common course of conduct complained of herein.

23. On information and belief, Dr. Kaufman also performed the infringing acts described herein including by manufacturing iPSCs with the claimed compositions or according to the claimed methods of the Asserted Patents, individually and for his own personal benefit and/or outside the scope of his agency or employment.

JURISDICTION AND VENUE

24. This is an action for patent infringement arising under the patent laws of the United States, 35 U.S.C. § 271.

25. This Court has subject matter jurisdiction over this action under 28 U.S.C. §§ 1331, 1332 and 1338(a).

26. This Court has personal jurisdiction over Defendants because they regularly conduct business within, and specifically direct their business activities to, the State of California and the Southern District of California ("this District"). Defendants have purposefully availed themselves of the opportunity to conduct business in this State through systematic and continuous dealings in this State.

27. Defendants' actions that give rise to personal jurisdiction include but are not limited to the following: making and using infringing products in this State and in this District, knowing and intending that the infringing products would be used in this District, deriving substantial revenue from the use of infringing products within this District, and expecting their infringing actions to have consequences in this District.

28. This Court also has personal jurisdiction over Dr. Kaufman because he is

domiciled in this District.

29. Venue is proper as to Defendants under 28 U.S.C. § 1400(b). Defendants have committed, induced others to commit, or contributed to others committing, acts of infringement in this District, including by residing in and/or having a regular and established place of business in this District at, for example, 11408 Sorrento Valley Road, San Diego, CA 92121.

FACTUAL ALLEGATIONS

A. Induced Pluripotent Stem Cells

30. Induced pluripotent stem cells ("iPSCs") are pluripotent stem cells generated from adult somatic cells by reprogramming. iPSCs have the same beneficial properties as embryonic stem cells, without the associated drawbacks, and therefore self-renew and can differentiate into all cell types of the body. iPSCs can enable the development of an unlimited source of any type of human cell needed for therapeutic purposes. For example, iPSC can be prodded into becoming beta islet cells to treat diabetes, blood cells to create new blood free of cancer cells for a leukemia patient, or neurons to treat neurological disorders.

31. Four specific genes—cMYC, OCT3/4, SOX2 and KLF4—encoding transcription factors play a role in converting or reprograming somatic cells into pluripotent stem cells. Of these four transcription factors, OCT4 is the most critical. OCT4 serves as a master regulator, playing an integral role in maintaining pluripotency and establishing the inner cell mass during development. OCT4 is expressed in the cell from nucleic acid encoding the OCT4 transcription factor. In fact, use of the exogenous OCT4 transcription factor (as opposed to nucleic acid encoding OCT4) is insufficient for producing viable, healthy, bona fide human iPSCs. And although SOX2, KLF4, and cMYC could be replaced by other members in its family of transcription factors, OCT4 cannot.

B. Dr. Kaufman's Double Dealing

32. Dr. Kaufman's obligations to Fate Therapeutics began on July 1, 2015 when

the parties entered into a Scientific Advisor Agreement (the "Agreement").

33. On August 21, 2019, Dr. Kaufman and Fate Therapeutics extended the Agreement through June 30, 2021. On information and belief, as Fate Therapeutics' Scientific Advisor, Dr. Kaufman had access to Fate Therapeutics' proprietary iPSC technology and was aware of the patents Fate Therapeutics licensed from Whitehead Institute, including the Asserted Patents.

34. On November 18, 2019, Dr. Kaufman asked Fate Therapeutics' Chief Science Officer for the details of Fate Therapeutics' Investor Dinner at the American Society of Hematology ("ASH") conference: "Let me know about the time and place for the Investor dinner at ASH. I can hide in the back…."

35. On December 4, 2019, Dr. Kaufman persisted in seeking details to attend the private Fate Investor Dinner. "Can you let me know the details (time and place) of the Fate investors dinner at ASH? As discussed, even if I am not presenting, it would be good if I could attend." With Dr. Kaufman under exclusivity obligations to Fate Therapeutics, Fate Therapeutics' Chief Science Officer provided Dr. Kaufman the details of the Fate Investor Dinner.

36. On December 6, 2019, Dr. Kaufman attended the private Fate Investor Dinner with Fate Therapeutics' investors and prospective investors. On information and belief, during the Fate Investor Dinner, Dr. Kaufman had access to information about Fate Therapeutics' iPSC technology and the Asserted Patents.

37. On information and belief, Dr. Kaufman met with Dr. Kleanthis Xanthopoulos to discuss founding a competitor to Fate Therapeutics around or after the time of Fate Therapeutics' Investor Dinner.

38. On February 6, 2020, Fate Therapeutics and Dr. Kaufman published research that demonstrated Fate Therapeutics' FT516 natural killer immunotherapy cells derived from induced pluripotent stem cells are effective against blood cancer. The publication disclosed that Dr. Kaufman "is a consultant for Fate Therapeutics, has equity and receives

1 income." The underlying research used iPSC cell lines reprogrammed using the
2 compositions and methods claimed in the Asserted Patents.
3 39. On information and belief, Dr. Kaufman and Dr. Xanthopoulos co-founded
4 Shoreline on or about May 14, 2020. Dr. Kaufman was consulting for Fate Therapeutics at
5 this time.
6 40. In violation of the Agreement, Dr. Kaufman failed to notify Fate Therapeutics
7 that he intended to be a co-founder, director of, and participant in Shoreline. He also failed
8 to notify Fate Therapeutics that as a co-founder, director of, and participant in, Shoreline,
9 he intended to help Shoreline pursue strategic partnerships totaling over $4 billion, all while
10 serving as Fate Therapeutics' Scientific Advisor.
11 41. On July 9, 2020, without notice to or approval from Fate Therapeutics, Dr.
12 Kaufman helped Shoreline raise $3 million through the sale of Shoreline equity to investors.
13 42. At least as early as August 2020 and after being reminded of his exclusivity
14 obligations to Fate Therapeutics under the Agreement, Dr. Kaufman helped Shoreline
15 pursue additional investors while denigrating Fate Therapeutics as purportedly having a
16 "short fall[] in treatment options." **Exhibit I**, August 2020 Shoreline "Corporate
17 Presentation" at p. 3 ("Shoreline is built by a very experienced team" including Dr.
18 Kaufman), and p. 17 (claiming Fate Therapeutics has an alleged "shortfall[] in treatment
19 options.").
20 43. Shoreline's August 2020 Corporate Presentation also contains five pages
21 whose contents are taken from a June 11, 2020 publication by Dr. Kaufman, but omits its
22 statement that Dr. Kaufman "is a consultant for Fate Therapeutics, has equity and receives
23 income." *Id.* at p. 9-13.
24 44. On September 4, 2020, Dr. Kaufman sought a written waiver of his exclusivity
25 obligations to Fate Therapeutics. But in his written waiver request Dr. Kaufman misled Fate
26 Therapeutics as to the true nature and extent of his relationship and involvement with
27 Shoreline. Dr. Kaufman failed to disclose that he had co-founded, directed, and participated
28

1 in Shoreline, already helped it raise millions of dollars in investor funds, was in the process
2 of helping it raise $43 million more, and intended to continue helping it secure strategic
3 partnerships, all while serving as Fate Therapeutics' Scientific Advisor.
4 45. In his waiver request, under "Detailed Description of Proposed Activities," Dr.
5 Kaufman only described his proposed activities with Shoreline as "Scientific Advisor" with
6 a "Maximum Time Commitment" of 4-6 hours per month. The same day, Fate Therapeutics
7 questioned Dr. Kaufman: "How/why is it distinct from what Fate is doing?"
8 46. On September 5, 2020, after being asked how and why Shoreline was distinct
9 from Fate Therapeutics, Dr. Kaufman responded that Shoreline would also develop natural
10 killer immunotherapy cells derived from induced pluripotent stem cells but (allegedly
11 unlike Fate Therapeutics) Shoreline would be "using new technology" so the cells had
12 "improved function." Dr. Kaufman's response was silent as to how the induced pluripotent
13 stem cells from which Shoreline was deriving its immunotherapy treatments were made.
14 On information and belief, Dr. Kaufman and Shoreline lacked the scientific expertise to
15 develop a method for making iPSCs suitable for developing immunotherapies, that did not
16 require the use of exogenously introduced nucleic acid encoding OCT4 and without
17 infringing the Asserted Patents.
18 47. Upon information and belief, Shoreline and Dr. Kaufman use Plaintiffs' iPSC
19 platform technology, including the use of exogenously introduced nucleic acid encoding
20 OCT4, to generate the induced pluripotent stem cells from which Shoreline derives its
21 immunotherapy treatments. On information and belief, Dr. Kaufman and Shoreline could
22 not have made human iPSCs suitable for generating immunotherapies, and could not have
23 done so as quickly as they did, without infringing the Asserted Patents.
24 48. On September 11, 2020, Fate Therapeutics informed Dr. Kaufman that he was
25 in breach of the Agreement by "providing services as an advisor" to Shoreline—the only
26 Shoreline-related activity for which Dr. Kaufman sought a waiver—as well as for serving
27 as a director of Shoreline and aiding in its formation. Fate Therapeutics demanded that Dr.
28

Kaufman terminate his relationship with Shoreline.

49. On October 12, 2020, Fate Therapeutics again informed Dr. Kaufman that his services to and participation in Shoreline breached the Agreement. Despite his breach and while still a Scientific Advisor for Fate Therapeutics, Dr. Kaufman continued to render services to Shoreline, including by securing investments totaling over $43 million, pursuing strategic partnerships with at least Kite Pharma, Inc. and BeiGene, Ltd., and planning to provide or develop competitive natural killer immunotherapy cells derived from induced pluripotent stem cells.

THE ASSERTED PATENTS

50. Fate Therapeutics is the exclusive licensee of the Asserted Patents, which were developed by inventors Rudolf Jaenisch and Konrad Hochedlinger at Whitehead Institute.

51. Although the invention(s) set forth in the Asserted Patents are best described by their claims, the Asserted Patents are generally directed to engineered somatic cells that are reprogrammed into a less differentiated state through, for example, the activation of a pluripotency gene(s), such as the OCT4 transgene.

52. For example, claim 1 of the ’369 Patent recites:

> A composition comprising an isolated primary somatic cell that comprises an exogenously introduced nucleic acid encoding an Oct4 protein operably linked to at least one regulatory sequence.

53. As an additional example, claim 1 of the ’856 Patent recites:

> A method of making a somatic cell more susceptible to reprogramming to a pluripotent state comprising introducing at least one exogenous nucleic acid encoding Oct4 operably linked to at least one regulatory sequence into the cell, thereby increasing expression of Oct4 protein in the somatic cell, wherein increased expression of Oct4 protein makes the cell more susceptible to reprogramming to a pluripotent state.

54. As another example, claim 1 of the ’797 Patent recites:

> A composition comprising an isolated primary somatic cell that comprises an exogenously introduced nucleic acid encoding Oct4, wherein the exogenously introduced nucleic acid increases Oct4 expression in the cell.

55. As an additional example, claim 1 of the '536 Patent recites:

> A method of making a primary somatic cell more susceptible to reprogramming to a less differentiated state, com-prising: introducing an exogenous nucleic acid encoding an Oct 4 protein operably linked to at least one regulatory sequence into the somatic cell, wherein expression of the exogenously introduced nucleic acid results in making the somatic cell more susceptible to reprogramming to a less differentiated state.

56. As another example, claim 1 of the '490 Patent recites:

> A somatic cell comprising an exogenous nucleic acid encoding Oct4 and an amount of Oct4 expression comparable to the amount of Oct4 expression in an embryonic stem cell.

57. As an additional example, claim 1 of the '917 Patent recites:

> A method of making a somatic cell more susceptible to reprogramming to a less differentiated state, comprising: introducing an exogenous nucleic acid encoding an Oct4 protein operably linked to at least one regulatory sequence into the somatic cell, thereby increasing expression of Oct4 protein in the somatic cell, wherein increased expression of Oct4 protein makes the cell more susceptible to reprogramming; and wherein the exogenous nucleic acid is transiently transfected into the somatic cell.

58. The Asserted Patents are related, share a common specification, and claim priority to at least November 26, 2003.

59. The Asserted Patents were duly issued by the United States Patent and Trademark Office and are presumed valid. The '369 Patent issued on December 6, 2011. The '856 Patent issued on January 13, 2015. The '797 Patent issued on February 10, 2015. The '536 Patent issued on January 27, 2015. The '490 Patent issued on October 27, 2015. The '917 Patent issued on October 29, 2019.

60. The groundbreaking iPSC reprogramming platform claimed in the Asserted Patents is not reasonably related to the development and submission of any information under the Federal Food, Drug, and Cosmetic Act ("FDCA"), including clinical and preclinical studies of patented compounds that are appropriate for submission to the U.S. Food and Drug Administration ("FDA"); pharmacological, toxicological, pharmacokinetic,

and biological qualities of drug substances or drug products; or the safety and/or clinical efficacy of drug substances or drug products. In other words, it is not reasonably related to the generation of the kinds of information submitted to support an investigational new drug application ("IND") or new drug application ("NDA"). Instead, the iPSC platform transforms somatic cells (such as skin fibroblast cells) into iPSC cells that have no therapeutic properties desired by Dr. Kaufman or Shoreline, including for the treatment of cancers or other conditions. On information and belief, at the time of infringement, Defendants did not and could not have reasonably believed they possessed a therapeutic with desired anti-cancer biological properties.

61. Because the iPSC reprogramming platform claimed in the Asserted Patents is not subject to FDA premarket approval, the Asserted Patents are also not eligible for patent term extension provided by 35 U.S.C. § 156(f).

DEFENDANTS' INFRINGING ACTIVITIES

62. On information and belief, Defendants, individually and acting in concert, make, use, sell, offer for sale, and/or import induced pluripotent stem cells that infringe one or more claims of the Asserted Patents.

63. On information and belief, Defendants have infringed the Asserted Patents in this District, including at Shoreline's corporate headquarters and at the Advanced Cell Therapy Laboratory of the University of California, San Diego.

64. Defendants' actions have irreparably harmed Plaintiffs and will continue to do so unless they permanently cease. At least the continued use of the infringing induced pluripotent stem cells will further damage Fate Therapeutics' market position and good reputation in the biotechnology/pharmaceutical industry. In addition, Defendants' continued knowing acts of infringement will frustrate Fate Therapeutics' ongoing and potential business relationships and contracts, with resulting lost sales and profits, and are otherwise causing or will cause substantial irreparable harm to Fate Therapeutics' business.

65. As a result of Defendants' actions, Plaintiffs are forced to file this lawsuit to

protect their patented inventions and reputation as a leader in the industry.

FIRST CLAIM FOR RELIEF

(Infringement of the ’369 Patent)

66. Plaintiffs restate and reallege each of the assertions set forth in the paragraphs above.

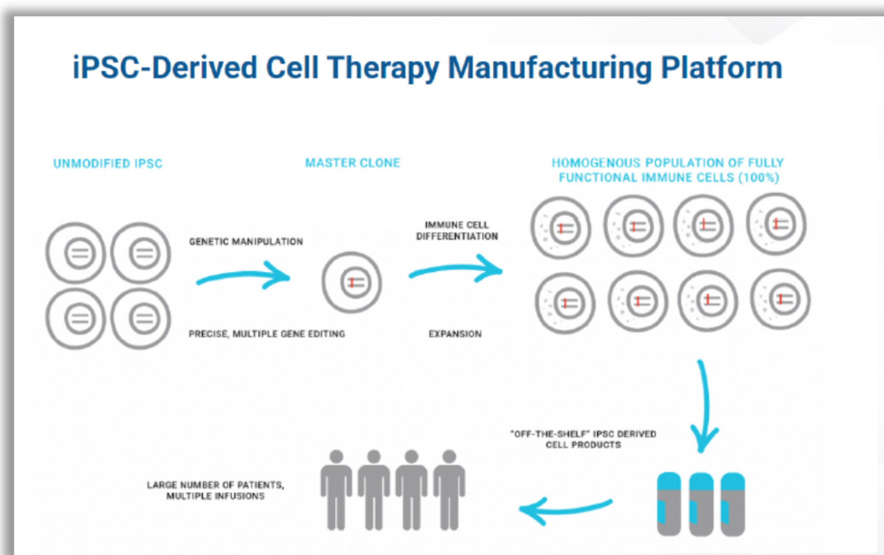
67. This is a claim for patent infringement and arises under the Patent Laws of the United States and, in particular, under 35 U.S.C. §§ 271, *et seq*.

68. Defendants have in the past infringed and continue to infringe the ’369 Patent in violation of at least 35 U.S.C. § 271(a) by making, using, offering to sell, and/or selling, in the United States, or importing into the United States induced pluripotent stem cells that infringe at least claim 1 of the ’369 Patent without Plaintiffs’ authorization or consent.

69. On information and belief, including the information regarding Defendants’ use of the infringing induced pluripotent stem cells, Defendants’ manufacture, use, offer to sell, or sale of the infringing products was and is not protected by the “safe harbor” provision of 35 U.S.C. § 271(e)(1).

70. Claim 1 of the ’369 Patent recites: “a composition comprising an isolated primary somatic cell that comprises an exogenously introduced nucleic acid encoding an Oct4 protein operably linked to at least one regulatory sequence.”

71. Defendants’ use of their “iPSC-derived cell therapy manufacturing platform” infringes at least claim 1 of the ’369 Patent. Defendants describe their process for making, iPSC-derived therapies as follows:



72. On information and belief, Defendants generate the iPSCs from isolated primary somatic cells (*e.g.*, human skin cells or fibroblasts). On information and belief, Defendants introduce into the somatic cells an exogenous nucleic acid (such as cDNA) encoding an OCT4 protein operably linked to at least one regulatory sequence.

73. On information and belief, there is no commercially suitable way to make healthy, viable, bona fide human iPSCs other than using isolated primary somatic cells (particularly, *e.g.*, fibroblasts). The iPSCs used in Defendants' manufacturing process thus originate from primary somatic cells.

74. On information and belief, the iPSCs used in Defendants' manufacturing process comprise an exogenously introduced nucleic acid (particularly, cDNA) encoding an OCT4 protein operably linked to at least one regulatory sequence. On information and belief, it would not have been practical or economical for Defendants to develop a method for making human iPSCs, suitable for their intended purposes of manufacturing healthy, viable immunotherapeutics (*e.g.*, NK cells), by using any method other than the invention disclosed in the '369 Patent, as no such method existed at the time of infringement and would have required expertise and an enormous and lengthy research effort, neither of which were within Defendants' capabilities.

75. OCT4 is the most critical transcription factor for making iPSCs. OCT4 serves as a master regulator, playing an integral role in maintaining pluripotency and establishing the inner cell mass during development. Healthy human iPSCs suitable for producing immunotherapies cannot be made without the introduction of exogenous nucleic acid encoding OCT4. The nucleic acid encoding OCT4 must be operably linked to one or more regulatory elements to affect the expression of the OCT4 transcription factor.

76. Indeed, Fate Therapeutics generates iPSCs through the activation/expression of OCT4 in accordance with the ’369 Patent.

77. As a Scientific Advisor for Fate Therapeutics, Dr. Kaufman was aware of Fate Therapeutics’ iPSCs and used such iPSCs in his consultation with Fate Therapeutics.

78. Defendants also infringe the ’369 Patent under at least 35 U.S.C. § 271(b).

79. On information and belief, Defendants have been aware of the ’369 Patent and that they infringe the ’369 Patent since at least May 14, 2020 by virtue of Dr. Kaufman’s position as a Scientific Advisor for Fate Therapeutics and the renown of the Asserted Patents in the industry.

80. On information and belief, Defendants intended to induce patent infringement by at least the Advanced Cell Therapy Laboratory of the University of California, San Diego to produce iPSCs claimed by the ’369 Patent and had knowledge that the inducing acts would cause infringement or were willfully blind to the possibility that their inducing acts would cause infringement. Indeed, Defendants prominently advertise on their website that they have “partnered with the Advanced Cell Therapy Laboratory (ACTL) of UC San Diego” to allow it “to bring in-house GMP grade iPSCs to bank and rapidly initiate preclinical development and IND-enabling studies.” **Exhibit J.** Defendants also “leverage an extensive network of leading CMC professionals to guide our manufacturing agenda.” **Exhibit K.** On information and belief, Dr. Kaufmann performed and/or directed the infringing work at ACTL.

81. On information and belief, Defendants performed acts that constitute

inducement of infringement, and would cause actual infringement, with the knowledge of the '369 Patent. For example, Defendants instructed and/or supervised the Advanced Cell Therapy Laboratory of the University of California, San Diego to produce iPSCs claimed by the '369 Patent.

82. The invention claimed by the '369 Patent does not require FDA approval for marketing.

83. On information and belief, Dr. Kaufman and Shoreline make and use the iPSCs of the '369 Patent in their cell therapy manufacturing platform. On information and belief, Dr. Kaufman carried out the infringing manufacture of iPSCs for the benefit of and in his role as an officer, employee, or agent of Shoreline.

84. Defendants' infringing conduct will continue unless enjoined by this Court.

85. On information and belief, Defendants became aware of the '369 Patent prior to their acts of infringement. As a result, the use of the iPSC compositions claimed in the '369 Patent by Defendants was made and will be made with full knowledge of the '369 Patent and without a reasonable basis for believing that Defendants would not be liable for infringing the '369 Patent.

86. Defendants have engaged in deliberate and willful behavior with knowledge of the '369 Patent and knew or should have known that their actions constituted direct and/or indirect infringement of the '369 Patent.

87. Defendants' acts of direct infringement have been, and continue to be, willful and deliberate, and Defendants' acts of indirect infringement were, and continue to be, knowing and intentional.

88. Plaintiffs are entitled to an award of damages adequate to compensate Fate Therapeutics for patent infringement, as well as prejudgment interest from the date the infringement began, but in no event less than a reasonable royalty as permitted by 35 U.S.C. § 284.

89. Plaintiffs are entitled to an award of treble damages for the period of any

1 willful infringement pursuant to 35 U.S.C. § 284.

2 90. Plaintiffs are entitled to a finding that this case is exceptional and an award of
3 interest, costs and attorneys' fees incurred by Fate Therapeutics in prosecuting this action
4 as provided by 35 U.S.C. § 285.

5 91. Plaintiffs are entitled to an award of pre-judgment and post-judgment interest
6 as provided by law.

7 92. Plaintiffs are entitled to such other and further relief as this Court or a jury may
8 deem just and proper.

9 **SECOND CLAIM FOR RELIEF**

10 **(Infringement of the '856 Patent)**

11 93. Plaintiffs restate and reallege each of the assertions set forth in the paragraphs
12 above.

13 94. This is a claim for patent infringement and arises under the Patent Laws of the
14 United States and, in particular, under 35 U.S.C. §§ 271, *et seq*.

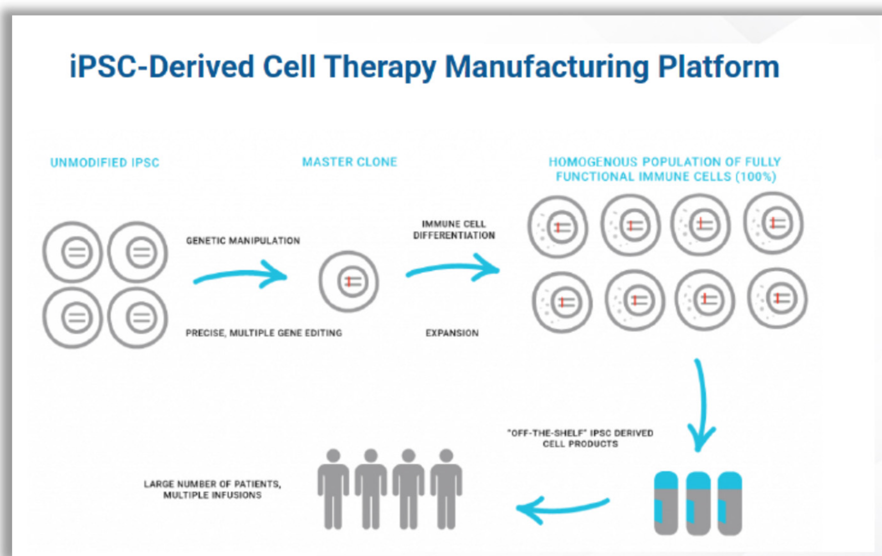
15 95. Defendants have in the past infringed and continue to infringe the '856 Patent
16 in violation of at least 35 U.S.C. § 271(a) by making, using, offering to sell, and/or selling,
17 in the United States, or importing into the United States induced pluripotent stem cells that
18 infringe at least claim 1 of the '856 Patent without Plaintiffs' authorization or consent.

19 96. On information and belief, including the information regarding Defendants'
20 use of the infringing induced pluripotent stem cells, Defendants' manufacture, use, offer to
21 sell, or sale of the infringing products was and is not protected by the "safe harbor"
22 provision of 35 U.S.C. § 271(e)(1).

23 97. Claim 1 of the '856 Patent recites: "A method of making a somatic cell more
24 susceptible to reprogramming to a pluripotent state comprising introducing at least one
25 exogenous nucleic acid encoding Oct4 operably linked to at least one regulatory sequence
26 into the cell, thereby increasing expression of Oct4 protein in the somatic cell, wherein
27 increased expression of Oct4 protein makes the cell more susceptible to reprogramming to
28

a pluripotent state."

98. Defendants' use of their "iPSC-derived cell therapy manufacturing platform" infringes at least claim 1 of the '856 Patent. Defendants describe their process for making, iPSC-derived therapies as follows:



99. On information and belief, Defendants manufacture iPSCs from isolated primary somatic cells (*e.g.*, human skin cells or fibroblasts). On information and belief, Defendants introduce into the somatic cells an exogenous nucleic acid (such as cDNA) encoding an OCT4 protein linked to at least one regulatory sequence. Doing so, necessarily increases expression of OCT4 protein in the cell and, in turn, makes the cell more susceptible to reprogramming to a pluripotent state.

100. On information and belief, there is no commercially suitable way to make healthy, viable, bona fide human iPSCs other than using isolated primary somatic cells (particularly, *e.g.*, fibroblasts). The iPSCs used in Defendants' manufacturing process thus originate from primary somatic cells.

101. On information and belief, Defendants make iPSCs by introducing nucleic acid (particularly, cDNA) encoding an OCT4 protein operably linked to at least one regulatory sequence into an isolated primary somatic cell (*e.g.*, a fibroblast). This

necessarily increases expression of OCT4 protein in the cell and, in turn, makes the cell more susceptible to reprogramming to a pluripotent state. On information and belief, it would not have been practical or economical for Defendants to develop a method for making human iPSCs, suitable for their intended purposes of manufacturing healthy, viable immunotherapeutics (*e.g.*, NK cells), by using any method other than the invention disclosed in the ’856 Patent, as no such method existed at the time of infringement and would have required expertise and an enormous and lengthy research effort, neither of which were within Defendants’ capabilities.

102. OCT4 is the most critical transcription factor for making iPSCs. OCT4 serves as a master regulator, playing an integral role in maintaining pluripotency and establishing the inner cell mass during development. Healthy human iPSCs suitable for producing immunotherapies cannot be made without the introduction of exogenous nucleic acid encoding OCT4. The nucleic acid encoding OCT4 must be operably linked to one or more regulatory elements to affect the expression of the OCT4 protein.

103. Indeed, Fate Therapeutics generates iPSCs through the activation/expression of OCT4 in accordance with the ’856 Patent.

104. As a Scientific Advisor for Fate Therapeutics, Dr. Kaufman was aware of Fate Therapeutics’ iPSCs, their manufacture, and used such iPSCs in his consultation with Fate Therapeutics.

105. Defendants also infringe the ’856 Patent under at least 35 U.S.C. § 271(b).

106. On information and belief, Defendants have been aware of the ’856 Patent and that they infringe the ’856 Patent since at least May 14, 2020 by virtue of Dr. Kaufman’s position as a Scientific Advisor for Fate Therapeutics and the renown of the Asserted Patents in the industry.

107. On information and belief, Defendants intended to induce patent infringement by at least the Advanced Cell Therapy Laboratory of the University of California, San Diego to produce iPSCs claimed by the ’856 Patent and had knowledge that the inducing acts

would cause infringement or were willfully blind to the possibility that their inducing acts would cause infringement. Indeed, Defendants prominently advertise on their website that they have "partnered with the Advanced Cell Therapy Laboratory (ACTL) of UC San Diego" to allow it "to bring in-house GMP grade iPSCs to bank and rapidly initiate preclinical development and IND-enabling studies." **Exhibit J.** Defendants also "leverage an extensive network of leading CMC professionals to guide our manufacturing agenda." **Exhibit K.**

108. On information and belief, Defendants performed acts that constitute inducement of infringement, and would cause actual infringement, with the knowledge of the '856 Patent. For example, Defendants instructed and/or supervised the Advanced Cell Therapy Laboratory of the University of California, San Diego to produce iPSCs claimed by the '856 Patent.

109. Defendants also infringe under 35 U.S.C. § 271(g) by offering to sell, selling, or using within the United States iPSCs which are made by a process patented in the '856 Patent.

110. Specifically, and as further detailed above, iPSCs used by Defendants to make at least the iPSC-derived natural killer (NK) cell platforms are made by a process that comprises at least each step of claim 1 of the '856 Patent.

111. Accordingly, Defendants offers for sale, sales and use of such iPSCs are infringing under § 271(g).

112. On information and belief, Dr. Kaufman and Shoreline make iPSCs according to at least the method of the '856 Patent in their cell therapy manufacturing platform. On information and belief, Dr. Kaufman carried out the infringing manufacture of iPSCs for the benefit of and in his role as an officer, employee, or agent of Shoreline.

113. The invention claimed by the '856 Patent does not require FDA approval for marketing.

114. Defendants' infringing conduct will continue unless enjoined by this Court.

115. On information and belief, Defendants became aware of the ’856 Patent prior to their acts of infringement. As a result, the use of the iPSC compositions claimed in the ’856 Patent by Defendants was made and will be made with full knowledge of the ’856 Patent and without a reasonable basis for believing that Defendants would not be liable for infringing the ’856 Patent.

116. Defendants have engaged in deliberate and willful behavior with knowledge of the ’856 Patent and knew or should have known that their actions constituted direct and/or indirect infringement of the ’856 Patent.

117. Defendants’ acts of direct infringement have been, and continue to be, willful and deliberate, and Defendants’ acts of indirect infringement were, and continue to be, knowing and intentional.

118. Plaintiffs are entitled to an award of damages adequate to compensate Fate Therapeutics for patent infringement, as well as prejudgment interest from the date the infringement began, but in no event less than a reasonable royalty as permitted by 35 U.S.C. § 284.

119. Plaintiffs are entitled to an award of treble damages for the period of any willful infringement pursuant to 35 U.S.C. § 284.

120. Plaintiffs are entitled to a finding that this case is exceptional and an award of interest, costs and attorneys’ fees incurred by Fate Therapeutics in prosecuting this action as provided by 35 U.S.C. § 285.

121. Plaintiffs are entitled to an award of pre-judgment and post-judgment interest as provided by law.

122. Plaintiffs are entitled to such other and further relief as this Court or a jury may deem just and proper.

THIRD CLAIM FOR RELIEF

(Infringement of the ’797 Patent)

123. Plaintiffs restate and reallege each of the assertions set forth in the paragraphs

1 above.

2 124. This is a claim for patent infringement and arises under the Patent Laws of the
3 United States and, in particular, under 35 U.S.C. §§ 271, *et seq*.

4 125. Defendants have in the past infringed and continue to infringe the ’797 Patent
5 in violation of at least 35 U.S.C. § 271(a) by making, using, offering to sell, and/or selling,
6 in the United States, or importing into the United States induced pluripotent stem cells that
7 infringe at least claim 1 of the ’797 Patent without Plaintiffs’ authorization or consent.

8 126. On information and belief, including the information regarding Defendants’
9 use of the infringing induced pluripotent stem cells, Defendants’ manufacture, use, offer to
10 sell, or sale of the infringing products was and is not protected by the “safe harbor”
11 provision of 35 U.S.C. § 271(e)(1).

12 127. Claim 1 of the ’797 Patent recites: “a composition comprising an isolated
13 primary somatic cell that comprises an exogenously introduced nucleic acid encoding
14 OCT4, wherein the exogenously introduced nucleic acid increases OCT4 expression in the
15 cell.”

16 128. Defendants’ use of their “iPSC-derived cell therapy manufacturing platform”
17 infringes at least claim 1 of the ’797 Patent. Defendants describe their process for making,
18 iPSC-derived therapies as follows:

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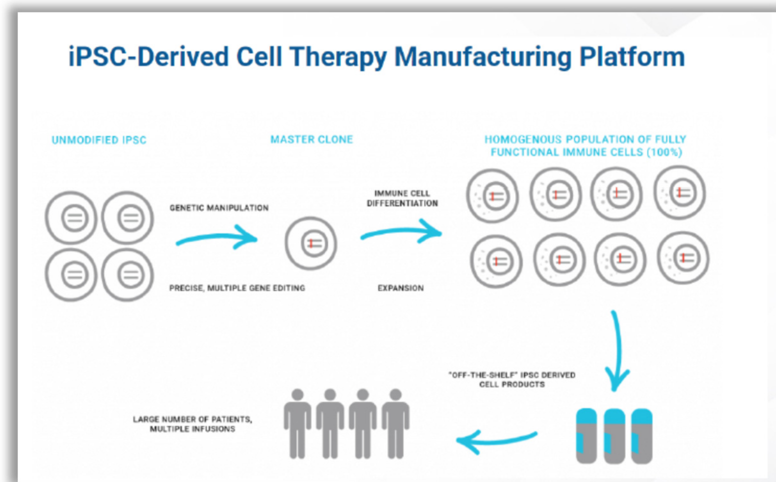


129. On information and belief, Defendants generate the iPSCs from isolated primary somatic cells (*e.g.*, human skin cells or fibroblasts). On information and belief, Defendants introduce into the somatic cells an exogenous nucleic acid (such as cDNA) encoding an OCT4 protein.

130. On information and belief, there is no commercially suitable way to make healthy, viable, bona fide human iPSCs other than using isolated primary somatic cells (particularly, *e.g.*, fibroblasts). The iPSCs used in Defendants' manufacturing process thus originate from primary somatic cells.

131. On information and belief, the iPSCs used in Defendants' manufacturing process comprise an exogenously introduced nucleic acid (particularly, cDNA) encoding an OCT4 protein. On information and belief, it would not have been practical or economical for Defendants to develop a method for making human iPSCs, suitable for their intended purposes of manufacturing healthy, viable immunotherapeutics (*e.g.*, NK cells), by using any method other than the invention disclosed in the '797 Patent, as no such method existed at the time of infringement and would have required expertise and an enormous and lengthy research effort, neither of which were within Defendants' capabilities.

132. OCT4 is the most critical transcription factor for making iPSCs. OCT4 serves as a master regulator, playing an integral role in maintaining pluripotency and establishing the inner cell mass during development. Healthy human iPSCs suitable for producing immunotherapies cannot be made without the introduction of exogenous nucleic acid encoding OCT4 protein. The nucleic acid encoding OCT4 must be operably linked to one or more regulatory elements to affect the expression of the OCT4 protein or transcription factor.

133. Indeed, Fate Therapeutics generates iPSCs through the activation/expression of OCT4 in accordance with the '797 Patent.

134. As a Scientific Advisor for Fate Therapeutics, Dr. Kaufman was aware of Fate Therapeutics' iPSCs and used such iPSCs in his consultation with Fate Therapeutics.

135. Defendants also infringe the ’797 Patent under at least 35 U.S.C. § 271(b).

136. On information and belief, Defendants have been aware of the ’797 Patent and that they infringe the ’797 Patent since at least May 14, 2020 by virtue of Dr. Kaufman’s position as a Scientific Advisor for Fate Therapeutics and the renown of the Asserted Patents in the industry.

137. On information and belief, Defendants intended to induce patent infringement by at least the Advanced Cell Therapy Laboratory of the University of California, San Diego to produce iPSCs claimed by the ’797 Patent and had knowledge that the inducing acts would cause infringement or were willfully blind to the possibility that their inducing acts would cause infringement. Indeed, Defendants prominently advertise on their website that they have “partnered with the Advanced Cell Therapy Laboratory (ACTL) of UC San Diego” to allow it “to bring in-house GMP grade iPSCs to bank and rapidly initiate preclinical development and IND-enabling studies.” **Exhibit J.** Defendants also “leverage an extensive network of leading CMC professionals to guide our manufacturing agenda.” **Exhibit K.**

138. On information and belief, Defendants performed acts that constitute inducement of infringement, and would cause actual infringement, with the knowledge of the ’797 Patent. For example, Defendants instructed and/or supervised the Advanced Cell Therapy Laboratory of the University of California, San Diego to produce iPSCs claimed by the ’797 Patent.

139. The invention claimed by the ’797 Patent does not require FDA approval for marketing.

140. On information and belief, Dr. Kaufman and Shoreline make and use the iPSCs of the ’797 Patent in their cell therapy manufacturing platform. On information and belief, Dr. Kaufman carried out the infringing manufacture of iPSCs for the benefit of and in his role as an officer, employee, or agent of Shoreline.

141. Defendants’ infringing conduct will continue unless enjoined by this Court.

142. On information and belief, Defendants became aware of the ’797 Patent prior to their acts of infringement. As a result, the use of the iPSC compositions claimed in the ’797 Patent by Defendants was made and will be made with full knowledge of the ’797 Patent and without a reasonable basis for believing that Defendants would not be liable for infringing the ’797 Patent.

143. Defendants have engaged in deliberate and willful behavior with knowledge of the ’797 Patent and knew or should have known that their actions constituted direct and/or indirect infringement of the ’797 Patent.

144. Defendants’ acts of direct infringement have been, and continue to be, willful and deliberate, and Defendants’ acts of indirect infringement were, and continue to be, knowing and intentional.

145. Plaintiffs are entitled to an award of damages adequate to compensate Fate Therapeutics for patent infringement, as well as prejudgment interest from the date the infringement began, but in no event less than a reasonable royalty as permitted by 35 U.S.C. § 284.

146. Plaintiffs are entitled to an award of treble damages for the period of any willful infringement pursuant to 35 U.S.C. § 284.

147. Plaintiffs are entitled to a finding that this case is exceptional and an award of interest, costs and attorneys’ fees incurred by Fate Therapeutics in prosecuting this action as provided by 35 U.S.C. § 285.

148. Plaintiffs are entitled to an award of pre-judgment and post-judgment interest as provided by law.

149. Plaintiffs are entitled to such other and further relief as this Court or a jury may deem just and proper.

FOURTH CLAIM FOR RELIEF

(Infringement of the ’536 Patent)

150. Plaintiffs restate and reallege each of the assertions set forth in the paragraphs

above.

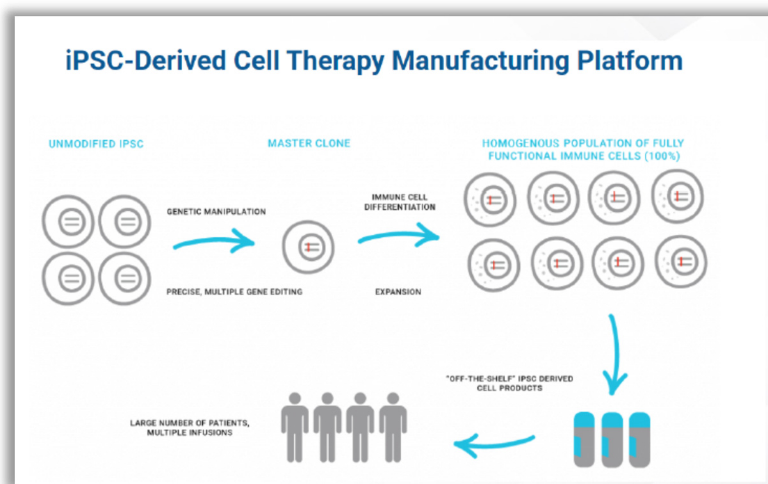
151. This is a claim for patent infringement and arises under the Patent Laws of the United States and, in particular, under 35 U.S.C. §§ 271, *et seq*.

152. Defendants have in the past infringed and continue to infringe the ’536 Patent in violation of at least 35 U.S.C. § 271(a) by making, using, offering to sell, and/or selling, in the United States, or importing into the United States induced pluripotent stem cells that infringe at least claim 1 of the ’536 Patent without Plaintiffs’ authorization or consent.

153. On information and belief, including the information regarding Defendants’ use of the infringing induced pluripotent stem cells, Defendants’ manufacture, use, offer to sell, or sale of the infringing products was and is not protected by the “safe harbor” provision of 35 U.S.C. § 271(e)(1).

154. Claim 1 of the ’536 Patent recites: “a method of making a primary somatic cell more susceptible to reprogramming to a less differentiated state, comprising: introducing an exogenous nucleic acid encoding an Oct4 protein operably linked to at least one regulatory sequence into the somatic cell, wherein expression of the exogenously introduced nucleic acid results in making the somatic cell more susceptible to reprogramming to a less differentiated state.”

155. Defendants’ use of their “iPSC-derived cell therapy manufacturing platform” infringes at least claim 1 of the ’536 Patent. Defendants describe their process for making, iPSC-derived therapies as follows:



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2 156. On information and belief, Defendants manufacture iPSCs from isolated
3 primary somatic cells (*e.g.*, human skin cells or fibroblasts). On information and belief,
4 Defendants introduce into the somatic cells an exogenous nucleic acid (such as cDNA)
5 encoding an OCT4 protein operably linked to at least one regulatory sequence. Doing so,
6 necessarily increases expression of OCT4 protein in the cell and, in turn, makes the cell
7 more susceptible to reprogramming to a less differentiated (*e.g.*, pluripotent) state.
8 157. On information and belief, there is no commercially suitable way to make
9 healthy, viable, bona fide human iPSCs other than using isolated primary somatic cells
10 (particularly, *e.g.*, fibroblasts). The iPSCs used in Defendants' manufacturing process thus
11 originate from primary somatic cells.
12 158. On information and belief, Defendants make iPSCs by introducing nucleic
13 acid (particularly, cDNA) encoding an OCT4 protein operably linked to at least one
14 regulatory sequence into an isolated primary somatic cell (*e.g.*, a fibroblast). This
15 necessarily increases expression of OCT4 protein in the cell and, in turn, makes the cell
16 more susceptible to reprogramming to a less differentiated (*e.g.*, pluripotent) state. On
17 information and belief, it would not have been practical or economical for Defendants to
18 develop a method for making human iPSCs, suitable for their intended purposes of
19 manufacturing healthy, viable immunotherapeutics (*e.g.*, NK cells), by using any method
20 other than the invention disclosed in the '536 Patent, as no such method existed at the time
21 of infringement and would have required expertise and an enormous and lengthy research
22 effort, neither of which were within Defendants' capabilities.
23 159. OCT4 is the most critical transcription factor for making iPSCs. OCT4 serves
24 as a master regulator, playing an integral role in maintaining pluripotency and establishing
25 the inner cell mass during development. Healthy human iPSCs suitable for producing
26 immunotherapies cannot be made without the introduction of exogenous nucleic acid
27 encoding OCT4. The nucleic acid encoding OCT4 must be operably linked to one or more
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1 regulatory elements to affect the expression of the OCT4 protein or transcription factor.
2 160. Indeed, Fate Therapeutics generates iPSCs through the activation/expression
3 of OCT4 in accordance with the '536 Patent.
4 161. As a Scientific Advisor for Fate Therapeutics, Dr. Kaufman was aware of Fate
5 Therapeutics' iPSCs, their manufacture, and used such iPSCs in his consultation with Fate
6 Therapeutics.
7 162. Defendants also infringe the '536 Patent under at least 35 U.S.C. § 271(b).
8 163. On information and belief, Defendants have been aware of the '536 Patent and
9 that they infringe the '536 Patent since at least May 14, 2020 by virtue of Dr. Kaufman's
10 position as a Scientific Advisor for Fate Therapeutics and the renown of the Asserted
11 Patents in the industry.
12 164. On information and belief, Defendants intended to induce patent infringement
13 by at least the Advanced Cell Therapy Laboratory of the University of California, San Diego
14 to produce iPSCs claimed by the '536 Patent and had knowledge that the inducing acts
15 would cause infringement or were willfully blind to the possibility that their inducing acts
16 would cause infringement. Indeed, Defendants prominently advertise on their website that
17 they have "partnered with the Advanced Cell Therapy Laboratory (ACTL) of UC San
18 Diego" to allow it "to bring in-house GMP grade iPSCs to bank and rapidly initiate
19 preclinical development and IND-enabling studies." **Exhibit J.** Defendants also "leverage
20 an extensive network of leading CMC professionals to guide our manufacturing agenda."
21 **Exhibit K.**
22 165. On information and belief, Defendants performed acts that constitute
23 inducement of infringement, and would cause actual infringement, with the knowledge of
24 the '536 Patent. For example, Defendants instructed and/or supervised the Advanced Cell
25 Therapy Laboratory of the University of California, San Diego to produce iPSCs claimed
26 by the '536 Patent.
27 166. Defendants also infringe under 35 U.S.C. § 271(g) by offering to sell, selling,
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1 or using within the United States iPSCs which are made by a process patented in the '536
2 Patent.
3 167. Specifically, and as further detailed above, iPSCs used by Defendants to make
4 at least the iPSC-derived natural killer (NK) cell platforms are made by a process that
5 comprises at least each step of claim 1 of the '536 Patent.
6 168. Accordingly, Defendants offers for sale, sales and use of such iPSCs are
7 infringing under § 271(g).
8 169. On information and belief, Dr. Kaufman and Shoreline make iPSCs according
9 to at least the method of the '536 Patent in their cell therapy manufacturing platform. On
10 information and belief, Dr. Kaufman carried out the infringing manufacture of iPSCs for
11 the benefit of and in his role as an officer, employee, or agent of Shoreline.
12 170. The invention claimed by the '536 Patent does not require FDA approval for
13 marketing.
14 171. Defendants' infringing conduct will continue unless enjoined by this Court.
15 172. On information and belief, Defendants became aware of the '536 Patent prior
16 to their acts of infringement. As a result, the use of the iPSC compositions claimed in the
17 '536 Patent by Defendants was made and will be made with full knowledge of the '536
18 Patent and without a reasonable basis for believing that Defendants would not be liable for
19 infringing the '536 Patent.
20 173. Defendants have engaged in deliberate and willful behavior with knowledge
21 of the '536 Patent and knew or should have known that their actions constituted direct
22 and/or indirect infringement of the '536 Patent.
23 174. Defendants' acts of direct infringement have been, and continue to be, willful
24 and deliberate, and Defendants' acts of indirect infringement were, and continue to be,
25 knowing and intentional.
26 175. Plaintiffs are entitled to an award of damages adequate to compensate Fate
27 Therapeutics for patent infringement, as well as prejudgment interest from the date the
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1 infringement began, but in no event less than a reasonable royalty as permitted by 35 U.S.C.
2 § 284.
3 176. Plaintiffs are entitled to an award of treble damages for the period of any
4 willful infringement pursuant to 35 U.S.C. § 284.
5 177. Plaintiffs are entitled to a finding that this case is exceptional and an award of
6 interest, costs and attorneys' fees incurred by Fate Therapeutics in prosecuting this action
7 as provided by 35 U.S.C. § 285.
8 178. Plaintiffs are entitled to an award of pre-judgment and post-judgment interest
9 as provided by law.
10 179. Plaintiffs are entitled to such other and further relief as this Court or a jury may
11 deem just and proper.
12 **FIFTH CLAIM FOR RELIEF**
13 **(Infringement of the '490 Patent)**
14 180. Plaintiffs restate and reallege each of the assertions set forth in the paragraphs
15 above.
16 181. This is a claim for patent infringement and arises under the Patent Laws of the
17 United States and, in particular, under 35 U.S.C. §§ 271, *et seq*.
18 182. Defendants have in the past infringed and continue to infringe the '490 Patent
19 in violation of at least 35 U.S.C. § 271(a) by making, using, offering to sell, and/or selling,
20 in the United States, or importing into the United States induced pluripotent stem cells that
21 infringe at least claim 1 of the '490 Patent without Plaintiffs' authorization or consent.
22 183. On information and belief, including the information regarding Defendants'
23 use of the infringing induced pluripotent stem cells, Defendants' manufacture, use, offer to
24 sell, or sale of the infringing products was and is not protected by the "safe harbor"
25 provision of 35 U.S.C. § 271(e)(1).
26 184. Claim 1 of the '490 Patent recites: "a somatic cell comprising an exogenous
27 nucleic acid encoding Oct4 and an amount of Oct4 expression comparable to the amount of
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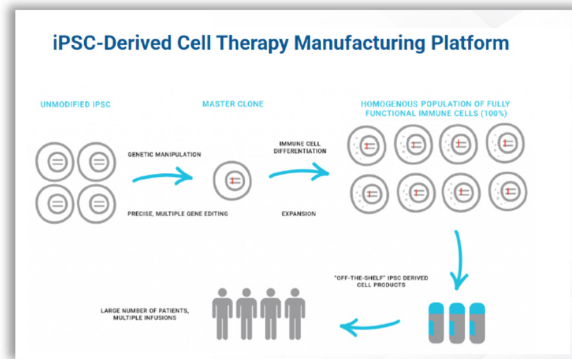
Oct4 expression in an embryonic stem cell."

185. Defendants' use of their "iPSC-derived cell therapy manufacturing platform" infringes at least claim 1 of the '490 Patent. Defendants describe their process for making, iPSC-derived therapies as follows:



186. On information and belief, Defendants generate the iPSCs from isolated primary somatic cells (*e.g.*, human skin cells or fibroblasts). On information and belief, Defendants introduce into the somatic cells an exogenous nucleic acid (such as cDNA) encoding an OCT4 protein.

187. On information and belief, there is no commercially suitable way to make healthy, viable, bona fide human iPSCs other than using isolated primary somatic cells (particularly, *e.g.*, fibroblasts). The iPSCs used in Defendants' manufacturing process thus originate from primary somatic cells.

188. On information and belief, Defendants make iPSCs by introducing nucleic acid (particularly, cDNA) encoding an OCT4 protein operably linked to at least one regulatory sequence into an isolated primary somatic cell (*e.g.*, a fibroblast). This necessarily increases expression of OCT4 protein in the cell and, in turn, makes the cell more susceptible to reprogramming to a less differentiated (*e.g.*, pluripotent) state. On information and belief, it would not have been practical or economical for Defendants to develop a method for making human iPSCs, suitable for their intended purposes of manufacturing healthy, viable immunotherapeutics (*e.g.*, NK cells), by using any method

other than the invention disclosed in the ’490 Patent, as no such method existed at the time of infringement and would have required expertise and an enormous and lengthy research effort, neither of which were within Defendants’ capabilities.

189. OCT4 is the most critical transcription factor for making iPSCs. OCT4 serves as a master regulator, playing an integral role in maintaining pluripotency and establishing the inner cell mass during development. Healthy human iPSCs suitable for producing immunotherapies cannot be made without the introduction of exogenous nucleic acid encoding OCT4. The nucleic acid encoding OCT4 must be operably linked to one or more regulatory elements to affect the expression of the OCT4 transcription factor.

190. Indeed, Fate Therapeutics generates iPSCs through the activation/expression of OCT4 in accordance with the ’490 Patent.

191. As a Scientific Advisor for Fate Therapeutics, Dr. Kaufman was aware of Fate Therapeutics’ iPSCs, their manufacture, and used such iPSCs in his consultation with Fate Therapeutics.

192. Defendants also infringe the ’490 Patent under at least 35 U.S.C. § 271(b).

193. On information and belief, Defendants have been aware of the ’490 Patent and that they infringe the ’490 Patent since at least May 14, 2020 by virtue of Dr. Kaufman’s position as a Scientific Advisor for Fate Therapeutics and the renown of the Asserted Patents in the industry.

194. On information and belief, Defendants intended to induce patent infringement by at least the Advanced Cell Therapy Laboratory of the University of California, San Diego to produce iPSCs claimed by the ’490 Patent and had knowledge that the inducing acts would cause infringement or were willfully blind to the possibility that their inducing acts would cause infringement. Indeed, Defendants prominently advertise on their website that they have “partnered with the Advanced Cell Therapy Laboratory (ACTL) of UC San Diego” to allow it “to bring in-house GMP grade iPSCs to bank and rapidly initiate preclinical development and IND-enabling studies.” **Exhibit J.** Defendants also “leverage

an extensive network of leading CMC professionals to guide our manufacturing agenda." **Exhibit K.**

195. On information and belief, Defendants performed acts that constitute inducement of infringement, and would cause actual infringement, with the knowledge of the '490 Patent. For example, Defendants instructed and/or supervised the Advanced Cell Therapy Laboratory of the University of California, San Diego to produce iPSCs claimed by the '490 Patent.

196. The invention claimed by the '490 Patent does not require FDA approval for marketing.

197. On information and belief, Dr. Kaufman and Shoreline make and use the iPSCs of the '490 Patent in their cell therapy manufacturing platform. On information and belief, Dr. Kaufman carried out the infringing manufacture of iPSCs for the benefit of and in his role as an officer, employee, or agent of Shoreline.

198. Defendants' infringing conduct will continue unless enjoined by this Court.

199. On information and belief, Defendants became aware of the '490 Patent prior to their acts of infringement. As a result, the use of the iPSC compositions claimed in the '490 Patent by Defendants was made and will be made with full knowledge of the '490 Patent and without a reasonable basis for believing that Defendants would not be liable for infringing the '490 Patent.

200. Defendants have engaged in deliberate and willful behavior with knowledge of the '490 Patent and knew or should have known that their actions constituted direct and/or indirect infringement of the '490 Patent.

201. Defendants' acts of direct infringement have been, and continue to be, willful and deliberate, and Defendants' acts of indirect infringement were, and continue to be, knowing and intentional.

202. Plaintiffs are entitled to an award of damages adequate to compensate Fate Therapeutics for patent infringement, as well as prejudgment interest from the date the

infringement began, but in no event less than a reasonable royalty as permitted by 35 U.S.C. § 284.

203. Plaintiffs are entitled to an award of treble damages for the period of any willful infringement pursuant to 35 U.S.C. § 284.

204. Plaintiffs are entitled to a finding that this case is exceptional and an award of interest, costs and attorneys' fees incurred by Fate Therapeutics in prosecuting this action as provided by 35 U.S.C. § 285.

205. Plaintiffs are entitled to an award of pre-judgment and post-judgment interest as provided by law.

206. Plaintiffs are entitled to such other and further relief as this Court or a jury may deem just and proper.

SIXTH CLAIM FOR RELIEF

(Infringement of the '917 Patent)

207. Plaintiffs restate and reallege each of the assertions set forth in the paragraphs above.

208. This is a claim for patent infringement and arises under the Patent Laws of the United States and, in particular, under 35 U.S.C. §§ 271, *et seq*.

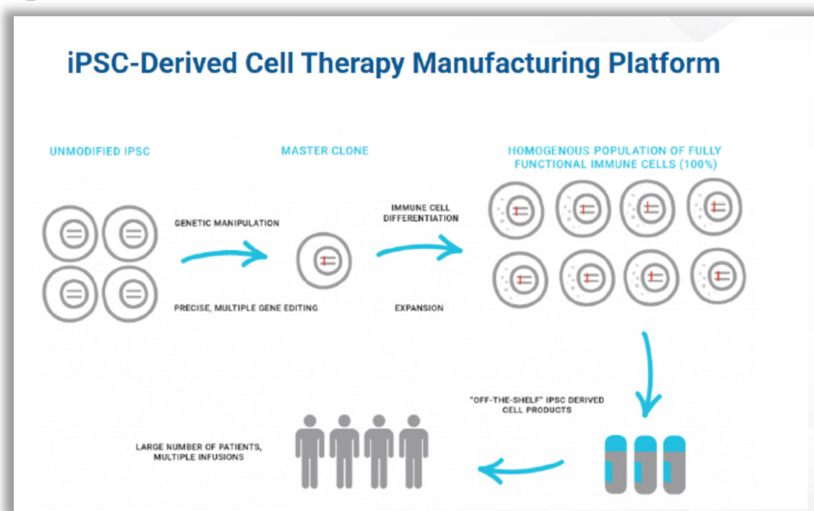
209. Defendants have in the past infringed and continue to infringe the '917 Patent in violation of at least 35 U.S.C. § 271(a) by making, using, offering to sell, and/or selling, in the United States, or importing into the United States induced pluripotent stem cells that infringe at least claim 1 of the '917 Patent without Plaintiffs' authorization or consent.

210. On information and belief, including the information regarding Defendants' use of the infringing induced pluripotent stem cells, Defendants' manufacture, use, offer to sell, or sale of the infringing products was and is not protected by the "safe harbor" provision of 35 U.S.C. § 271(e)(1).

211. Claim 1 of the '917 Patent recites: "A method of making a somatic cell more susceptible to reprogramming to a less differentiated state, comprising: introducing an

exogenous nucleic acid encoding an Oct4 protein operably linked to at least one regulatory sequence into the somatic cell, thereby increasing expression of Oct4 protein in the somatic cell, wherein increased expression of Oct4 protein makes the cell more susceptible to reprogramming; and wherein the exogenous nucleic acid is transiently transfected into the somatic cell."

212. Defendants' use of their "iPSC-derived cell therapy manufacturing platform" infringes at least claim 1 of the '917 Patent. Defendants describe their process for making, iPSC-derived therapies as follows:



213. On information and belief, Defendants manufacture iPSCs from isolated primary somatic cells (*e.g.*, human skin cells or fibroblasts). On information and belief, Defendants transiently transfect the somatic cells with an exogenous nucleic acid (such as cDNA) that encodes an OCT4 protein operably linked to at least one regulatory sequence. Doing so, necessarily increases expression of OCT4 protein in the cell and, in turn, makes the cell more susceptible to reprogramming to a less differentiated (*e.g.*, pluripotent) state.

214. On information and belief, there is no commercially suitable way to make healthy, viable, bona fide human iPSCs other than using isolated primary somatic cells (particularly, *e.g.*, fibroblasts). The iPSCs used in Defendants' manufacturing process thus originate from primary somatic cells.

215. On information and belief, Defendants make iPSCs by introducing, via

transient transfection, nucleic acids (particularly, cDNA) encoding an OCT4 protein operably linked to at least one regulatory sequence into an isolated primary somatic cell (*e.g.*, a fibroblast). This necessarily increases expression of OCT4 protein in the cell and, in turn, makes the cell more susceptible to reprogramming to a less differentiated (*e.g.*, pluripotent) state. On information and belief, it would not have been practical or economical for Defendants to develop a method for making human iPSCs, suitable for their intended purposes of manufacturing healthy, viable immunotherapeutics (*e.g.*, NK cells), by using any method other than the invention disclosed in the '917 Patent, as no such method existed at the time of infringement and would have required expertise and an enormous and lengthy research effort, neither of which were within Defendants' capabilities.

216. OCT4 is the most critical transcription factor for making iPSCs. OCT4 serves as a master regulator, playing an integral role in maintaining pluripotency and establishing the inner cell mass during development. Healthy human iPSCs suitable for producing immunotherapies cannot be made without the introduction of exogenous nucleic acid encoding OCT4. The nucleic acid encoding OCT4 must be operably linked to one or more regulatory elements to affect the expression of the OCT4 transcription factor.

217. Indeed, Fate Therapeutics generates iPSCs through the activation/expression of OCT4 in accordance with the '917 Patent.

218. As a Scientific Advisor for Fate Therapeutics, Dr. Kaufman was aware of Fate Therapeutics' iPSCs, their manufacture, and used such iPSCs in his consultation with Fate Therapeutics.

219. Defendants also infringe the '917 Patent under at least 35 U.S.C. § 271(b).

220. On information and belief, Defendants have been aware of the '917 Patent and that they infringe the '917 Patent since at least May 14, 2020 by virtue of Dr. Kaufman's position as a Scientific Advisor for Fate Therapeutics and the renown of the Asserted Patents in the industry.

221. On information and belief, Defendants intended to induce patent infringement

1 by at least the Advanced Cell Therapy Laboratory of the University of California, San Diego
2 to produce iPSCs claimed by the ’917 Patent and had knowledge that the inducing acts
3 would cause infringement or were willfully blind to the possibility that their inducing acts
4 would cause infringement. Indeed, Defendants prominently advertise on their website that
5 they have “partnered with the Advanced Cell Therapy Laboratory (ACTL) of UC San
6 Diego” to allow it “to bring in-house GMP grade iPSCs to bank and rapidly initiate
7 preclinical development and IND-enabling studies.” **Exhibit J.** Defendants also “leverage
8 an extensive network of leading CMC professionals to guide our manufacturing agenda.”
9 **Exhibit K.**

10 222. On information and belief, Defendants performed acts that constitute
11 inducement of infringement, and would cause actual infringement, with the knowledge of
12 the ’917 Patent. For example, Defendants instructed and/or supervised the Advanced Cell
13 Therapy Laboratory of the University of California, San Diego to produce iPSCs claimed
14 by the ’917 Patent.

15 223. Defendants also infringe under 35 U.S.C. § 271(g) by offering to sell, selling,
16 or using within the United States iPSCs which are made by a process patented in the ’917
17 Patent.

18 224. Specifically, and as further detailed above, iPSCs used by Defendants to make
19 at least the iPSC-derived natural killer (NK) cell platforms are made by a process that
20 comprises at least each step of claim 1 of the ’917 Patent.

21 225. Accordingly, Defendants offers for sale, sales and use of such iPSCs are
22 infringing under § 271(g).

23 226. On information and belief, Dr. Kaufman and Shoreline make iPSCs according
24 to at least the method of the ’917 Patent in their cell therapy manufacturing platform. On
25 information and belief, Dr. Kaufman carried out the infringing manufacture of iPSCs for
26 the benefit of and in his role as an officer, employee, or agent of Shoreline.

27 227. The invention claimed by the ’917 Patent does not require FDA approval for
28

marketing.

228. Defendants' infringing conduct will continue unless enjoined by this Court.

229. On information and belief, Defendants became aware of the '917 Patent prior to their acts of infringement. As a result, the use of the iPSC compositions claimed in the '536 Patent by Defendants was made and will be made with full knowledge of the '917 Patent and without a reasonable basis for believing that Defendants would not be liable for infringing the '917 Patent.

230. Defendants have engaged in deliberate and willful behavior with knowledge of the '917 Patent and knew or should have known that their actions constituted direct and/or indirect infringement of the '917 Patent.

231. Defendants' acts of direct infringement have been, and continue to be, willful and deliberate, and Defendants' acts of indirect infringement were, and continue to be, knowing and intentional.

232. Plaintiffs are entitled to an award of damages adequate to compensate Fate Therapeutics for patent infringement, as well as prejudgment interest from the date the infringement began, but in no event less than a reasonable royalty as permitted by 35 U.S.C. § 284.

233. Plaintiffs are entitled to an award of treble damages for the period of any willful infringement pursuant to 35 U.S.C. § 284.

234. Plaintiffs are entitled to a finding that this case is exceptional and an award of interest, costs and attorneys' fees incurred by Fate Therapeutics in prosecuting this action as provided by 35 U.S.C. § 285.

235. Plaintiffs are entitled to an award of pre-judgment and post-judgment interest as provided by law.

236. Plaintiffs are entitled to such other and further relief as this Court or a jury may deem just and proper.

PRAYER FOR RELIEF

WHEREFORE, Plaintiffs respectfully requests the following relief:

A. A judgment finding that the Asserted Patents have been infringed by Defendants in violation of 35 U.S.C. §271;

B. A judgment finding that Defendants' infringement of the Asserted Patents is willful;

C. An award of damages adequate to compensate Plaintiffs for patent infringement, but in no event less than a reasonable royalty as permitted by 35 U.S.C. § 284;

D. An award of treble damages for the period of any willful infringement pursuant to 35 U.S.C. § 284;

E. A finding that this case is exceptional and an award of interest, costs and attorneys' fees incurred by Fate Therapeutics in prosecuting this action as provided by 35 U.S.C. § 285;

F. An award of pre-judgment and post-judgment interest as provided by law;

G. A permanent injunction as provided by 35 U.S.C. § 283; and

H. Such other and further relief as this Court or a jury may deem just and proper.

DEMAND FOR JURY TRIAL

Pursuant to Fed. R. Civ. P. 38(b), Plaintiffs demand a trial by jury on all issues and claims so triable.

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DATED: May 13, 2022

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EXHIBIT A

(12) **United States Patent**
Jaenisch et al.

(10) **Patent No.: US 8,071,369 B2**
(45) **Date of Patent: *Dec. 6, 2011**

(54) **COMPOSITIONS FOR REPROGRAMMING SOMATIC CELLS**

(75) Inventors: **Rudolf Jaenisch**, Brookline, MA (US); **Konrad Hochedlinger**, Cambridge, MA (US)

(73) Assignee: **Whitehead Institute for Biomedical Research**, Cambridge, MA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **12/703,015**

(22) Filed: **Feb. 9, 2010**

(65) **Prior Publication Data**

US 2010/0221827 A1 Sep. 2, 2010

Related U.S. Application Data

(63) Continuation of application No. 10/997,146, filed on Nov. 24, 2004, now Pat. No. 7,682,828.

(60) Provisional application No. 60/525,612, filed on Nov. 26, 2003, provisional application No. 60/530,042, filed on Dec. 15, 2003.

(51) **Int. Cl.**
C12N 5/00 (2006.01)
C12N 5/07 (2006.01)

(52) **U.S. Cl.** **435/325**; 435/354; 435/366

(58) **Field of Classification Search** 435/325, 435/354, 366

See application file for complete search history.

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Primary Examiner — Marcia S Noble

(74) *Attorney, Agent, or Firm* — Morse, Barnes-Brown & Pendleton, PC; Lisa M. Treannie, Esq.

(57) **ABSTRACT**

The invention provides methods for reprogramming somatic cells to generate multipotent or pluripotent cells. Such methods are useful for a variety of purposes, including treating or preventing a medical condition in an individual. The invention further provides methods for identifying an agent that reprograms somatic cells to a less differentiated state.

9 Claims, 2 Drawing Sheets

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Figure 1. Inducible *Oct4* allele

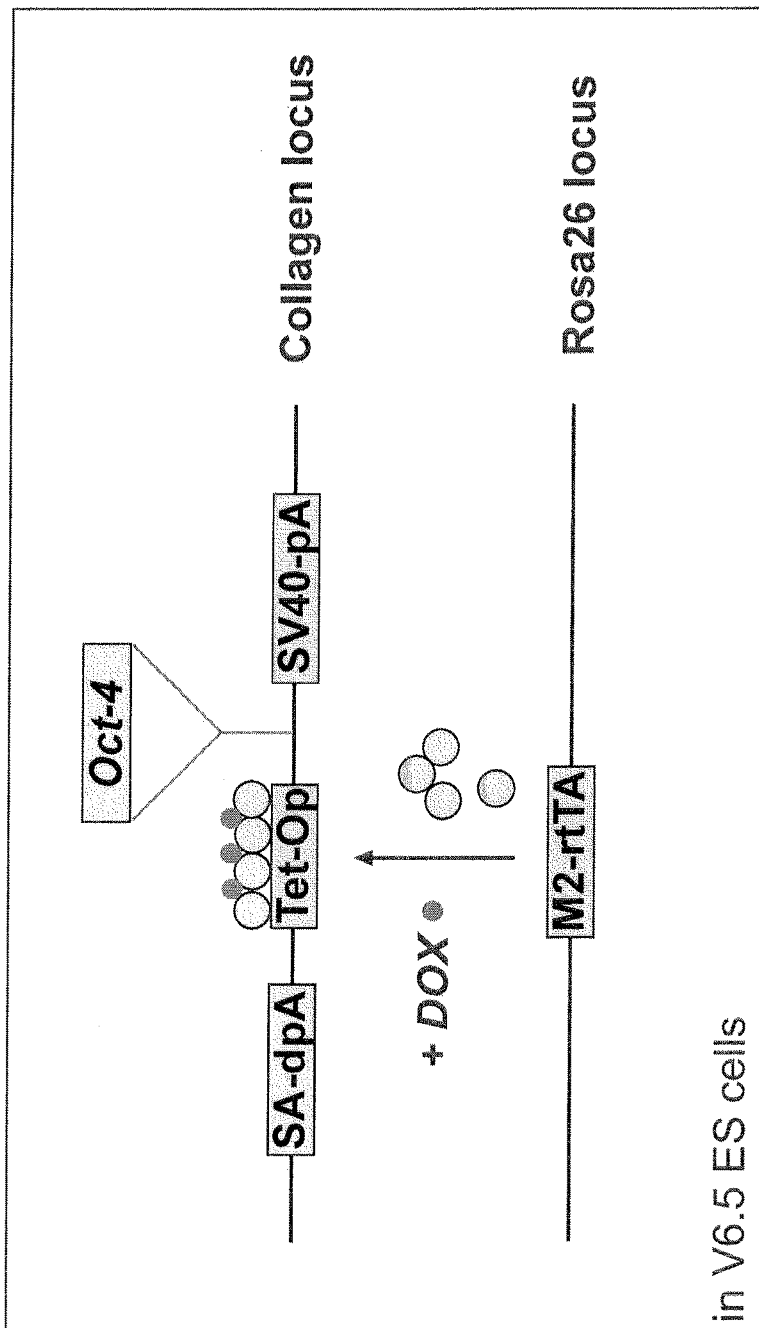

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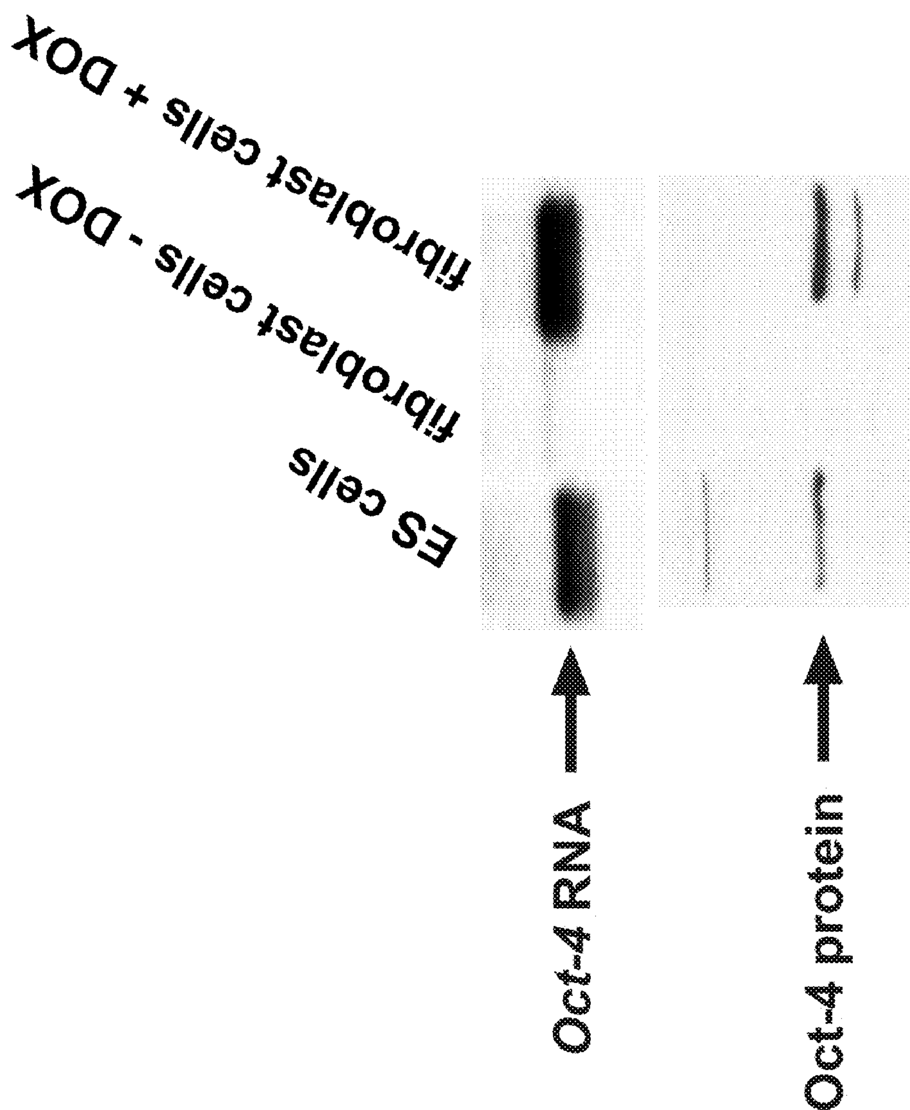
Figure 2. The system works...



COMPOSITIONS FOR REPROGRAMMING SOMATIC CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 10/997,146, filed Nov. 24, 2004, now U.S. Pat. No 7,682, 828 which claims the benefit of U.S. Provisional Application No. 60/525,612, filed Nov. 26, 2003, and U.S. Provisional Application No. 60/530,042, filed Dec. 15, 2003, the specifications of which are incorporated herein by reference in their entirety.

GOVERNMENT FUNDING

The invention described herein was supported, in whole or in part, by Grant R37 CA84198 from the National Institutes of Health. The United States government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Pluripotent stem cells have the potential to differentiate into the full range of daughter cells having distinctly different morphological, cytological or functional phenotypes unique to a specific tissue. By contrast, descendants of pluripotent cells are restricted progressively in their differentiation potential, with some cells having only one fate. Pluripotent cells have extraordinary scientific and therapeutic potential, as they can be differentiated along the desired differentiation pathway in a precisely controlled manner and used in cell-based therapy.

Two categories of pluripotent stem cells are known to date: embryonic stem cells and embryonic germ cells. Embryonic stem cells are pluripotent stem cells that are derived directly from an embryo. Embryonic germ cells are pluripotent stem cells that are derived directly from the fetal tissue of aborted fetuses. For purposes of simplicity, embryonic stem cells and embryonic germ cells will be collectively referred to as "ES" cells herein.

ES cells are presently obtained via several methods. In a first method, an ES cell line is derived from the inner cell mass of a normal embryos in the blastocyst stage (See U.S. Pat. No. 6,200,806, Thompson, J. A. et al. Science, 282:1145-7, 1998 and Hogan et al., 2003). A second method for creating pluripotent ES cells utilizes the technique of somatic cell nuclear transfer (SCNT). In this technique, the nucleus is removed from a normal egg, thus removing the genetic material. Next, a donor diploid somatic cell is placed next to the enucleated egg and the two cells are fused, or the nucleus is introduced directly into the oocyte by micromanipulation. The fused cell has the potential to develop into a viable embryo, which may then be sacrificed to remove that portion of the embryo containing the stem cell producing inner cell mass.

In a third method, the nucleus of a human cell is transplanted into an entirely enucleated animal oocyte of a species different from the donor cell (referred to herein as animal stem cell nuclear transfer, or "ASCNT"). See U.S. Pat. application Ser. No. 20010012513 (2001). The resultant chimeric cells are used for the production of pluripotent ES cells, in particular human-like pluripotent ES cells. One disadvantage of this technique is that these chimeric cells may contain unknown non-human viruses and still contain the mitochondria of the animal species. Thus, there would be substantial risks of immune rejection if such cells were used in cell transplantation therapies.

In a fourth method, ES cells can be isolated from the primordial germ cells found in the genital ridges of post-implanted embryos.

As described above, all presently available methods depend on controversial sources—embryos (either created naturally or via cloning), fetal tissue and via the mixing of materials of multiple species. The controversy surrounding the sources for such cells, according to many leading scientists and public and private organizations including the NIH, has greatly compromised and slowed the study of such cells and their application.

There is thus a great demand for alternative methods of generating pluripotent cells.

SUMMARY OF THE INVENTION

The present invention provides engineered somatic cells, in which one or more endogenous pluripotency gene(s) is operably linked to a selectable marker in such a manner that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene to which the marker is linked. The invention also provides transgenic mice containing these engineered somatic cells.

The present invention also provides methods for reprogramming somatic cells to a less differentiated state. In the methods, engineered somatic cells of the invention are treated with an agent. Cells that express the selectable marker are then selected, and assessed for pluripotency characteristics. The treatment with an agent may be contacting the cells with an agent which alters chromatin structure, or may be transfecting the cells with at least one pluripotency gene, or both.

The present invention further provides methods for identifying an agent that reprograms somatic cells to a less differentiated state. In the methods, the engineered somatic cells described above are contacted with a candidate agent. Cells that express the selectable marker are then selected, and assessed for pluripotency characteristics. The presence of at least a subset of pluripotency characteristics indicates that the agent is capable of reprogramming somatic cells to a less-differentiated state. The agents identified by the present invention can then by used to reprogram somatic cells by contacting somatic cells with the agents.

The present invention also provides methods for identifying a gene that causes the expression of at least one endogenous pluripotency gene in somatic cells. In the methods, the engineered somatic cells are transfected with a cDNA library prepared from a pluripotent cell, such as an ES cell. The cells that express the appropriate selectable marker are then selected, and the expression of the appropriate endogenous pluripotency gene is examined. The expression of an endogenous pluripotency gene indicates that the cDNA encodes a protein whose expression in the cell results in, directly or indirectly, expression of the endogenous pluripotency gene.

The present invention further provides methods for treating a condition in an individual in need of such treatment. In certain embodiments, somatic cells are obtained from the individual and reprogrammed by the methods of the invention under conditions suitable for the cells to develop into cells of a desired cell type. The reprogrammed cells of a desired cell type are then harvested and introduced into the individual to treat the condition. In certain further embodiments, the somatic cells obtained from the individual contains a mutation in one or more genes. In these instances, the methods are modified so that the somatic cells obtained from the individual are first treated to restore the one or more normal gene(s) to the cells such that the resulting cells carry the normal endogenous gene, which are then introduced into the

individual. In certain other embodiments, methods of the invention can be used to treat individuals in need of a functional organ. In the methods, somatic cells are obtained from an individual in need of a functional organ, and reprogrammed by the methods of the invention to produce reprogrammed somatic cells. Such reprogrammed somatic cells are then cultured under conditions suitable for development of the reprogrammed somatic cells into a desired organ, which is then introduced into the individual. The methods are useful for treating any one of the following conditions: a neurological, endocrine, structural, skeletal, vascular, urinary, digestive, integumentary, blood, autoimmune, inflammatory, or muscular condition.

The present invention also provides methods for producing a cloned animal. In the methods, a somatic cell is isolated from an animal having desired characteristics, and reprogrammed using the methods of the invention to produce one or more reprogrammed pluripotent somatic cell ("RPSC"). The RPSCs are then inserted into a recipient embryo, and the resulting embryo is cultured to produce an embryo of suitable size for implantation into a recipient female, which is then transferred into a recipient female to produce a pregnant female. The pregnant female is maintained under conditions appropriate for carrying the embryo to term to produce chimeric animal progeny, which is then bred with a wild type animal to produce a cloned animal.

In certain embodiments, the RPSCs may alternatively be cryopreserved for future cloning uses. In certain other embodiments, genetic modification, such as a targeted mutation, may be introduced into the RPSCs prior to its insertion into a recipient embryo.

The present invention also provides methods for producing a cloned avian. In the methods, a somatic cell is isolated from an avian having desired characteristics, and reprogrammed using the methods of the invention to produce one or more reprogrammed pluripotent somatic cell ("RPSC"). The RPSCs are then inserted into eggs that are unable to develop into an embryo, and the resulting eggs are then incubated to produce avian offspring having the genotype of the RPSC, thereby producing a cloned avian.

It is contemplated that all embodiments described above are applicable to all different aspects of the invention. It is also contemplated that any of the above embodiments can be freely combined with one or more other such embodiments whenever appropriate.

Specific embodiments of the invention are described in more detail below. However, these are illustrative embodiments, and should not be construed as limiting in any respect.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts an inducible Oct4 allele.

FIG. **2** shows the expression of the inducible Oct4 transgene by Northern blot and Western blot analysis.

DETAILED DESCRIPTION OF THE INVENTION

Overview

Presently, human ES cells or ES-like cells can only be generated from controversial sources. It would be useful to reprogram somatic cells directly into pluripotent cells. Nuclei from somatic cells retain the totopotency potential to direct development of an animal, as demonstrated by nuclear transfer technology. It would be useful to reprogram somatic cells directly into ES cells without the use of oocytes and nuclear transfer technology.

Applicants have devised novel methods of reprogramming somatic cells to generate pluripotent cells or multipotent cells. Applicants have also devised novel methods to identify agents that reprogram somatic cells. The methods take advantage of the engineered somatic cells designed by Applicants, in which an endogenous gene typically associated with pluripotency ("pluripotency gene") is engineered to be operably linked to a selectable marker in a manner that the expression the endogenous pluripotency gene substantially matches the expression of the selectable marker. Because pluripotency genes are generally expressed only in pluripotent cells and not in somatic cells, the expression of an endogenous pluripotent gene(s) is an indication of successful reprogramming. Having a selectable marker operably linked to an endogenous pluripotency gene gives one a powerful mechanism to select for potentially reprogrammed somatic cells, which likely is a rare occurrence. The resulting cells may be further assessed for pluripotency characteristics to confirm whether a somatic cell has been successfully reprogrammed to pluripotency.

Generating pluripotent or multipotent cells by somatic cell reprogramming using the methods of the present invention has at least two advantages. First, the methods of the present invention allow one to generate autologous pluripotent cells, which are cells specific to a patient. The use of autologous cells in cell therapy offers a major advantage over the use of non-autologous cells, which are likely to be subject to immunological rejection. In contrast, autologous cells are unlikely to elicit significant immunological responses (See Munsie et al, 2000). Second, the methods of the present invention allow one to generate pluripotent without using embryos, oocytes and/or nuclear transfer technology.

A pluripotent cell is a cell that has the potential to divide in vitro for a long period of time (greater than one year) and has the unique ability to differentiate into cells derived from all three embryonic germ layers—endoderm, mesoderm and ectoderm.

A multipotent cell is a cell that is able to differentiate into some but not all of the cells derived from all three germ layers. Thus, a multipotent cell is a partially differentiated cell. Adult stem cells are multipotent cells. Known adult stem cells include, for example, hematopoietic stem cells and neural stem cells. A hematopoietic stem cell is multipotent because it has the ability to differentiate into all types of specific blood cells, but it is unlikely that they can differentiate into all cells of a given animal or human. Multipotent/adult stem cells have a great deal of promise in research and in the area of therapeutic applications. For example, multipotent/adult stem cells have already been used in humans in attempts to treat certain blood, neural and cancer diseases.

The term "pluripotency gene", as used herein, refers to a gene that is associated with pluripotency. The expression of a pluripotency gene is typically restricted to pluripotent stem cells, and is crucial for the functional identity of pluripotent stem cells. The transcription factor Oct-4 (also called Pou5fl, Oct-3, Oct3/4) is an example of a pluripotency gene. Oct-4 has been shown to be required for establishing and maintaining the undifferentiated phenotype of ES cells and plays a major role in determining early events in embryogenesis and cellular-.differentiation (Nichols et al., 1998, Cell 95:379-391; Niwa et al., 2000, Nature Genet. 24:372-376). Oct-4 is down-regulated as stem cells differentiate into specialised cells. Other exemplary pluripotency genes include Nanog, and Stella (See Chambers et al., 2003, Cell 113: 643-655; Mitsui et al., Cell. 2003, 113(5):631-42; Bortvin et al. Development. 2003, 130(8):1673-80; Saitou et al., Nature. 2002, 418 (6895):293-300.

Engineered Somatic Cells and Transgenic Mice Comprising Such Cells

The present invention provides somatic cells comprising an endogenous pluripotency gene linked to DNA encoding a selectable marker in such a manner that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene. In one embodiment, the somatic cells of the present invention comprise a first endogenous pluripotency gene linked to DNA encoding a first selectable marker in such a manner that the expression of the first selectable marker substantially matches the expression of the first endogenous pluripotency gene. The somatic cells may also be engineered to comprise any number of endogenous pluripotency genes respectively linked to a distinct selectable marker. Thus, in another embodiment, the somatic cells of the present invention comprise two endogenous pluripotency genes, each of which is linked to DNA encoding a distinct selectable marker. In a further embodiment, the somatic cells of the present invention comprise three endogenous pluripotency genes, each of which is linked to DNA encoding a distinct selectable marker. The somatic cells described above will be collectively referred in this application as "engineered somatic cells." The engineered somatic cells may be further engineered to have one or more pluripotency gene expressed as a transgene under an inducible promoter.

The selectable marker is linked to an appropriate endogenous pluripotency gene such that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene. By "substantially match", it is meant that the expression of the selectable marker substantially reflects the expression pattern of the endogenous pluripotency gene. In other words, the selectable marker and the endogenous pluripotency gene are co-expressed. For purpose of the present invention, it is not necessary that the expression level of the endogenous gene and the selectable marker is the same or even similar. It is only necessary that the cells in which an endogenous pluripotency gene is activated will also express the selectable marker at a level sufficient to confer a selectable phenotype on the reprogrammed cells. For example, when the selectable marker is a marker that confers resistance to a lethal drug (a "drug resistance marker"), the cells are engineered in a way that allows cells in which an endogeneous pluripotency gene is activated to also express the drug resistance marker at a sufficient level to confer on reprogrammed cells resistance to lethal drugs. Thus, reprogrammed cells will survive and proliferate whereas non-reprogrammed cells will die.

The DNA encoding a selectable marker may be inserted downstream from the end of the open reading frame (ORF) encoding the desired endogenous pluripotency gene, anywhere between the last nucleotide of the ORF and the first nucleotide of the polyadenylation site. An internal ribosome entry site (IRES) may be placed in front of the DNA encoding the selectable marker. Alternatively, the DNA encoding a selectable marker may be inserted anywhere within the ORF of the desired endogenous pluripotency gene, downstream of the promoter, with a termination signal. An internal ribosome entry site (IRES) may be placed in front of the DNA encoding the selectable marker. The selectable marker may be inserted into only one allele, or both alleles, of the endogenous pluripotency gene.

The somatic cells in the invention may be primary cells or immortalized cells. Such cells may be primary cells (non-immortalized cells), such as those freshly isolated from an animal, or may be derived from a cell line (immortalized cells).

The somatic cells in the present invention are mammalian cells, such as, for example, human cells or mouse cells. They may be obtained by well-known methods, from different organs, e.g., skin, lung, pancreas, liver, stomach, intestine, heart, reproductive organs, bladder, kidney, urethra and other urinary organs, etc., generally from any organ or tissue containing live somatic cells. Mammalian somatic cells useful in the present invention include, by way of example, adult stem cells, sertoli cells, endothelial cells, granulosa epithelial, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc. generally any live somatic cells. The term "somatic cells", as used herein, also includes adult stem cells. An adult stem cell is a cell that is capable of giving rise to all cell types of a particular tissue. Exemplary adult stem cells include hematopoietic stem cells, neural stem cells, and mesenchymal stem cells.

In one embodiment, the engineered somatic cells are obtained from a transgenic mouse comprising such engineered somatic cells. Such transgenic mouse can be produced using standard techniques known in the art. For example, Bronson et al. describe a technique for inserting a single copy of a transgene into a chosen chromosomal site. See Bronson et al., 1996. Briefly, a vector containing the desired integration construct (for example, a construct containing a selectable marker linked to a pluripotency gene) is introduced into ES cells by standard techniques known in the art. The resulting ES cells are screened for the desired integration event, in which the knock-in vector is integrated into the desired endogenous pluripotency gene locus such that the selectable marker is integrated into the genomic locus of the pluripotency gene and is under the control of the pluripotency gene promoter. The desired ES cell is then used to produce transgenic mouse in which all cell types contain the correct integration event. Desired types of cells may be selectively obtained from the transgenic mouse and maintained in vitro. In one embodiment, two or more transgenic mice may be created, each carrying a distinct integration construct. These mice may then be bred to generate mice that carry multiple desired integration construct. For example, one type of transgenic mouse may be created to carry an endogenous pluripotency gene linked to a selectable marker, while a second type of transgenic mouse may be created to carry a pluripotency gene expressed as a transgene under an inducible promoter. These two types of mice may then be bred to generate transgenic mice that have both a selectable marker linked to an endogenous pluripotency gene and an additional pluripotency gene expressed as a transgene under an inducible promoter. These two pluripotency genes may or may not be the same. Many variables are contemplated: the identity of the endogenous pluripotency gene linked to marker, the identity of the pluripotency gene expressed as a transgene, and the number of the endogenous pluripotency gene linked to a selectable marker, and the number of pluripotency gene expressed as a transgene. The present invention encompasses all possible combinations of these variables.

Alternatively, engineered somatic cells of the present invention may be produced by direct introduction of the desired construct into somatic cells. DNA construct may be introduced into cells by any standard technique known in the art, such as viral transfection (eg. using an adenoviral system) or liposome-mediated transfection. Any means known in the art to generate somatic cells with targeted integration can be used to produce somatic cells of the invention. In mammalian

cells, homologous recombination occurs at much lower frequency compared to non-homologous recombination. To facilitate the selection of homologous recombination events over the non-homologous recombination events, at least two enrichment methods have been developed: the positive-negative selection (PNS) method and the "promoterless" selection method (Sedivy and Dutriaux, 1999). Briefly, PNS, the first method, is in genetic terms a negative selection: it selects against recombination at the incorrect (non-homologous) loci by relying on the use of a negatively selectable gene that is placed on the flanks of a targeting vector. On the other hand, the second method, the "promoterless" selection, is a positive selection in genetic terms: it selects for recombination at the correct (homologous) locus by relying on the use of a positively selectable gene whose expression is made conditional on recombination at the homologous target site. The disclosure of Sedivy and Dutriaux is incorporated herein.

A selectable marker, as used herein, is a marker that, when expressed, confers upon recipient cells a selectable phenotype, such as antibiotic resistance, resistance to a cytotoxic agent, nutritional prototrophy or expression of a surface protein. The presence of a selectable marker linked to an endogenous pluripotency gene makes it possible to identify and select reprogrammed cells in which the endogenous pluripotency gene is expressed. A variety of selectable marker genes can be used, such as neomycin resistance gene (neo), puromycin resistance gene (puro), guanine phosphoribosyl transferase (gpt), dihydrofolate reductase (DHFR), adenosine deaminase (ada), puromycin-N-acetyltransferase (PAC), hygromycin resistance gene (hyg), multidrug resistance gene (mdr), and hisD gene.

The present invention further provides transgenic mice comprising the somatic cells of the invention.

Methods for Reprogramming Somatic Cells

The present invention further provides methods for reprogramming somatic cells to a less differentiated state. The resulting cells are termed "reprogrammed somatic cells" ("RSC") herein. A RSC may be a reprogrammed pluripotent somatic cell ("RPSC"), a reprogrammed multipotent somatic cell ("RMSC"), or a reprogrammed somatic cell of varying differentiation status.

In general, the methods comprise treating the engineered somatic cells with an agent. The treatment with an agent may be contacting the cells with an agent which alters chromatin structure, or may be transfecting the cells with one or more pluripotency gene, or both. The above two treatments may be concurrent, or may be sequential, with no particular preference for order. In a further embodiment, reprogrammed somatic cells are identified by selecting for cells that express the appropriate selectable marker. In still a further embodiment, reprogrammed somatic cells are further assessed for pluripotency characteristics. The presence of pluripotency characteristics indicates that the somatic cells have been reprogrammed to a pluripotent state.

Differentiation status of cells is a continuous spectrum, with terminally differentiated state at one end of this spectrum and de-differentiated state (pluripotent state) at the other end. Reprogramming, as used herein, refers to a process that alters or reverses the differentiation status of a somatic cell, which can be either partially or terminally differentiated. Reprogramming includes complete reversion, as well as partial reversion, of the differentiation status of a somatic cell. In other words, the term "reprogramming", as used herein, encompasses any movement of the differentiation status of a cell along the spectrum toward a less-differentiated state. For example, reprogramming includes reversing a multipotent cell back to a pluripotent cell, reversing a terminally differentiated cell back to either a multipotent cell or a pluripotent cell. In one embodiment, reprogramming of a somatic cell turns the somatic cell all the way back to a pluripotent state. In another embodiment, reprogramming of a somatic cell turns the somatic cell back to a multipotent state. The term "less-differentiated state", as used herein, is thus a relative term and includes a completely de-differentiated state and a partially differentiated state.

The term "pluripotency characteristics", as used herein, refers to many characteristics associated with pluripotency, including, for example, the ability to differentiate into all types of cells and an expression pattern distinct for a pluripotent cell, including expression of pluripotency genes, expression of other ES cell markers, and on a global level, a distinct expression profile known as "stem cell molecular signature" or "sternness."

Thus, to assess reprogrammed somatic cells for pluripotency characteristics, one may analyze such cells for different growth characteristics and ES cell-like morphology. Cells may be injected subcutaneously into immunocompromised SCID mice to induce teratomas (a standard assay for ES cells). ES-like cells can be differentiated into embryoid bodies (another ES specific feature). Moreover, ES-like cells can be differentiated in vitro by adding certain growth factors known to drive differentiation into specific cell types. Self-renewing capacity, marked by induction of telomerase activity, is another pluripotency characteristics that can be monitored. One may carry out functional assays of the reprogrammed somatic cells by introducing them into blastocysts and determine whether the cells are capable of giving rise to all cell types. See Hogan et al., 2003. If the reprogrammed cells are capable of forming a few cell types of the body, they are multipotent; if the reprogrammed cells are capable of forming all cell types of the body including germ cells, they are pluripotent.

One may also examine the expression of an individual pluripotency gene in the reprogrammed somatic cells to assess their pluripotency characteristics. Additionally, one may assess the expression of other ES cell markers. Stage-specific embryonic 1 5 antigens-1, -3, and -4 (SSEA-1, SSEA-3, SSEA-4) are glycoproteins specifically expressed in early embryonic development and are markers for ES cells (Solter and Knowles, 1978, Proc. Natl. Acad. Sci. USA 75:5565-5569; Kannagi et al., 1983, EMBO J 2:2355-2361). Elevated expression of the enzyme Alkaline Phosphatase (AP) is another marker associated with undifferentiated embryonic stem cells (Wobus et al., 1984, Exp. Cell 152:212-219; Pease et al., 1990, Dev. Biol. 141:322-352). Other stem/progenitor cells markers include the intermediate neurofilament nestin (Lendahl et al., 1990, Cell 60:585-595; Dah-Istrand et al., 1992, J.

Cell Sci. 103:589-597), the membrane glycoprotein prominin/AC133 (Weigmann et al., 1997, Proc. Natl. Acad. USA 94:12425-12430; Corbeil et al., 1998, Blood 91:2625-22626), the transcription factor Tcf-4 (Korinek et al, 1998, Nat. Genet. 19: 379-383; Lee et al., 1999, J. Biol. Chem. 274.1 566-1 572), and the transcription factor Cdx1 (Duprey et al., 1988, Genes Dev. 2:1647-1654; Subramania'n et al., 1998, Differentiation 64:11-1 8).

One may additionally conduct expression profiling of the reprogrammed somatic cells to assess their pluripotency characteristics. Pluripotent cells, such as embryonic stem cells, and multipotent cells, such as adult stem cells, are known to have a distinct pattern of global gene expression profile. This distinct pattern is termed "stem cell molecular

signature", or "stemness". See, for example, Ramalho-Santos et al., Science 298: 597-600 (2002); Ivanova et al., Science 298: 601-604.

Somatic cells may be reprogrammed to gain either a complete set of the pluripotency characteristics and are thus pluripotent. Alternatively, somatic cells may be reprogrammed to gain only a subset of the pluripotency characteristics. In another alternative, somatic cells may be reprogrammed to be multipotent.

In a further embodiment, in conjunction with contacting the somatic cells of the invention with an agent which alters chromatin structure, at least one gene that affects pluripotent state of a cell may be further introduced into the same cells. This may be carried out sequentially. For example, the somatic cells of the invention may be first contacted with an agent which alters chromatin structure. Then at least one pluripotency gene can be introduced into the same cells, or vice versa. Alternatively, the two steps may be carried out simultaneously.

Genes that affect pluripotent state of a cell includes pluripotency genes, genes involved in chromatin remodeling, and genes that are important for maintaining pluripotency, such as LIF, BMP, and PD098059 (See Cell, 115: 281-292 (2003); Philos Trans R Soc Lond B Biol Sci. 2003 Aug. 29; 358 (1436):1397-402).

The exogenously introduced pluripotency gene may be carried out in several ways. In one embodiment, the exogenously introduced pluripotency gene may be expressed from a chromosomal locus different from the endogenous chromosomal locus of the pluripotency gene. Such chromosomal locus may be a locus with open chromatin structure, and contain gene(s) dispensible for a somatic cell. In other words, the desirable chromosomal locus contains gene(s) whose disruption will not cause cells to die. Exemplary chromosomal loci include, for example, the mouse ROSA 26 locus and type II collagen (Col2a1) locus (See Zambrowicz et al., 1997) The exogenously introduced pluripotency gene may be expressed from an inducible promoter such that their expression can be regulated as desired.

In an alternative embodiment, the exogenously introduced pluripotency gene may be transiently transfected into cells, either individually or as part of a cDNA expression library, prepared from pluripotent cells. Such pluripotent cells may be embryonic stem cells, oocytes, blastomeres, inner cell mass cells, embryonic germ cells, embryoid body (embryonic) cells, morula-derived cells, teratoma (teratocarcinoma) cells, and multipotent partially differentiated embryonic stem cells taken from later in the embryonic development process.

The cDNA library is prepared by conventional techniques. Briefly, mRNA is isolated from an organism of interest. An RNA-directed DNA polymerase is employed for first strand synthesis using the mRNA as template. Second strand synthesis is carried out using a DNA-directed DNA polymerase which results in the cDNA product. Following conventional processing to facilitate cloning of the cDNA, the cDNA is inserted into an expression vector such that the cDNA is operably linked to at least one regulatory sequence. The choice of expression vectors for use in connection with the cDNA library is not limited to a particular vector. Any expression vector suitable for use in mouse cells is appropriate. In one embodiment, the promoter which drives expression from the cDNA expression construct is an inducible promoter. The term regulatory sequence includes promoters, enhancers and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology*, Academic Press, San Diego, Calif. (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express cDNAs. Such useful expression control sequences, include, for example, the early and late promoters of SV40, tet promoter, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

The exogenously introduced pluripotency gene may be expressed from an inducible promoter. The term "inducible promoter", as used herein, refers to a promoter that, in the absence of an inducer (such as a chemical and/or biological agent), does not direct expression, or directs low levels of expression of an operably linked gene (including cDNA), and, in response to an inducer, its ability to direct expression is enhanced. Exemplary inducible promoters include, for example, promoters that respond to heavy metals (CRC Boca Raton, Fla. (1991), 167-220; Brinster et al. Nature (1982), 296, 39-42), to thermal shocks, to hormones (Lee et al. P.N.A.S. USA (1988), 85, 1204-1208; (1981), 294, 228-232; Klock et al. Nature (1987), 329, 734-736; Israel and Kaufman, Nucleic Acids Res. (1989), 17, 2589-2604), promoters that respond to chemical agents, such as glucose, lactose, galactose or antibiotic.

A tetracycline-inducible promoter is an example of an inducible promoter that responds to an antibiotics. See Gossen et al., 2003. The tetracycline-inducible promoter comprises a minimal promoter linked operably to one or more tetracycline operator(s). The presence of tetracycline or one of its analogues leads to the binding of a transcription activator to the tetracycline operator sequences, which activates the minimal promoter and hence the transcription of the associated cDNA. Tetracycline analogue includes any compound that displays structural homologies with tetracycline and is capable of activating a tetracycline-inducible promoter. Exemplary tetracycline analogues includes, for example, doxycycline, chlorotetracycline and anhydrotetracycline.

Thus, in one embodiment, the present invention provides mice and somatic cells carrying at least one pluripotency gene expressed as a transgene under an inducible promoter. It is possible that somatic cells with such inducible pluripotency transgene(s) are more prone to be reprogrammed.

Any of the engineered somatic cells of the present invention may be used in the methods. In one embodiment, somatic cells used in the methods comprise only one endogenous pluripotency gene linked to a first selectable marker, and the selection step is carried out to select for the expression of the first selectable marker. In an alternative embodiment, the somatic cells used in the methods comprise any number of endogenous pluripotency genes, each of which is linked to a distinct selectable marker respectively, and the selection step is carried out to select for at least a subset of the selectable markers. For example, the selection step may be carried out to

select for all the selectable markers linked to the various endogenous pluripotency genes.

In an alternative embodiment, somatic cells used in the method comprise a selectable marker linked to an endogenous pluripotency gene and an additional pluripotency gene expressed as a transgene under an inducible promoter. For these cells, the method of reprogramming may comprises induce the expression of the pluripotency transgene and select for the expression of the selectable marker. The method may further comprise contacting the somatic cells with an agent that alter chromatic structure.

Without wishing to be bound by theory, the agents used in the method may cause chromatin to take on a more open structure, which is more permissive for gene expression. DNA methylation and histone acetylation are two known events that alter chromatin toward a more closed structure. For example, loss of methylation by genetic deletion of DNA methylation enzyme Dnmt1 in fibroblasts results in reactivation of endogenous Oct4 gene. See J. Biol. Chem. 277: 34521-30, 2002; and Bergman and Mostoslaysky, Biol. Chem. 1990. Thus, DNA methylation inhibitors and histone deacetyation inhibitors are two classes of agents that may be used in the methods of the invention. Exemplary agents include 5-aza-cytidine, TSA and valproic acid.

In another embodiment, methods of the invention may further include repeating the steps of treating the cells with an agent. The agent used in the repeating treatment may be the same as, or different from, the one used during the first treatment.

Methods for Screening for an Agent that Reprograms Somatic Cells

The present invention also provides methods for identifying an agent that reprograms somatic cells to a less-differentiated state, as well as the agents thus identified. In one embodiment, the methods comprise contacting the engineered somatic cells of the invention with a candidate agent, selecting for cells that express the appropriate selectable marker. The presence of cells that express the appropriate selectable marker indicates that the agent reprograms somatic cells. Such an agent is referred as a "reprogramming agent" for purpose of this application.

In a further embodiment, the methods comprise contacting the engineered somatic cells of the invention with a candidate agent, selecting for cells that express the appropriate selectable marker, and assessing the cells so selected for pluripotency characteristics. The presence of a complete set of pluripotency characteristics indicates that the agent reprograms somatic cells to become pluripotent.

Candidate agents used in the invention encompass numerous chemical classes, though typically they are organic molecules, including small organic compounds. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, nucleic acids and derivatives, structural analogs or combinations thereof.

Candidate agents may be naturally arising, recombinant or designed in the laboratory. The candidate agents may be isolated from microorganisms, animals, or plants, or may be produced recombinantly, or synthesized by chemical methods known in the art. In some embodiments, candidate agents are isolated from libraries of synthetic or natural compounds using the methods of the present invention. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, including acylation, alkylation, esterification, amidification, to produce structural analogs.

There are numerous commercially available compound libraries, including, for example, the Chembridge DIVERSet. Libraries are also available from academic investigators, such as the Diversity set from the NCI developmental therapeutics program.

The screening methods mentioned above are based on assays performed on cells. These cell-based assays may be performed in a high throughput screening (HTS) format, which has been described in the art. For example, Stockwell et al. described a high-throughput screening of small molecules in miniaturized mammalian cell-based assays involving post-translational modifications (Stockwell et al., 1999). Likewise, Qian et al. described a leukemia cell-based assay for high-throughput screening for anti-cancer agents (Qian et al., 2001). Both references are incorporated herein in their entirety.

A reprogramming agent may belong to any one of many different categories. For example, a reprogramming agent may be a chromatin remodeling agent. A chromatin remodeling agent may be a protein involved in chromatin remodeling or an agent known to alter chromatin toward a more open structure, such as a DNA methylation inhibitor or a histone deacetyation inhibitor. Exemplary compounds include 5-aza-cytidine, TSA and valproic acid. For another example, such an agent may be a pluripotency protein, including, for example, Nanog, Oct-4 and Stella. Such an agent may also be a gene essential for pluripotency, including, for example, Sox2, FoxD3, and LIF, and Stat3. See Smith et al. 1988, William et al., 1988, Ihle, 1996, Avilion et al., 2003, and Hanna et al., 2002)

Methods for Reprogramming Somatic Cells with a Reprogramming Agent

The reprogramming agent identified by the methods of the present invention is useful for reprogramming somatic cells into pluripotent or multipotent cells. Accordingly, the present invention provides methods for reprogramming somatic cells to a less differentiated state, comprising contacting somatic cells with a reprogramming agent. The somatic cells used may be native somatic cells, or engineered somatic cells. It is not necessary for these cells to carry a selectable marker integrated into the endogenous locus of a pluripotency gene.

Reprogrammed Somatic Cells and These Uses

The present invention also provides reprogrammed somatic cells (RSCs), including reprogrammed pluripotent somatic cells (RPSCs), produced by the methods of the invention. These methods, useful for the generation of cells of a desired cell type, have wide range of applications. For one example, these methods have applications in livestock management, involving the precise genetic manipulation of animals for economic or health purposes. For another example, these methods have medical application in treating or preventing a condition.

Accordingly, the invention provides methods for the treatment or prevention of a condition in a mammal. In one embodiment, the methods start with obtaining somatic cells from the individual, reprogramming the somatic cells so obtained by methods of the present invention to obtain RPSCs. The RPSCs are then cultured under conditions suitable for development of the RPSCs into cells of a desired cell

type. The developed cells of the desired cell type are harvested and introduced into the individual to treat the condition. In an alternative embodiment, the methods start with obtaining somatic cells from the individual, reprogramming the somatic cells so obtained by methods of the present invention. The RPSCs are then cultured under conditions suitable for development of the RPSCs into a desired organ, which is harvested and introduced into the individual to treat the condition.

The RPSCs of the present invention are ES-like cells, and thus may be induced to differentiate to obtain the desired cell types according to known methods to differentiate ES cells. For example, the RPSCs may be induced to differentiate into hematopoietic stem cells, muscle cells, cardiac muscle cells, liver cells, cartilage cells, epithelial cells, urinary tract cells, etc., by culturing such cells in differentiation medium and under conditions which provide for cell differentiation. Medium and methods which result in the differentiation of embryonic stem cells are known in the art as are suitable culturing conditions.

For example, Palacios et al., Proc. Natl. Acad. Sci., USA, 92: 7530-37 (1995) teaches the production of hematopoietic stem cells from an embryonic cell line by subjecting stem cells to an induction procedure comprising initially culturing aggregates of such cells in a suspension culture medium lacking retinoic acid followed by culturing in the same medium containing retinoic acid, followed by transferral of cell aggregates to a substrate which provides for cell attachment.

Moreover, Pedersen, J. Reprod. Fertil. Dev., 6: 543-52 (1994) is a review article which references numerous articles disclosing methods for in vitro differentiation of embryonic stem cells to produce various differentiated cell types including hematopoietic cells, muscle, cardiac muscle, nerve cells, among others.

Further, Bain et al., Dev. Biol., 168:342-357 (1995) teaches in vitro differentiation of embryonic stem cells to produce neural cells which possess neuronal properties. These references are exemplary of reported methods for obtaining differentiated cells from embryonic or stem-like cells. These references and in particular the disclosures therein relating to methods for differentiating embryonic stem cells are incorporated by reference in their entirety herein.

Thus, using known methods and culture medium, one skilled in the art may culture the subject embryonic or stem-like cells to obtain desired differentiated cell types, e.g., neural cells, muscle cells, hematopoietic cells, etc. In addition, the use of inducible Bcl-2 or Bcl-x1 might be useful for enhancing in vitro development of specific cell lineages. In vivo, BcI-2 prevents many, but not all, forms of apoptotic cell death that occur during lymphoid and neural development. A thorough discussion of how Bcl-2 expression might be used to inhibit apoptosis of relevant cell lineages following transfection of donor cells is disclosed in U.S. Pat. No. 5,646,008, which is herein incorporated by reference.

The subject RPSCs may be used to obtain any desired differentiated cell type. Therapeutic usages of such differentiated human cells are unparalleled. For example, human hematopoietic stem cells may be used in medical treatments requiring bone marrow transplantation. Such procedures are used to treat many diseases, e.g., late stage cancers such as ovarian cancer and leukemia, as well as diseases that compromise the immune system, such as AIDS. Hematopoietic stem cells can be obtained, e.g., by fusing adult somatic cells of a cancer or AIDS patient, e.g., epithelial cells or lymphocytes with an enucleated oocyte, e.g., bovine oocyte, obtaining embryonic or stem-like cells as described above, and culturing such cells under conditions which favor differentiation, until hematopoietic stem cells are obtained. Such hematopoietic cells may be used in the treatment of diseases including cancer and AIDS.

The methods of the present invention can also be used to treat, prevent, or stabilize a neurological disease such as Alzheimer's disease, Parkinson's disease, Huntington's disease, or ALS, lysosomal storage diseases, multiple sclerosis, or a spinal cord injury. For example, somatic cells may be obtained from the individual in need of treatment, and reprogrammed to gain pluripotency, and cultured to derive neurectoderm cells that may be used to replace or assist the normal function of diseased or damaged tissue.

For the treatment or prevention of endocrine conditions, RPSCs that produce a hormone, such as a growth factor, thyroid hormone, thyroid-stimulating hormone, parathyroid hormone, steroid, serotonin, epinephrine, or norepinephrine may be administered to a mammal. Additionally, reprogrammed epithelial cells may be administered to repair damage to the lining of a body cavity or organ, such as a lung, gut, exocrine gland, or urogenital tract. It is also contemplated that RPSCs may be administered to a mammal to treat damage or deficiency of cells in an organ such as the bladder, brain, esophagus, fallopian tube, heart, intestines, gallbladder, kidney, liver, lung, ovaries, pancreas, prostate, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, ureter, urethra, or uterus.

The great advantage of the present invention is that it provides an essentially limitless supply of isogenic or synegenic human cells suitable for transplantation. Therefore, it will obviate the significant problem associated with current transplantation methods, i.e., rejection of the transplanted tissue which may occur because of host versus graft or graft versus host rejection. Conventionally, rejection is prevented or reduced by the administration of anti-rejection drugs such as cyclosporin. However, such drugs have significant adverse side-effects, e.g., immunosuppression, carcinogenic properties, as well as being very expensive. The present invention should eliminate, or at least greatly reduce, the need for anti-rejection drugs, such as cyclosporine, imulan, FK-506, glucocorticoids, and rapamycin, and derivatives thereof.

RPSCs may also be combined with a matrix to form a tissue or organ in vitro or in vivo that may be used to repair or replace a tissue or organ in a recipient mammal. For example, RPSCs may be cultured in vitro in the presence of a matrix to produce a tissue or organ of the urogenital system, such as the bladder, clitoris, corpus cavermosum, kidney, testis, ureter, uretal valve, or urethra, which may then be transplanted into a mammal (Atala, Curr. Opin. Urol. 9(6):517-526, 1999). In another transplant application, synthetic blood vessels are formed in vitro by culturing reprogrammed cells in the presence of an appropriate matrix, and then the vessels are transplanted into a mammal for the treatment or prevention of a cardiovascular or circulatory condition. For the generation of donor cartilage or bone tissue, RPSCs such as chondrocytes or osteocytes are cultured in vitro in the presence of a matrix under conditions that allow the formation of cartilage or bone, and then the matrix containing the donor tissue is administered to a mammal. Alternatively, a mixture of the cells and a matrix may be administered to a mammal for the formation of the desired tissue in vivo. Preferably, the cells are attached to the surface of the matrix or encapsulated by the matrix. Examples of matrices that may be used for the formation of donor tissues or organs include collagen matrices, carbon fibers, polyvinyl alcohol sponges, acrylateamide sponges, fibrin-thrombin gels, hyaluronic acid-based polymers, and synthetic polymer matrices containing polyanhydride, poly-

orthoester, polyglycolic acid, or a combination thereof (see, for example, U.S. Pat. Nos. 4,846,835; 4,642,120; 5,786,217; and 5,041,138).

The RPSCs produced according to the invention may be used to produce genetically engineered or transgenic differentiated cells. Essentially, this will be effected by introducing a desired gene or genes, or removing all or part of an endogenous gene or genes of RPSCs produced according to the invention, and allowing such cells to differentiate into the desired cell type. A preferred method for achieving such modification is by homologous recombination because such technique can be used to insert, delete or modify a gene or genes at a specific site or sites in the stem-like cell genome.

This methodology can be used to replace defective genes, e.g., defective immune system genes, cystic fibrosis genes, or to introduce genes which result in the expression of therapeutically beneficial proteins such as growth factors, lymphokines, cytokines, enzymes, etc. For example, the gene encoding brain derived growth factor maybe introduced into human embryonic or stem-like cells, the cells differentiated into neural cells and the cells transplanted into a Parkinson's patient to retard the loss of neural cells during such disease. Examples of mutations that may be rescued using these methods include mutations in the cystic fibrosis gene; mutations associated with Dunningan's disease such as the R482W, R482Q, and R584H mutations in the lamin A gene; and mutations associated with the autosomal-dominant form of Emery Deyfuss muscular dystrophy such as the R249Q, R453W, and Q6STOP mutations in the lamin A gene. In the Q6STOP mutation, the codon for G1n6 is mutated to a stop codon.

Previously, cell types transfected with BDNF varied from primary cells to immortalized cell lines, either neural or non-neural (myoblast and fibroblast) derived cells. For example, astrocytes have been transfected with BDNF gene using retroviral vectors, and the cells grafted into a rat model of Parkinson's disease (Yoshimoto et al., Brain Research, 691:25-36, (1995)). This ex vivo therapy reduced Parkinson's-like symptoms in the rats up to 45% 32 days after transfer. Also, the tyrosine hydroxylase gene has been placed into astrocytes with similar results (Lundberg et al., Develop. Neurol., 139: 39-53 (1996) and references cited therein).

However, such ex vivo systems have problems. In particular, retroviral vectors currently used are down-regulated in vivo and the transgene is only transiently expressed (review by Mulligan, Science, 260: 926-932 (1993)). Also, such studies used primary cells, astrocytes, which have finite life span and replicate slowly. Such properties adversely affect the rate of transfection and impede selection of stably transfected cells. Moreover, it is almost impossible to propagate a large population of gene targeted primary cells to be used in homologous recombination techniques.

By contrast, the difficulties associated with retroviral systems should be eliminated by the use of RPSCs of the present invention, which are ES-like cells. Using known methods to introduced desired genes/mutations into ES cells, RPSCs may be genetically engineered, and the resulting engineered cells differentiated into desired cell types, e.g., heniatopoietic cells, neural cells, pancreatic cells, cartilage cells, etc. Genes which may be introduced into the RPSCs include, for example, epidermal growth factor, basic fibroblast growth factor, glial derived neurotrophic growth factor, insulin-like growth factor (I and II), neurotrophin3, neurotrophin-4/5, ciliary neurotrophic factor, AFT-1, cytokine genes (interleukins, interferons, colony stimulating factors, tumor necrosis factors (alpha and beta), etc.), genes encoding therapeutic enzymes, collagen, human serum albumin, etc.

In addition, it is also possible to use one of the negative selection systems now known in the art for eliminating therapeutic cells from a patient if necessary. For example, donor cells transfected with the thymidine kinase (TK) gene will lead to the production of embryonic cells containing the TK gene. Differentiation of these cells will lead to the isolation of therapeutic cells of interest which also express the TK gene. Such cells may be selectively eliminated at any time from a patient upon gancyclovir administration. Such a negative selection system is described in U.S. Pat. No. 5,698,446, and is herein incorporated by reference.

Examples of diseases, disorders, or conditions that may be treated or prevented include neurological, endocrine, structural, skeletal, vascular, urinary, digestive, integumentary, blood, immune, auto-immune, inflammatory, endocrine, kidney, bladder, cardiovascular, cancer, circulatory, digestive, hematopoeitic, and muscular diseases, disorders, and conditions. In addition, reprogrammed cells may be used for reconstructive applications, such as for repairing or replacing tissues or organs.

With respect to the therapeutic methods of the invention, it is not intended that the administration of RPSCs to a mammal be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration, including intramuscular, intravenous, intraarticular, intralesional, subcutaneous, or any other route sufficient to provide a dose adequate to prevent or treat a disease. The RPSCs may be administered to the mammal in a single dose or multiple doses. When multiple doses are administered, the doses may be separated from one another by, for example, one week, one month, one year, or ten years. One or more growth factors, hormones, interleukins, cytokines, or other cells may also be administered before, during, or after administration of the cells to further bias them towards a particular cell type.

The RPSCs of the present invention may be used as an in vitro model of differentiation, in particular for the study of genes which are involved in the regulation of early development. Differentiated cell tissues and organs using the RPSCs may be used in drug studies.

Furthermore, the RPSCs produced according to the invention maybe introduced into animals, e.g., SCID mice, cows, pigs, e.g., under the renal capsule or intramuscularly and used to produce a teratoma therein. This teratoma can be used to derive different tissue types. Also, the inner cell mass produced, by X-species nuclear transfer may be introduced together with a biodegradable, biocompatible polymer matrix that provides for the formation of 3-dimensional tissues. After tissue formation, the polymer degrades, ideally just leaving the donor tissue, e.g., cardiac, pancreatic, neural, lung, liver. In some instances, it may be advantageous to include growth factors and proteins that promote angiogenesis. Alternatively, the formation of tissues can be effected totally in vitro, with appropriate culture media and conditions, growth factors, and biodegradable polymer matrices.

Applications of the Somatic Cell Reprogramming Methods and RPSCs in Animals

The reprogramming methods disclosed herein may be used to generate RPSCs for a variety of animal species. The RPSCs generated can be useful to produce desired animals. Animals include, for example, avians and mammals as well as any animal that is an endangered species. Exemplary birds include domesticated birds (e.g., quail, chickens, ducks, geese, turkeys, and guinea hens) as well as other birds such as birds of prey (e.g., hawks, falcons, ospreys, condors, etc.), endangered birds (e.g., parrots, California condor, etc.), ostriches etc. Exemplary mammals include murine, caprine,

ovine, bovine, porcine, canine, feline and primate. Of these, preferred members include domesticated animals, including, for examples, cattle, buffalo, pigs, horses, cows, rabbits, guinea pigs, sheep, and goats.

RPSCs generated by the reprogramming methods of the present invention allows one, for the first time, to genetically engineer animals other than mouse and human. RPSCs are ES-like cells, and are thus amenable to genetic manipulation. To date, no ES cells are available for animals other than mouse and human. As a result, for these animals, it is currently practically impossible to create genetically modified animals having targeted mutations. The ES-cell like RPSCs can be manipulated to introduce desired targeted genetic modifications. The resulting engineered RPSCs can then be used to generate a cloned animal with the desired genetic modifications in its germ line, using methods described for ES cells in mouse. See Capecchi and Thomas, U.S. Pat. Nos. 5,487,997, 5,627,059, 5,631,153, and 6,204,061. Genetic engineering in animals has potentially great applications in a variety of animals, especially farm animals.

The somatic cell reprogramming methods of the present invention provides at least two methods for delivering optimized farm animals. In the first, somatic cell reprogramming can be used to capture the best available phenotype for a farm animal stock. The current technologies used to deliver optimized farm animals are based on selective breeding, and expansion from preferred breeding stocks. Animals that have been selected on the basis of superior characteristics, including, for example, meat content, egg production (in the case of poultry), feed conversion ratio, are used to breed large numbers of animals that are in turn used in the human food supply. This traditional process has profound inherent inefficiencies. The phenotype observed in an individual animal is often only partially transmitted in the progeny of that animal. Therefore, traditional breeding schemes are inefficient in capturing the very best phenotype in all of the progeny animals. In contrast, the reprogramming methods of the present invention provides a controlled and efficient way to achieve the same goal, by generating RPSCs from somatic cells of an animal with the desired characteristics. The RPSCs generated may be used immediately to generate cloned animals derived from the RPSCs. Known methods for generating mice from ES cells can be used for this procedure. Alternatively, the RPSCs generated may be cryopreserved and thawed in response to a grower's needs.

In the second method, somatic cells from an animal with the desired characteristics are reprogrammed to produce RPSCs. The RPSCs are further genetically engineered to introduce desired genetic modification(s), before being placed into a recipient embryo to produce desired progeny.

The reprogramming methods can also be used to rescue endangered species. Somatic cell reprogramming provides an efficient method to generate RPSCs from somatic cells of an endangered animal. The resulting RPSCs can be used immediately to expand the numbers of the endangered animal. Alternatively, the RPSCs can be cryopreserved to generate a RPSC stock for the endangered species, as a safeguard measure against extinction of the endangered species.

Methods for Gene Identification

The present invention provides methods for identifying a gene that activates the expression of an endogenous pluripotency gene in somatic cells. The methods comprise: transfecting the somatic cells of the present invention with a cDNA library prepared from ES cells or oocytes, selecting for cells that express the first selectable marker, and assessing the expression of the first endogenous pluripotency gene in the transfected cells that express the first selectable marker. The expression of the first endogenous pluripotency gene indicates that the cDNA encodes a gene that activates the expression of an endogenous pluripotency gene in somatic cells.

The methods are applicable for identifying a gene that activates the expression of at least two endogenous pluripotency genes in somatic cells. The somatic cells used in the methods further comprise a second endogenous pluripotency gene linked to a second selectable marker. The methods are modified to select for transfected cells that express both selectable markers, among which the expression of the first and the second endogenous pluripotency genes are assessed. The expression of both the first and the second endogenous pluripotency genes indicates that the cDNA encodes a gene that activates the expression of at least two pluripotency genes in somatic cells.

The methods are further applicable for identifying a gene that activates the expression of at least three endogenous pluripotency genes in somatic cells. The somatic cells used in the methods further comprise a third endogenous pluripotency gene linked to a third selectable marker. The methods are modified to select for transfected cells that express all three selectable markers, among which the expression of all three endogenous pluripotency genes are assessed. The expression of all three endogenous pluripotency genes indicates that the cDNA encodes a gene that activates the expression of at least three pluripotency genes in somatic cells.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of mouse genetics, developmental biology, cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, Current Protocols in Cell Biology, ed. by Bonifacino, Dasso, Lippincott-Schwartz, Harford, and Yamada, John Wiley and Sons, Inc., New York, 1999; Manipulating the Mouse Embryos, A Laboratory Manual, 3rd Ed., by Hogan et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2003; Gene Targeting: A Practical Approach, IRL Press at Oxford University Press, Oxford, 1993; and Gene Targeting Protocols, Human Press, Totowa, N.J., 2000. All patents, patent applications and references cited herein are incorporated in their entirety by reference.

EXEMPLIFICATION

The invention now being generally described, it will be more readily understood by reference to the following example, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

EXAMPLE

Oct4-Induced Fibroblasts are More Susceptible to Reprogramming than Unduced Fibroblasts as Demonstrated by Nuclear Transfer Experiment

A. Generation of Transgenic Mouse Carrying an Inducible Oct4 Transgene

An inducible Oct4 allele is constructed as the following: first, two integration vectors are constructed. The first integration vector, inducible Oct4 integration vector, contains an Oct4 gene driven by a tetracycline-inducible promoter (Tet-Op). The Tet-Op-Oct4 cassette is flanked by a splice-acceptor double poly-A signal (SA-dpA) at its 5' end and a SV40 polyA tail (SV40-pA) at its 3' end. The second integration vector, tetracycline activator integration vector, contains a

mutant form of tetracycline activator, M2-rtTA, which is more responsive to doxycycline (Dox) induction than the wild type activator. (Urlinger S. et al., 2000)

The two integration vectors are introduced into V6.5 ES cells: the inducible Oct4 integration vector and the tetracycline activator integration vector are introduced into the Collagen locus and the Rosa26 locus respectively via site-specific integration, as shown in FIG. **1**. The resulting ES cells are used to make Oct4-inducible mice by tetraploid complementation.

B. Expression of the Inducible Oct4 Transgene

Fibroblasts derived from tail biopsies of the Oct4-inducible mice were cultured. A fraction of the cultured fibroblasts were induced with doxycycline for 3 days (at 2 microgram/ml), and Oct4 expression was detected by Northern blot and Western blot analysis. As shown in FIG. **2**, the Oct4 expression level in fibroblasts treated with doxycycline is comparable to the Oct4 expression level in ES cells, and undetectable in fibroblasts not treated with doxycycline. The expression results demonstrate that the inducible Oct4 transgene is expressed as planned.

C. Nuclear Transfer Experiment

Nuclear transfer was performed on fibroblasts derived from tail biopsies of mice that carry the inducible Oct4 transgene. Dox induction was for 24 hours prior to nuclear transfer. Cloned embryos were then activated and cultured to the blastocyst stage to derive ES cells as described previously (Hochedlinger and Jaenisch, 2002). As shown in Table 1, on average, blastocyst formation and ES cell derivation (as measured as a fraction of eggs with pronucleus formation) is more efficient from Oct4 induced fibroblast than from uninduced fibroblasts. This result demonstrated that induced Oct4 expression in somatic cells such as fibroblasts make these cells more susceptible to reprogramming.

One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods, systems and kits are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention and are defined by the scope of the claims. It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

TABLE 1

In vitro development of clones derived from Oct4-induced fibroblasts

Expt.	Oct4	eggs w/ PN	Blastocysts (% PN)		ES lines (% PN)	
#1	–	22	5 (23%)	19%	0 (0%)	3%
#2	–	35	5 (14%)		2 (6%)	
#3	+	37	10 (27%)	24%	2 (5%)	7%
#4	+	47	10 (21%)		4 (9%)	

PN . . . ProNucleus formation

Nuclear transfer was performed on fibroblasts derived from tail biopsies of mice that carry the inducible Oct4 transgene. Dox induction was for 24 hours prior to nuclear transfer. Cloned embryos were then activated and cultured to the blastocyst stage to derive ES cells as described previously (Hochedlinger and Jaenisch, Nature, 2002). These preliminary results show that on average blastocyst formation and ES cell derivation is more efficient from Oct4 induced than from uninduced fibroblasts.

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What is claimed is:

1. A composition comprising an isolated primary somatic cell that comprises an exogenously introduced nucleic acid encoding an Oct4 protein operably linked to at least one regulatory sequence.

2. The composition of claim **1**, wherein the isolated primary somatic cell is a mammalian cell.

3. The composition of claim **2**, wherein the isolated primary somatic cell is a human cell or a mouse cell.

4. The composition of claim **2**, wherein the isolated primary somatic cell is an adult stem cell.

5. The composition of claim **4**, wherein the adult stem cell is selected from the group consisting of: a hematopoietic stem cell, a neural stem cell, and a mesenchymal stem cell.

6. The composition of claim **1**, further comprising a candidate agent of interest with respect to its potential to reprogram a somatic cell.

7. The composition of claim **6**, wherein the agent is a DNA methylation inhibitor, a histone deacetylase inhibitor or PD098059.

8. The composition according to claim **6**, wherein the agent is Sox-2.

9. The composition of claim **1**, wherein the isolated primary somatic cell does not comprise a selectable marker integrated into an endogenous locus of a pluripotency gene.

* * * * *

EXHIBIT B

US008932856B2

(12) **United States Patent**
Jaenisch et al.

(10) **Patent No.:** **US 8,932,856 B2**
(45) **Date of Patent:** ***Jan. 13, 2015**

(54) **METHODS FOR REPROGRAMMING SOMATIC CELLS**

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This patent is subject to a terminal disclaimer.

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USPC **435/377**; 435/325

(58) **Field of Classification Search**
USPC 435/377, 325
See application file for complete search history.

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(57) **ABSTRACT**

The invention provides methods for reprogramming somatic cells to generate multipotent or pluripotent cells. Such methods are useful for a variety of purposes, including treating or preventing a medical condition in an individual. The invention further provides methods for identifying an agent that reprograms somatic cells to a less differentiated state.

8 Claims, 2 Drawing Sheets

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Figure 1. Inducible *Oct4* allele

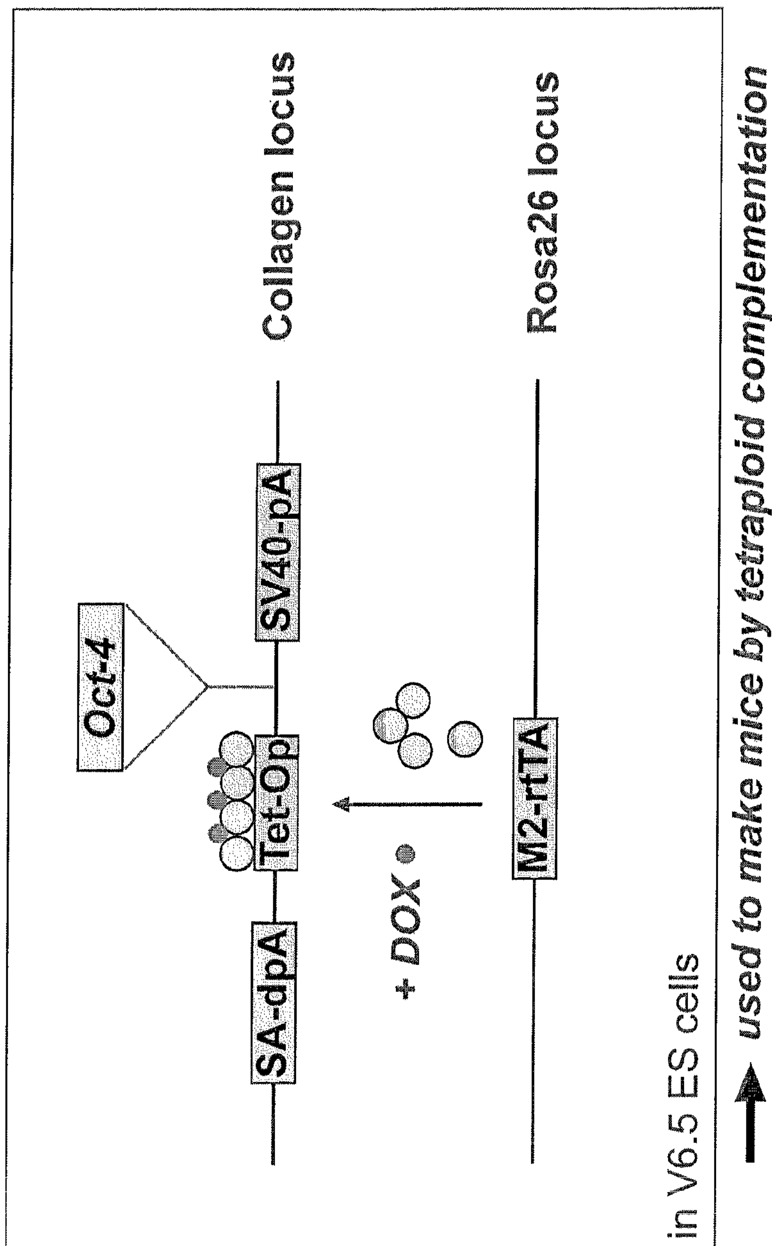
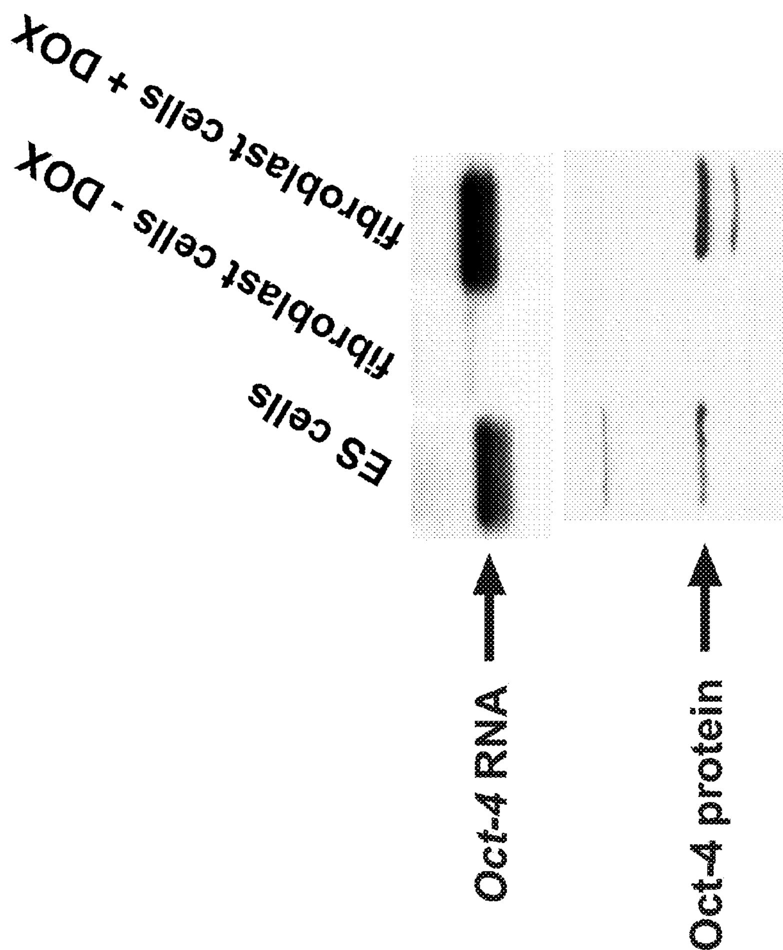


Figure 2. The system works...



METHODS FOR REPROGRAMMING SOMATIC CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 12/703,061, filed Feb. 9, 2010, which is a divisional of U.S. application Ser. No. 10/997,146, filed Nov. 24, 2004 (U.S. Pat. No. 7,682,828), which claims the benefit of U.S. Provisional Application No. 60/525,612, filed Nov. 26, 2003, and U.S. Provisional Application No. 60/530,042, filed Dec. 15, 2003, the specifications of which are incorporated herein by reference in their entirety.

GOVERNMENTAL FUNDING

This invention was made with government support under R37 CA84198 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Pluripotent stem cells have the potential to differentiate into the full range of daughter cells having distinctly different morphological, cytological or functional phenotypes unique to a specific tissue. By contrast, descendants of pluripotent cells are restricted progressively in their differentiation potential, with some cells having only one fate. Pluripotent cells have extraordinary scientific and therapeutic potential, as they can be differentiated along the desired differentiation pathway in a precisely controlled manner and used in cell-based therapy.

Two categories of pluripotent stem cells are known to date: embryonic stem cells and embryonic germ cells. Embryonic stem cells are pluripotent stem cells that are derived directly from an embryo. Embryonic germ cells are pluripotent stem cells that are derived directly from the fetal tissue of aborted fetuses. For purposes of simplicity, embryonic stem cells and embryonic germ cells will be collectively referred to as "ES" cells herein.

ES cells are presently obtained via several methods. In a first method, an ES cell line is derived from the inner cell mass of a normal embryos in the blastocyst stage (See U.S. Pat. No. 6,200,806, Thompson, J. A. et al. Science, 282:1145-7, 1998 and Hogan et al., 2003). A second method for creating pluripotent ES cells utilizes the technique of somatic cell nuclear transfer (SCNT). In this technique, the nucleus is removed from a normal egg, thus removing the genetic material. Next, a donor diploid somatic cell is placed next to the enucleated egg and the two cells are fused, or the nucleus is introduced directly into the oocyte by micromanipulation. The fused cell has the potential to develop into a viable embryo, which may then be sacrificed to remove that portion of the embryo containing the stem cell producing inner cell mass.

In a third method, the nucleus of a human cell is transplanted into an entirely enucleated animal oocyte of a species different from the donor cell (referred to herein as animal stem cell nuclear transfer, or "ASCNT"). See U.S. Pat. application Ser. No. 20010012513 (2001). The resultant chimeric cells are used for the production of pluripotent ES cells, in particular human-like pluripotent ES cells. One disadvantage of this technique is that these chimeric cells may contain unknown non-human viruses and still contain the mitochondria of the animal species. Thus, there would be substantial risks of immune rejection if such cells were used in cell transplantation therapies.

In a fourth method, ES cells can be isolated from the primordial germ cells found in the genital ridges of post-implanted embryos.

As described above, all presently available methods depend on controversial sources—embryos (either created naturally or via cloning), fetal tissue and via the mixing of materials of multiple species. The controversy surrounding the sources for such cells, according to many leading scientists and public and private organizations including the NIH, has greatly compromised and slowed the study of such cells and their application.

There is thus a great demand for alternative methods of generating pluripotent cells.

SUMMARY OF THE INVENTION

The present invention provides engineered somatic cells, in which one or more endogenous pluripotency gene(s) is operably linked to a selectable marker in such a manner that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene to which the marker is linked. The invention also provides transgenic mice containing these engineered somatic cells.

The present invention also provides methods for reprogramming somatic cells to a less differentiated state. In the methods, engineered somatic cells of the invention are treated with an agent. Cells that express the selectable marker are then selected, and assessed for pluripotency characteristics. The treatment with an agent may be contacting the cells with an agent which alters chromatin structure, or may be transfecting the cells with at least one pluripotency gene, or both.

The present invention further provides methods for identifying an agent that reprograms somatic cells to a less differentiated state. In the methods, the engineered somatic cells described above are contacted with a candidate agent. Cells that express the selectable marker are then selected, and assessed for pluripotency characteristics. The presence of at least a subset of pluripotency characteristics indicates that the agent is capable of reprogramming somatic cells to a less-differentiated state. The agents identified by the present invention can then by used to reprogram somatic cells by contacting somatic cells with the agents.

The present invention also provides methods for identifying a gene that causes the expression of at least one endogenous pluripotency gene in somatic cells. In the methods, the engineered somatic cells are transfected with a cDNA library prepared from a pluripotent cell, such as an ES cell. The cells that express the appropriate selectable marker are then selected, and the expression of the appropriate endogenous pluripotency gene is examined. The expression of an endogenous pluripotency gene indicates that the cDNA encodes a protein whose expression in the cell results in, directly or indirectly, expression of the endogenous pluripotency gene.

The present invention further provides methods for treating a condition in an individual in need of such treatment. In certain embodiments, somatic cells are obtained from the individual and reprogrammed by the methods of the invention under conditions suitable for the cells to develop into cells of a desired cell type. The reprogrammed cells of a desired cell type are then harvested and introduced into the individual to treat the condition. In certain further embodiments, the somatic cells obtained from the individual contains a mutation in one or more genes. In these instances, the methods are modified so that the somatic cells obtained from the individual are first treated to restore the one or more normal gene(s) to the cells such that the resulting cells carry the normal endogenous gene, which are then introduced into the

individual. In certain other embodiments, methods of the invention can be used to treat individuals in need of a functional organ. In the methods, somatic cells are obtained from an individual in need of a functional organ, and reprogrammed by the methods of the invention to produce reprogrammed somatic cells. Such reprogrammed somatic cells are then cultured under conditions suitable for development of the reprogrammed somatic cells into a desired organ, which is then introduced into the individual. The methods are useful for treating any one of the following conditions: a neurological, endocrine, structural, skeletal, vascular, urinary, digestive, integumentary, blood, autoimmune, inflammatory, or muscular condition.

The present invention also provides methods for producing a cloned animal. In the methods, a somatic cell is isolated from an animal having desired characteristics, and reprogrammed using the methods of the invention to produce one or more reprogrammed pluripotent somatic cell ("RPSC"). The RPSCs are then inserted into a recipient embryo, and the resulting embryo is cultured to produce an embryo of suitable size for implantation into a recipient female, which is then transferred into a recipient female to produce a pregnant female. The pregnant female is maintained under conditions appropriate for carrying the embryo to term to produce chimeric animal progeny, which is then bred with a wild type animal to produce a cloned animal.

In certain embodiments, the RPSCs may alternatively be cryopreserved for future cloning uses. In certain other embodiments, genetic modification, such as a targeted mutation, may be introduced into the RPSCs prior to its insertion into a recipient embryo.

The present invention also provides methods for producing a cloned avian. In the methods, a somatic cell is isolated from an avian having desired characteristics, and reprogrammed using the methods of the invention to produce one or more reprogrammed pluripotent somatic cell ("RPSC"). The RPSCs are then inserted into eggs that are unable to develop into an embryo, and the resulting eggs are then incubated to produce avian offspring having the genotype of the RPSC, thereby producing a cloned avian.

It is contemplated that all embodiments described above are applicable to all different aspects of the invention. It is also contemplated that any of the above embodiments can be freely combined with one or more other such embodiments whenever appropriate.

Specific embodiments of the invention are described in more detail below. However, these are illustrative embodiments, and should not be construed as limiting in any respect.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts an inducible Oct4 allele.

FIG. **2** shows the expression of the inducible Oct4 transgene by Northern blot and Western blot analysis.

DETAILED DESCRIPTION OF THE INVENTION

Overview

Presently, human ES cells or ES-like cells can only be generated from controversial sources. It would be useful to reprogram somatic cells directly into pluripotent cells. Nuclei from somatic cells retain the totopotency potential to direct development of an animal, as demonstrated by nuclear transfer technology. It would be useful to reprogram somatic cells directly into ES cells without the use of oocytes and nuclear transfer technology.

Applicants have devised novel methods of reprogramming somatic cells to generate pluripotent cells or multipotent cells. Applicants have also devised novel methods to identify agents that reprogram somatic cells. The methods take advantage of the engineered somatic cells designed by Applicants, in which an endogenous gene typically associated with pluripotency ("pluripotency gene") is engineered to be operably linked to a selectable marker in a manner that the expression the endogenous pluripotency gene substantially matches the expression of the selectable marker. Because pluripotency genes are generally expressed only in pluripotent cells and not in somatic cells, the expression of an endogenous pluripotent gene(s) is an indication of successful reprogramming. Having a selectable marker operably linked to an endogenous pluripotency gene gives one a powerful mechanism to select for potentially reprogrammed somatic cells, which likely is a rare occurrence. The resulting cells may be further assessed for pluripotency characteristics to confirm whether a somatic cell has been successfully reprogrammed to pluripotency.

Generating pluripotent or multipotent cells by somatic cell reprogramming using the methods of the present invention has at least two advantages. First, the methods of the present invention allow one to generate autologous pluripotent cells, which are cells specific to a patient. The use of autologous cells in cell therapy offers a major advantage over the use of non-autologous cells, which are likely to be subject to immunological rejection. In contrast, autologous cells are unlikely to elicit significant immunological responses (See Munsie et al, 2000). Second, the methods of the present invention allow one to generate pluipotent without using embryos, oocytes and/or nuclear transfer technology.

A pluripotent cell is a cell that has the potential to divide in vitro for a long period of time (greater than one year) and has the unique ability to differentiate into cells derived from all three embryonic germ layers—endoderm, mesoderm and ectoderm.

A multipotent cell is a cell that is able to differentiate into some but not all of the cells derived from all three germ layers. Thus, a multipotent cell is a partially differentiated cell. Adult stem cells are multipotent cells. Known adult stem cells include, for example, hematopoietic stem cells and neural stem cells. A hematopoietic stem cell is multipotent because it has the ability to differentiate into all types of specific blood cells, but it is unlikely that they can differentiate into all cells of a given animal or human. Multipotent/adult stem cells have a great deal of promise in research and in the area of therapeutic applications. For example, multipotent/adult stem cells have already been used in humans in attempts to treat certain blood, neural and cancer diseases.

The term "pluripotency gene", as used herein, refers to a gene that is associated with pluripotency. The expression of a pluripotency gene is typically restricted to pluripotent stem cells, and is crucial for the functional identity of pluripotent stem cells. The transcription factor Oct-4 (also called Pou5fl, Oct-3, Oct3/4) is an example of a pluripotency gene. Oct-4 has been shown to be required for establishing and maintaining the undifferentiated phenotype of ES cells and plays a major role in determining early events in embryogenesis and cellular-.differentiation (Nichols et al., 1998, Cell 95:379-391; Niwa et al., 2000, Nature Genet. 24:372-376). Oct-4 is down-regulated as stem cells differentiate into specialised cells. Other exemplary pluripotency genes include Nanog, and Stella (See Chambers et al., 2003, Cell 113: 643-655; Mitsui et al., Cell. 2003, 113(5):631-42; Bortvin et al. Development. 2003, 130(8):1673-80; Saitou et al., Nature. 2002, 418 (6895):793-300.

Engineered Somatic Cells and Transgenic Mice Comprising Such Cells

The present invention provides somatic cells comprising an endogenous pluripotency gene linked to DNA encoding a selectable marker in such a manner that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene. In one embodiment, the somatic cells of the present invention comprise a first endogenous pluripotency gene linked to DNA encoding a first selectable marker in such a manner that the expression of the first selectable marker substantially matches the expression of the first endogenous pluripotency gene. The somatic cells may also be engineered to comprise any number of endogenous pluripotency genes respectively linked to a distinct selectable marker. Thus, in another embodiment, the somatic cells of the present invention comprise two endogenous pluripotency genes, each of which is linked to DNA encoding a distinct selectable marker. In a further embodiment, the somatic cells of the present invention comprise three endogenous pluripotency genes, each of which is linked to DNA encoding a distinct selectable marker. The somatic cells described above will be collectively referred in this application as "engineered somatic cells." The engineered somatic cells may be further engineered to have one or more pluripotency gene expressed as a transgene under an inducible promoter.

The selectable marker is linked to an appropriate endogenous pluripotency gene such that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene. By "substantially match", it is meant that the expression of the selectable marker substantially reflects the expression pattern of the endogenous pluripotency gene. In other words, the selectable marker and the endogenous pluripotency gene are co-expressed. For purpose of the present invention, it is not necessary that the expression level of the endogenous gene and the selectable marker is the same or even similar. It is only necessary that the cells in which an endogenous pluripotency gene is activated will also express the selectable marker at a level sufficient to confer a selectable phenotype on the reprogrammed cells. For example, when the selectable marker is a marker that confers resistance to a lethal drug (a "drug resistance marker"), the cells are engineered in a way that allows cells in which an endogeneous pluripotency gene is activated to also express the drug resistance marker at a sufficient level to confer on reprogrammed cells resistance to lethal drugs. Thus, reprogrammed cells will survive and proliferate whereas non-reprogrammed cells will die.

The DNA encoding a selectable marker may be inserted downstream from the end of the open reading frame (ORF) encoding the desired endogenous pluripotency gene, anywhere between the last nucleotide of the ORF and the first nucleotide of the polyadenylation site. An internal ribosome entry site (IRES) may be placed in front of the DNA encoding the selectable marker. Alternatively, the DNA encoding a selectable marker may be inserted anywhere within the ORF of the desired endogenous pluripotency gene, downstream of the promoter, with a termination signal. An internal ribosome entry site (IRES) may be placed in front of the DNA encoding the selectable marker. The selectable marker may be inserted into only one allele, or both alleles, of the endogenous pluripotency gene.

The somatic cells in the invention may be primary cells or immortalized cells. Such cells may be primary cells (non-immortalized cells), such as those freshly isolated from an animal, or may be derived from a cell line (immortalized cells).

The somatic cells in the present invention are mammalian cells, such as, for example, human cells or mouse cells. They may be obtained by well-known methods, from different organs, e.g., skin, lung, pancreas, liver, stomach, intestine, heart, reproductive organs, bladder, kidney, urethra and other urinary organs, etc., generally from any organ or tissue containing live somatic cells. Mammalian somatic cells useful in the present invention include, by way of example, adult stem cells, sertoli cells, endothelial cells, granulosa epithelial, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc. generally any live somatic cells. The term "somatic cells", as used herein, also includes adult stem cells. An adult stem cell is a cell that is capable of giving rise to all cell types of a particular tissue. Exemplary adult stem cells include hematopoietic stem cells, neural stem cells, and mesenchymal stem cells.

In one embodiment, the engineered somatic cells are obtained from a transgenic mouse comprising such engineered somatic cells. Such transgenic mouse can be produced using standard techniques known in the art. For example, Bronson et al. describe a technique for inserting a single copy of a transgene into a chosen chromosomal site. See Bronson et al., 1996. Briefly, a vector containing the desired integration construct (for example, a construct containing a selectable marker linked to a pluripotency gene) is introduced into ES cells by standard techniques known in the art. The resulting ES cells are screened for the desired integration event, in which the knock-in vector is integrated into the desired endogenous pluripotency gene locus such that the selectable marker is integrated into the genomic locus of the pluripotency gene and is under the control of the pluripotency gene promoter. The desired ES cell is then used to produce transgenic mouse in which all cell types contain the correct integration event. Desired types of cells may be selectively obtained from the transgenic mouse and maintained in vitro. In one embodiment, two or more transgenic mice may be created, each carrying a distinct integration construct. These mice may then be bred to generate mice that carry multiple desired integration construct. For example, one type of transgenic mouse may be created to carry an endogenous pluripotency gene linked to a selectable marker, while a second type of transgenic mouse may be created to carry a pluripotency gene expressed as a transgene under an inducible promoter. These two types of mice may then be bred to generate transgenic mice that have both a selectable marker linked to an endogenous pluripotency gene and an additional pluripotency gene expressed as a transgene under an inducible promoter. These two pluripotency genes may or may not be the same. Many variables are contemplated: the identity of the endogenous pluripotency gene linked to marker, the identity of the pluripotency gene expressed as a transgene, and the number of the endogenous pluripotency gene linked to a selectable marker, and the number of pluripotency gene expressed as a transgene. The present invention encompasses all possible combinations of these variables.

Alternatively, engineered somatic cells of the present invention may be produced by direct introduction of the desired construct into somatic cells. DNA construct may be introduced into cells by any standard technique known in the art, such as viral transfection (eg. using an adenoviral system) or liposome-mediated transfection. Any means known in the art to generate somatic cells with targeted integration can be used to produce somatic cells of the invention. In mammalian

cells, homologous recombination occurs at much lower frequency compared to non-homologous recombination. To facilitate the selection of homologous recombination events over the non-homologous recombination events, at least two enrichment methods have been developed: the positive-negative selection (PNS) method and the "promoterless" selection method (Sedivy and Dutriaux, 1999). Briefly, PNS, the first method, is in genetic terms a negative selection: it selects against recombination at the incorrect (non-homologous) loci by relying on the use of a negatively selectable gene that is placed on the flanks of a targeting vector. On the other hand, the second method, the "promoterless" selection, is a positive selection in genetic terms: it selects for recombination at the correct (homologous) locus by relying on the use of a positively selectable gene whose expression is made conditional on recombination at the homologous target site. The disclosure of Sedivy and Dutriaux is incorporated herein.

A selectable marker, as used herein, is a marker that, when expressed, confers upon recipient cells a selectable phenotype, such as antibiotic resistance, resistance to a cytotoxic agent, nutritional prototrophy or expression of a surface protein. The presence of a selectable marker linked to an endogenous pluripotency gene makes it possible to identify and select reprogrammed cells in which the endogenous pluripotency gene is expressed. A variety of selectable marker genes can be used, such as neomycin resistance gene (neo), puromycin resistance gene (puro), guanine phosphoribosyl transferase (gpt), dihydrofolate reductase (DHFR), adenosine deaminase (ada), puromycin-N-acetyltransferase (PAC), hygromycin resistance gene (hyg), multidrug resistance gene (mdr), and hisD gene.

The present invention further provides transgenic mice comprising the somatic cells of the invention.

Methods for Reprogramming Somatic Cells

The present invention further provides methods for reprogramming somatic cells to a less differentiated state. The resulting cells are termed "reprogrammed somatic cells" ("RSC") herein. A RSC may be a reprogrammed pluripotent somatic cell ("RPSC"), a reprogrammed multipotent somatic cell ("RMSC"), or a reprogrammed somatic cell of varying differentiation status.

In general, the methods comprise treating the engineered somatic cells with an agent. The treatment with an agent may be contacting the cells with an agent which alters chromatin structure, or may be transfecting the cells with one or more pluripotency gene, or both. The above two treatments may be concurrent, or may be sequential, with no particular preference for order. In a further embodiment, reprogrammed somatic cells are identified by selecting for cells that express the appropriate selectable marker. In still a further embodiment, reprogrammed somatic cells are further assessed for pluripotency characteristics. The presence of pluripotency characteristics indicates that the somatic cells have been reprogrammed to a pluripotent state.

Differentiation status of cells is a continuous spectrum, with terminally differentiated state at one end of this spectrum and de-differentiated state (pluripotent state) at the other end. Reprogramming, as used herein, refers to a process that alters or reverses the differentiation status of a somatic cell, which can be either partially or terminally differentiated. Reprogramming includes complete reversion, as well as partial reversion, of the differentiation status of a somatic cell. In other words, the term "reprogramming", as used herein, encompasses any movement of the differentiation status of a cell along the spectrum toward a less-differentiated state. For example, reprogramming includes reversing a multipotent cell back to a pluripotent cell, reversing a terminally differentiated cell back to either a multipotent cell or a pluripotent cell. In one embodiment, reprogramming of a somatic cell turns the somatic cell all the way back to a pluripotent state. In another embodiment, reprogramming of a somatic cell turns the somatic cell back to a multipotent state. The term "less-differentiated state", as used herein, is thus a relative term and includes a completely de-differentiated state and a partially differentiated state.

The term "pluripotency characteristics", as used herein, refers to many characteristics associated with pluripotency, including, for example, the ability to differentiate into all types of cells and an expression pattern distinct for a pluripotent cell, including expression of pluripotency genes, expression of other ES cell markers, and on a global level, a distinct expression profile known as "stem cell molecular signature" or "sternness."

Thus, to assess reprogrammed somatic cells for pluripotency characteristics, one may analyze such cells for different growth characteristics and ES cell-like morphology. Cells may be injected subcutaneously into immunocompromised SCID mice to induce teratomas (a standard assay for ES cells). ES-like cells can be differentiated into embryoid bodies (another ES specific feature). Moreover, ES-like cells can be differentiated in vitro by adding certain growth factors known to drive differentiation into specific cell types. Self-renewing capacity, marked by induction of telomerase activity, is another plutipotency characteristics that can be monitored. One may carry out functional assays of the reprogrammed somatic cells by introducing them into blastocysts and determine whether the cells are capable of giving rise to all cell types. See Hogan et al., 2003. If the reprogrammed cells are capable of forming a few cell types of the body, they are multipotent; if the reprogrammed cells are capable of forming all cell types of the body including germ cells, they are pluripotent.

One may also examine the expression of an individual pluripotency gene in the reprogrammed somatic cells to assess their pluripotency characteristics. Additionally, one may assess the expression of other ES cell markers. Stage-specific embryonic 1 5 antigens-1, -3, and -4 (SSEA-1, SSEA-3, SSEA-4) are glycoproteins specifically expressed in early embryonic development and are markers for ES cells (Solter and Knowles, 1978, Proc. Natl. Acad. Sci. USA 75:5565-5569; Kannagi et al., 1983, EMBO J 2:2355-2361). Elevated expression of the enzyme Alkaline Phosphatase (AP) is another marker associated with undifferentiated embryonic stem cells (Wobus et al., 1 984, Exp. Cell 152:212-219; Pease et al., 1990, Dev. Biol. 141:322-352). Other stem/progenitor cells markers include the intermediate neurofilament nestin (Lendahl et al., 1990, Cell 60:585-595; Dah-Istrand et al., 1992, J.

Cell Sci. 103:589-597), the membrane glycoprotein prominin/AC133 (Weigmann et al., 1997, Proc. Natl. Acad. USA 94:12425-12430; Corbeil et al., 1 998, Blood 91:2625-22626), the transcription factor Tcf-4 (Korinek et al, 1998, Nat. Genet. 19: 379-383; Lee et al., 1999, J. Biol. Chem. 274.1 566-1 572), and the transcription factor Cdx1 (Duprey et al., 1 988, Genes Dev. 2:1647-1654; Subramania'n et al., 1998, Differentiation 64:11-1 8).

One may additionally conduct expression profiling of the reprogrammed somatic cells to assess their pluripotency characteristics. Pluripotent cells, such as embryonic stem cells, and multipotent cells, such as adult stem cells, are known to have a distinct pattern of global gene expression profile. This distinct pattern is termed "stem cell molecular

signature", or "sternness". See, for example, Ramalho-Santos et al., Science 298: 597-600 (2002); Ivanova et al., Science 298: 601-604.

Somatic cells may be reprogrammed to gain either a complete set of the pluripotency characteristics and are thus pluripotent. Alternatively, somatic cells may be reprogrammed to gain only a subset of the pluripotency characteristics. In another alternative, somatic cells may be reprogrammed to be multipotent.

In a further embodiment, in conjunction with contacting the somatic cells of the invention with an agent which alters chromatin structure, at least one gene that affects pluripotent state of a cell may be further introduced into the same cells. This may be carried out sequentially. For example, the somatic cells of the invention may be first contacted with an agent which alters chromatin structure. Then at least one pluripotency gene can be introduced into the same cells, or vice versa. Alternatively, the two steps may be carried out simultaneously.

Genes that affect pluripotent state of a cell includes pluripotency genes, genes involved in chromatin remodeling, and genes that are important for maintaining pluripotency, such as LIF, BMP, and PD098059 (See Cell, 115: 281-292 (2003); Philos Trans R Soc Lond B Biol Sci. 2003 Aug. 29; 358 (1436):1397-402).

The exogenously introduced pluripotency gene may be carried out in several ways. In one embodiment, the exogenously introduced pluripotency gene may be expressed from a chromosomal locus different from the endogenous chromosomal locus of the pluripotency gene. Such chromosomal locus may be a locus with open chromatin structure, and contain gene(s) dispensible for a somatic cell. In other words, the desirable chromosomal locus contains gene(s) whose disruption will not cause cells to die. Exemplary chromosomal loci include, for example, the mouse ROSA 26 locus and type II collagen (Col2a1) locus (See Zambrowicz et al., 1997) The exogenously introduced pluripotency gene may be expressed from an inducible promoter such that their expression can be regulated as desired.

In an alternative embodiment, the exogenously introduced pluripotency gene may be transiently transfected into cells, either individually or as part of a cDNA expression library, prepared from pluripotent cells. Such pluripotent cells may be embryonic stem cells, oocytes, blastomeres, inner cell mass cells, embryonic germ cells, embryoid body (embryonic) cells, morula-derived cells, teratoma (teratocarcinoma) cells, and multipotent partially differentiated embryonic stem cells taken from later in the embryonic development process.

The cDNA library is prepared by conventional techniques. Briefly, mRNA is isolated from an organism of interest. An RNA-directed DNA polymerase is employed for first strand synthesis using the mRNA as template. Second strand synthesis is carried out using a DNA-directed DNA polymerase which results in the cDNA product. Following conventional processing to facilitate cloning of the cDNA, the cDNA is inserted into an expression vector such that the cDNA is operably linked to at least one regulatory sequence. The choice of expression vectors for use in connection with the cDNA library is not limited to a particular vector. Any expression vector suitable for use in mouse cells is appropriate. In one embodiment, the promoter which drives expression from the cDNA expression construct is an inducible promoter. The term regulatory sequence includes promoters, enhancers and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology*, Academic Press, San Diego, Calif. (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express cDNAs. Such useful expression control sequences, include, for example, the early and late promoters of SV40, tet promoter, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

The exogenously introduced pluripotency gene may be expressed from an inducible promoter. The term "inducible promoter", as used herein, refers to a promoter that, in the absence of an inducer (such as a chemical and/or biological agent), does not direct expression, or directs low levels of expression of an operably linked gene (including cDNA), and, in response to an inducer, its ability to direct expression is enhanced. Exemplary inducible promoters include, for example, promoters that respond to heavy metals (CRC Boca Raton, Fla. (1991), 167-220; Brinster et al. Nature (1982), 296, 39-42), to thermal shocks, to hormones (Lee et al. P.N.A.S. USA (1988), 85, 1204-1208; (1981), 294, 228-232; Klock et al. Nature (1987), 329, 734-736; Israel and Kaufman, Nucleic Acids Res. (1989), 17, 2589-2604), promoters that respond to chemical agents, such as glucose, lactose, galactose or antibiotic.

A tetracycline-inducible promoter is an example of an inducible promoter that responds to an antibiotics. See Gossen et al., 2003. The tetracycline-inducible promoter comprises a minimal promoter linked operably to one or more tetracycline operator(s). The presence of tetracycline or one of its analogues leads to the binding of a transcription activator to the tetracycline operator sequences, which activates the minimal promoter and hence the transcription of the associated cDNA. Tetracycline analogue includes any compound that displays structural homologies with tetracycline and is capable of activating a tetracycline-inducible promoter. Exemplary tetracycline analogues includes, for example, doxycycline, chlorotetracycline and anhydrotetracycline.

Thus, in one embodiment, the present invention provides mice and somatic cells carrying at least one pluripotency gene expressed as a transgene under an inducible promoter. It is possible that somatic cells with such inducible pluripotency transgene(s) are more prone to be reprogrammed.

Any of the engineered somatic cells of the present invention may be used in the methods. In one embodiment, somatic cells used in the methods comprise only one endogenous pluripotency gene linked to a first selectable marker, and the selection step is carried out to select for the expression of the first selectable marker. In an alternative embodiment, the somatic cells used in the methods comprise any number of endogenous pluripotency genes, each of which is linked to a distinct selectable marker respectively, and the selection step is carried out to select for at least a subset of the selectable markers. For example, the selection step may be carried out to

select for all the selectable markers linked to the various endogenous pluripotency genes.

In an alternative embodiment, somatic cells used in the method comprise a selectable marker linked to an endogenous pluripotency gene and an additional pluripotency gene expressed as a transgene under an inducible promoter. For these cells, the method of reprogramming may comprises induce the expression of the pluripotency transgene and select for the expression of the selectable marker. The method may further comprise contacting the somatic cells with an agent that alter chromatic structure.

Without wishing to be bound by theory, the agents used in the method may cause chromatin to take on a more open structure, which is more permissive for gene expression. DNA methylation and histone acetylation are two known events that alter chromatin toward a more closed structure. For example, loss of methylation by genetic deletion of DNA methylation enzyme Dnmt1 in fibroblasts results in reactivation of endogenous Oct4 gene. See J. Biol, Chem. 277: 34521-30, 2002; and Bergman and Mostoslaysky, Biol. Chem. 1990. Thus, DNA methylation inhibitors and histone deacelyation inhibitors are two classes of agents that may be used in the methods of the invention. Exemplary agents include 5-aza-cytidine, TSA and valproic acid.

In another embodiment, methods of the invention may further include repeating the steps of treating the cells with an agent. The agent used in the repeating treatment may be the same as, or different from, the one used during the first treatment.

Methods for Screening for an Agent that Reprograms Somatic Cells

The present invention also provides methods for identifying an agent that reprograms somatic cells to a less-differentiated state, as well as the agents thus identified. In one embodiment, the methods comprise contacting the engineered somatic cells of the invention with a candidate agent, selecting for cells that express the appropriate selectable marker. The presence of cells that express the appropriate selectable marker indicates that the agent reprograms somatic cells. Such an agent is referred as a "reprogramming agent" for purpose of this application.

In a further embodiment, the methods comprise contacting the engineered somatic cells of the invention with a candidate agent, selecting for cells that express the appropriate selectable marker, and assessing the cells so selected for pluripotency characteristics. The presence of a complete set of pluripotency characteristics indicates that the agent reprograms somatic cells to become pluripotent.

Candidate agents used in the invention encompass numerous chemical classes, though typically they are organic molecules, including small organic compounds. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, nucleic acids and derivatives, structural analogs or combinations thereof.

Candidate agents may be naturally arising, recombinant or designed in the laboratory. The candidate agents may be isolated from microorganisms, animals, or plants, or may be produced recombinantly, or synthesized by chemical methods known in the art. In some embodiments, candidate agents are isolated from libraries of synthetic or natural compounds using the methods of the present invention. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, including acylation, alkylation, esterification, amidification, to produce structural analogs.

There are numerous commercially available compound libraries, including, for example, the Chembridge DIVERSet. Libraries are also available from academic investigators, such as the Diversity set from the NCI developmental therapeutics program.

The screening methods mentioned above are based on assays performed on cells. These cell-based assays may be performed in a high throughput screening (HTS) format, which has been described in the art. For example, Stockwell et al. described a high-throughput screening of small molecules in miniaturized mammalian cell-based assays involving post-translational modifications (Stockwell et al., 1999). Likewise, Qian et al. described a leukemia cell-based assay for high-throughtput screening for anti-cancer agents (Qian et al., 2001). Both references are incorporated herein in their entirety.

A reprogramming agent may belong to any one of many different categories. For example, a reprogramming agent may be a chromatin remodeling agent. A chromatin remodeling agent may be a protein involved in chromatin remodeling or an agent known to alter chromatin toward a more open structure, such as a DNA methylation inhibitor or a histone deacelyation inhibitor. Exemplary compounds include 5-aza-cytidine, TSA and valproic acid. For another example, such an agent may be a pluripotency protein, including, for example, Nanog, Oct-4 and Stella. Such an agent may also be a gene essential for pluripotency, including, for example, Sox2, FoxD3, and LIF, and Stat3. See Smith et al. 1988, William et al., 1988, Ihle, 1996, Avilion et al., 2003, and Hanna et al., 2002)

Methods for Reprogramming Somatic Cells with a Reprogramming Agent

The reprogramming agent identified by the methods of the present invention is useful for reprogramming somatic cells into pluripotent or multipotent cells. Accordingly, the present invention provides methods for reprogramming somatic cells to a less differentiated state, comprising contacting somatic cells with a reprogramming agent. The somatic cells used may be native somatic cells, or engineered somatic cells. It is not necessary for these cells to carry a selectable marker integrated into the endogenous locus of a pluripotency gene.

Reprogrammed Somatic Cells and These Uses

The present invention also provides reprogrammed somatic cells (RSCs), including reprogrammed pluripotent somatic cells (RPSCs), produced by the methods of the invention. These methods, useful for the generation of cells of a desired cell type, have wide range of applications. For one example, these methods have applications in livestock management, involving the precise genetic manipulation of animals for economic or health purposes. For another example, these methods have medical application in treating or preventing a condition.

Accordingly, the invention provides methods for the treatment or prevention of a condition in a mammal. In one embodiment, the methods start with obtaining somatic cells from the individual, reprogramming the somatic cells so obtained by methods of the present invention to obtain RPSCs. The RPSCs are then cultured under conditions suitable for development of the RPSCs into cells of a desired cell

type. The developed cells of the desired cell type are harvested and introduced into the individual to treat the condition. In an alternative embodiment, the methods start with obtaining somatic cells from the individual, reprogramming the somatic cells so obtained by methods of the present invention. The RPSCs are then cultured under conditions suitable for development of the RPSCs into a desired organ, which is harvested and introduced into the individual to treat the condition.

The RPSCs of the present invention are ES-like cells, and thus may be induced to differentiate to obtain the desired cell types according to known methods to differentiate ES cells. For example, the RPSCs may be induced to differentiate into hematopoietic stem cells, muscle cells, cardiac muscle cells, liver cells, cartilage cells, epithelial cells, urinary tract cells, etc., by culturing such cells in differentiation medium and under conditions which provide for cell differentiation. Medium and methods which result in the differentiation of embryonic stem cells are known in the art as are suitable culturing conditions.

For example, Palacios et al., Proc. Natl. Acad. Sci., USA, 92: 7530-37 (1995) teaches the production of hematopoietic stem cells from an embryonic cell line by subjecting stem cells to an induction procedure comprising initially culturing aggregates of such cells in a suspension culture medium lacking retinoic acid followed by culturing in the same medium containing retinoic acid, followed by transferral of cell aggregates to a substrate which provides for cell attachment.

Moreover, Pedersen, J. Reprod. Fertil. Dev., 6: 543-52 (1994) is a review article which references numerous articles disclosing methods for in vitro differentiation of embryonic stem cells to produce various differentiated cell types including hematopoietic cells, muscle, cardiac muscle, nerve cells, among others.

Further, Bain et al., Dev. Biol., 168:342-357 (1995) teaches in vitro differentiation of embryonic stem cells to produce neural cells which possess neuronal properties. These references are exemplary of reported methods for obtaining differentiated cells from embryonic or stem-like cells. These references and in particular the disclosures therein relating to methods for differentiating embryonic stem cells are incorporated by reference in their entirety herein.

Thus, using known methods and culture medium, one skilled in the art may culture the subject embryonic or stem-like cells to obtain desired differentiated cell

types, e.g., neural cells, muscle cells, hematopoietic cells, etc. In addition, the use of inducible Bcl-2 or Bcl-x1 might be useful for enhancing in vitro development of specific cell lineages. In vivo, BcI-2 prevents many, but not all, forms of apoptotic cell death that occur during lymphoid and neural development. A thorough discussion of how Bcl-2 expression might be used to inhibit apoptosis of relevant cell lineages following transfection of donor cells is disclosed in U.S. Pat. No. 5,646,008, which is herein incorporated by reference.

The subject RPSCs may be used to obtain any desired differentiated cell type. Therapeutic usages of such differentiated human cells are unparalleled. For example, human hematopoietic stem cells may be used in medical treatments requiring bone marrow transplantation. Such procedures are used to treat many diseases, e.g., late stage cancers such as ovarian cancer and leukemia, as well as diseases that compromise the immune system, such as AIDS. Hematopoietic stem cells can be obtained, e.g., by fusing adult somatic cells of a cancer or AIDS patient, e.g., epithelial cells or lymphocytes with an enucleated oocyte, e.g., bovine oocyte, obtaining embryonic or stem-like cells as described above, and

culturing such cells under conditions which favor differentiation, until hematopoietic stem cells are obtained. Such hematopoietic cells may be used in the treatment of diseases including cancer and AIDS.

The methods of the present invention can also be used to treat, prevent, or stabilize a neurological disease such as Alzheimer's disease, Parkinson's disease, Huntington's disease, or ALS, lysosomal storage diseases, multiple sclerosis, or a spinal cord injury. For example, somatic cells may be obtained from the individual in need of treatment, and reprogrammed to gain pluripotency, and cultured to derive neurectoderm cells that may be used to replace or assist the normal function of diseased or damaged tissue.

For the treatment or prevention of endocrine conditions, RPSCs that produce a hormone, such as a growth factor, thyroid hormone, thyroid-stimulating hormone, parathyroid hormone, steroid, serotonin, epinephrine, or norepinephrine may be administered to a mammal. Additionally, reprogrammed epithelial cells may be administered to repair damage to the lining of a body cavity or organ, such as a lung, gut, exocrine gland, or urogenital tract. It is also contemplated that RPSCs may be administered to a mammal to treat damage or deficiency of cells in an organ such as the bladder, brain, esophagus, fallopian tube, heart, intestines, gallbladder, kidney, liver, lung, ovaries, pancreas, prostate, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, ureter, urethra, or uterus.

The great advantage of the present invention is that it provides an essentially limitless supply of isogenic or synegenic human cells suitable for transplantation. Therefore, it will obviate the significant problem associated with current transplantation methods, i.e., rejection of the transplanted tissue which may occur because of host versus graft or graft versus host rejection. Conventionally, rejection is prevented or reduced by the administration of anti-rejection drugs such as cyclosporin. However, such drugs have significant adverse side-effects, e.g., immunosuppression, carcinogenic properties, as well as being very expensive. The present invention should eliminate, or at least greatly reduce, the need for anti-rejection drugs, such as cyclosporine, imulan, FK-506, glucocorticoids, and rapamycin, and derivatives thereof.

RPSCs may also be combined with a matrix to form a tissue or organ in vitro or in vivo that may be used to repair or replace a tissue or organ in a recipient mammal. For example, RPSCs may be cultured in vitro in the presence of a matrix to produce a tissue or organ of the urogenital system, such as the bladder, clitoris, corpus cavermosum, kidney, testis, ureter, uretal valve, or urethra, which may then be transplanted into a mammal (Atala, Curr. Opin. Urol. 9(6):517-526, 1999). In another transplant application, synthetic blood vessels are formed in vitro by culturing reprogrammed cells in the presence of an appropriate matrix, and then the vessels are transplanted into a mammal for the treatment or prevention of a cardiovascular or circulatory condition. For the generation of donor cartilage or bone tissue, RPSCs such as chondrocytes or osteocytes are cultured in vitro in the presence of a matrix under conditions that allow the formation of cartilage or bone, and then the matrix containing the donor tissue is administered to a mammal. Alternatively, a mixture of the cells and a matrix may be administered to a mammal for the formation of the desired tissue in vivo. Preferably, the cells are attached to the surface of the matrix or encapsulated by the matrix. Examples of matrices that may be used for the formation of donor tissues or organs include collagen matrices, carbon fibers, polyvinyl alcohol sponges, acrylateamide sponges, fibrin-thrombin gels, hyaluronic acid-based polymers, and synthetic polymer matrices containing polyanhydride, poly-

orthoester, polyglycolic acid, or a combination thereof (see, for example, U.S. Pat. Nos. 4,846,835; 4,642,120; 5,786,217; and 5,041,138).

The RPSCs produced according to the invention may be used to produce genetically engineered or transgenic differentiated cells. Essentially, this will be effected by introducing a desired gene or genes, or removing all or part of an endogenous gene or genes of RPSCs produced according to the invention, and allowing such cells to differentiate into the desired cell type. A preferred method for achieving such modification is by homologous recombination because such technique can be used to insert, delete or modify a gene or genes at a specific site or sites in the stem-like cell genome.

This methodology can be used to replace defective genes, e.g., defective immune system genes, cystic fibrosis genes, or to introduce genes which result in the expression of therapeutically beneficial proteins such as growth factors, lymphokines, cytokines, enzymes, etc. For example, the gene encoding brain derived growth factor maybe introduced into human embryonic or stem-like cells, the cells differentiated into neural cells and the cells transplanted into a Parkinson's patient to retard the loss of neural cells during such disease. Examples of mutations that may be rescued using these methods include mutations in the cystic fibrosis gene; mutations associated with Dunningan's disease such as the R482W, R482Q, and R584H mutations in the lamin A gene; and mutations associated with the autosomal-dominant form of Emery Deyfuss muscular dystrophy such as the R249Q, R453W, and Q6STOP mutations in the lamin A gene. In the Q6STOP mutation, the codon for Gln6 is mutated to a stop codon.

Previously, cell types transfected with BDNF varied from primary cells to immortalized cell lines, either neural or non-neural (myoblast and fibroblast) derived cells. For example, astrocytes have been transfected with BDNF gene using retroviral vectors, and the cells grafted into a rat model of Parkinson's disease (Yoshimoto et al., Brain Research, 691:25-36, (1995)). This ex vivo therapy reduced Parkinson's-like symptoms in the rats up to 45% 32 days after transfer. Also, the tyrosine hydroxylase gene has been placed into astrocytes with similar results (Lundberg et al., Develop. Neurol., 139: 39-53 (1996) and references cited therein).

However, such ex vivo systems have problems. In particular, retroviral vectors currently used are down-regulated in vivo and the transgene is only transiently expressed (review by Mulligan, Science, 260: 926-932 (1993)). Also, such studies used primary cells, astrocytes, which have finite life span and replicate slowly. Such properties adversely affect the rate of transfection and impede selection of stably transfected cells. Moreover, it is almost impossible to propagate a large population of gene targeted primary cells to be used in homologous recombination techniques.

By contrast, the difficulties associated with retroviral systems should be eliminated by the use of RPSCs of the present invention, which are ES-like cells. Using known methods to introduced desired genes/mutations into ES cells, RPSCs may be genetically engineered, and the resulting engineered cells differentiated into desired cell types, e.g., heniatopoietic cells, neural cells, pancreatic cells, cartilage cells, etc. Genes which may be introduced into the RPSCs include, for example, epidermal growth factor, basic fibroblast growth factor, glial derived neurotrophic growth factor, insulin-like growth factor (I and II), neurotrophin3, neurotrophin-4/5, ciliary neurotrophic factor, AFT-1, cytokine genes (interleukins, interferons, colony stimulating factors, tumor necrosis factors (alpha and beta), etc.), genes encoding therapeutic enzymes, collagen, human serum albumin, etc.

In addition, it is also possible to use one of the negative selection systems now known in the art for eliminating therapeutic cells from a patient if necessary. For example, donor cells transfected with the thymidine kinase (TK) gene will lead to the production of embryonic cells containing the TK gene. Differentiation of these cells will lead to the isolation of therapeutic cells of interest which also express the TK gene. Such cells may be selectively eliminated at any time from a patient upon gancyclovir administration. Such a negative selection system is described in U.S. Pat. No. 5,698,446, and is herein incorporated by reference.

Examples of diseases, disorders, or conditions that may be treated or prevented include neurological, endocrine, structural, skeletal, vascular, urinary, digestive, integumentary, blood, immune, auto-immune, inflammatory, endocrine, kidney, bladder, cardiovascular, cancer, circulatory, digestive, hematopoeitic, and muscular diseases, disorders, and conditions. In addition, reprogrammed cells may be used for reconstructive applications, such as for repairing or replacing tissues or organs.

With respect to the therapeutic methods of the invention, it is not intended that the administration of RPSCs to a mammal be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration, including intramuscular, intravenous, intraarticular, intralesional, subcutaneous, or any other route sufficient to provide a dose adequate to prevent or treat a disease. The RPSCs may be administered to the mammal in a single dose or multiple doses. When multiple doses are administered, the doses may be separated from one another by, for example, one week, one month, one year, or ten years. One or more growth factors, hormones, interleukins, cytokines, or other cells may also be administered before, during, or after administration of the cells to further bias them towards a particular cell type.

The RPSCs of the present invention may be used as an in vitro model of differentiation, in particular for the study of genes which are involved in the regulation of early development. Differentiated cell tissues and organs using the RPSCs may be used in drug studies.

Furthermore, the RPSCs produced according to the invention maybe introduced into animals, e.g., SCID mice, cows, pigs, e.g., under the renal capsule or intramuscularly and used to produce a teratoma therein. This teratoma can be used to derive different tissue types. Also, the inner cell mass produced by X-species nuclear transfer may be introduced together with a biodegradable, biocompatible polymer matrix that provides for the formation of 3-dimensional tissues. After tissue formation, the polymer degrades, ideally just leaving the donor tissue, e.g., cardiac, pancreatic, neural, lung, liver. In some instances, it may be advantageous to include growth factors and proteins that promote angiogenesis. Alternatively, the formation of tissues can be effected totally in vitro, with appropriate culture media and conditions, growth factors, and biodegradable polymer matrices.

Applications of the Somatic Cell Reprogramming Methods and RPSCs in Animals

The reprogramming methods disclosed herein may be used to generate RPSCs for a variety of animal species. The RPSCs generated can be useful to produce desired animals. Animals include, for example, avians and mammals as well as any animal that is an endangered species. Exemplary birds include domesticated birds (e.g., quail, chickens, ducks, geese, turkeys, and guinea hens) as well as other birds such as birds of prey (e.g., hawks, falcons, ospreys, condors, etc.), endangered birds (e.g., parrots, California condor, etc.), ostriches etc. Exemplary mammals include murine, caprine,

ovine, bovine, porcine, canine, feline and primate. Of these, preferred members include domesticated animals, including, for examples, cattle, buffalo, pigs, horses, cows, rabbits, guinea pigs, sheep, and goats.

RPSCs generated by the reprogramming methods of the present invention allows one, for the first time, to genetically engineer animals other than mouse and human. RPSCs are ES-like cells, and are thus amenable to genetic manipulation. To date, no ES cells are available for animals other than mouse and human. As a result, for these animals, it is currently practically impossible to create genetically modified animals having targeted mutations. The ES-cell like RPSCs can be manipulated to introduce desired targeted genetic modifications. The resulting engineered RPSCs can then be used to generate a cloned animal with the desired genetic modifications in its germ line, using methods described for ES cells in mouse. See Capecchi and Thomas, U.S. Pat. Nos. 5,487,992, 5,627,059, 5,631,153, and 6,204,061. Genetic engineering in animals has potentially great applications in a variety of animals, especially farm animals.

The somatic cell reprogramming methods of the present invention provides at least two methods for delivering optimized farm animals. In the first, somatic cell reprogramming can be used to capture the best available phenotype for a farm animal stock. The current technologies used to deliver optimized farm animals are based on selective breeding, and expansion from preferred breeding stocks. Animals that have been selected on the basis of superior characteristics, including, for example, meat content, egg production (in the case of poultry), feed conversion ratio, are used to breed large numbers of animals that are in turn used in the human food supply. This traditional process has profound inherent inefficiencies. The phenotype observed in an individual animal is often only partially transmitted in the progeny of that animal. Therefore, traditional breeding schemes are inefficient in capturing the very best phenotype in all of the progeny animals. In contrast, the reprogramming methods of the present invention provides a controlled and efficient way to achieve the same goal, by generating RPSCs from somatic cells of an animal with the desired characteristics. The RPSCs generated may be used immediately to generate cloned animals derived from the RPSCs. Known methods for generating mice from ES cells can be used for this procedure. Alternatively, the RPSCs generated may be cryopreserved and thawed in response to a grower's needs.

In the second method, somatic cells from an animal with the desired characteristics are reprogrammed to produce RPSCs. The RPSCs are further genetically engineered to introduce desired genetic modification(s), before being placed into a recipient embryo to produce desired progeny.

The reprogramming methods can also be used to rescue endangered species. Somatic cell reprogramming provides an efficient method to generate RPSCs from somatic cells of an endangered animal. The resulting RPSCs can be used immediately to expand the numbers of the endangered animal. Alternatively, the RPSCs can be cryopreserved to generate a RPSC stock for the endangered species, as a safeguard measure against extinction of the endangered species.

Methods for Gene Identification

The present invention provides methods for identifying a gene that activates the expression of an endogenous pluripotency gene in somatic cells. The methods comprise: transfecting the somatic cells of the present invention with a cDNA library prepared from ES cells or oocytes, selecting for cells that express the first selectable marker, and assessing the expression of the first endogenous pluripotency gene in the transfected cells that express the first selectable marker. The expression of the first endogenous pluripotency gene indicates that the cDNA encodes a gene that activates the expression of an endogenous pluripotency gene in somatic cells.

The methods are applicable for identifying a gene that activates the expression of at least two endogenous pluripotency genes in somatic cells. The somatic cells used in the methods further comprise a second endogenous pluripotency gene linked to a second selectable marker. The methods are modified to select for transfected cells that express both selectable markers, among which the expression of the first and the second endogenous pluripotency genes are assessed. The expression of both the first and the second endogenous pluripotency genes indicates that the cDNA encodes a gene that activates the expression of at least two pluripotency genes in somatic cells.

The methods are further applicable for identifying a gene that activates the expression of at least three endogenous pluripotency genes in somatic cells. The somatic cells used in the methods further comprise a third endogenous pluripotency gene linked to a third selectable marker. The methods are modified to select for transfected cells that express all three selectable markers, among which the expression of all three endogenous pluripotency genes are assessed. The expression of all three endogenous pluripotency genes indicates that the cDNA encodes a gene that activates the expression of at least three pluripotency genes in somatic cells.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of mouse genetics, developmental biology, cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, Current Protocols in Cell Biology, ed. by Bonifacino, Dasso, Lippincott-Schwartz, Harford, and Yamada, John Wiley and Sons, Inc., New York, 1999; Manipulating the Mouse Embryos, A Laboratory Manual, 3rd Ed., by Hogan et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2003; Gene Targeting: A Practical Approach, IRL Press at Oxford University Press, Oxford, 1993; and Gene Targeting Protocols, Human Press, Totowa, N.J., 2000. All patents, patent applications and references cited herein are incorporated in their entirety by reference.

EXEMPLIFICATION

The invention now being generally described, it will be more readily understood by reference to the following example, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example. Oct4-Induced Fibroblasts are More Susceptible to Reprogramming than Unduced Fibroblasts as Demonstrated by Nuclear Transfer Experiment

A. Generation of Transgenic Mouse Carrying an Inducible Oct4 Transgene

An inducible Oct4 allele is constructed as the following: first, two integration vectors are constructed. The first integration vector, inducible Oct4 integration vector, contains an Oct4 gene driven by a tetracycline-inducible promoter (Tet-Op). The Tet-Op-Oct4 cassette is flanked by a splice-acceptor double poly-A signal (SA-dpA) at its 5' end and a SV40 polyA tail (SV40-pA) at its 3' end. The second integration vector, tetracycline activator integration vector, contains a mutant form of tetracycline activator, M2-rtTA, which is

more responsive to doxycycline (Dox) induction than the wild type activator. (Urlinger S. et al., 2000)

The two integration vectors are introduced into V6.5 ES cells: the inducible Oct4 integration vector and the tetracycline activator integration vector are introduced into the Collagen locus and the Rosa26 locus respectively via site-specific integration, as shown in FIG. **1**. The resulting ES cells are used to make Oct4-inducible mice by tetraploid complementation.

B. Expression of the Inducible Oct4 Transgene

Fibroblasts derived from tail biopsies of the Oct4-inducible mice were cultured. A fraction of the cultured fibroblasts were induced with doxycycline for 3 days (at 2 microgram/ml), and Oct4 expression was detected by Northern blot and Western blot analysis. As shown in FIG. **2**, the Oct4 expression level in fibroblasts treated with doxycycline is comparable to the Oct4 expression level in ES cells, and undetectable in fibroblasts not treated with doxycycline. The expression results demonstrate that the inducible Oct4 transgene is expressed as planned.

C. Nuclear Transfer Experiment

Nuclear transfer was performed on fibroblasts derived from tail biopsies of mice that carry the inducible Oct4 transgene. Dox induction was for 24 hours prior to nuclear transfer. Cloned embryos were then activated and cultured to the blastocyst stage to derive ES cells as described previously (Hochedlinger and Jaenisch, 2002). As shown in Table 1, on average, blastocyst formation and ES cell derivation (as measured as a fraction of eggs with pronucleus formation) is more efficient from Oct4 induced fibroblast than from uninduced fibroblasts. This result demonstrated that induced Oct4 expression in somatic cells such as fibroblasts make these cells more susceptible to reprogramming.

One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods, systems and kits are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention and are defined by the scope of the claims. It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

TABLE 1

In vitro development of clones derived from Oct4-induced fibroblasts

Expt.	Oct4	eggs w/PN	Blastocysts (% PN)		ES lines (% PN)	
#1	−	22	5 (23%)	19%	0 (0%)	3%
#2	−	35	5 (14%)		2 (6%)	
#3	+	37	10 (27%)	24%	2 (5%)	7%
#4	+	47	10 (21%)		4 (9%)	

PN . . . ProNucleus formation

Nuclear transfer was performed on fibroblasts derived from tail biopsies of mice that carry the inducible Oct4 transgene. Dox induction was for 24 hours prior to nuclear transfer, Cloned embryos were then activated and cultured to the blastocyst stage to derive ES cells as described previously (Hochedlinger and Jaenisch, Nature, 2002). These preliminary results show that on average blastocyst formation and ES cell derivation is more efficient from Oct4 induced than from uninduced fibroblasts.

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What is claimed is:

1. A method of making a somatic cell more susceptible to reprogramming to a pluripotent state comprising introducing at least one exogenous nucleic acid encoding Oct 4 operably linked to at least one regulatory sequence into the cell, thereby increasing expression of Oct4 protein in the somatic cell, wherein increased expression of Oct4 protein makes the cell more susceptible to reprogramming to a pluripotent state.

2. The method of claim **1**, further comprising introducing an exogenous nucleic acid encoding Nanog or Sox2 operably linked to at least one regulatory sequence.

3. The method of claim **1**, further comprising introducing an exogenous nucleic acid encoding Nanog operably linked to at least one regulatory sequence.

4. The method of claim **1**, wherein the somatic cell does not comprise a selectable marker integrated into the endogenous locus of the pluripotency gene.

5. The method of claim **1**, wherein the somatic cell is a human cell or a mouse cell.

6. The method of claim **1**, wherein the somatic cell is an adult stem cell.

7. The method of claim **6**, wherein the adult stem cell is a hematopoietic stem cell, neural stem cell, or mesenchymal stem cell.

8. The method of claim **1**, further comprising introducing an endogenous nucleic acid encoding Sox2 operably linked to at least one regulatory sequence.

* * * * *

EXHIBIT C

US008951797B2

(12) **United States Patent**
Jaenisch et al.

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(45) **Date of Patent: *Feb. 10, 2015**

(54) **COMPOSITIONS FOR IDENTIFYING REPROGRAMMING FACTORS**

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This patent is subject to a terminal disclaimer.

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(52) **U.S. Cl.**

CPC ***C12N 15/85*** (2013.01); ***A01K 67/0273*** (2013.01); ***A01K 67/0275*** (2013.01); ***C07K 14/4702*** (2013.01); ***C12N 15/8509*** (2013.01); ***C12N 15/8775*** (2013.01); ***C12N 5/0696*** (2013.01); *A01K 2217/05* (2013.01); *A01K 2227/105* (2013.01); *C12N 2830/003* (2013.01); *C12N 2830/006* (2013.01)

USPC **435/377**; 435/325

(58) **Field of Classification Search**

USPC 435/377, 325

See application file for complete search history.

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(57) **ABSTRACT**

The invention provides methods for reprogramming somatic cells to generate multipotent or pluripotent cells. Such methods are useful for a variety of purposes, including treating or preventing a medical condition in an individual. The invention further provides methods for identifying an agent that reprograms somatic cells to a less differentiated state.

10 Claims, 2 Drawing Sheets

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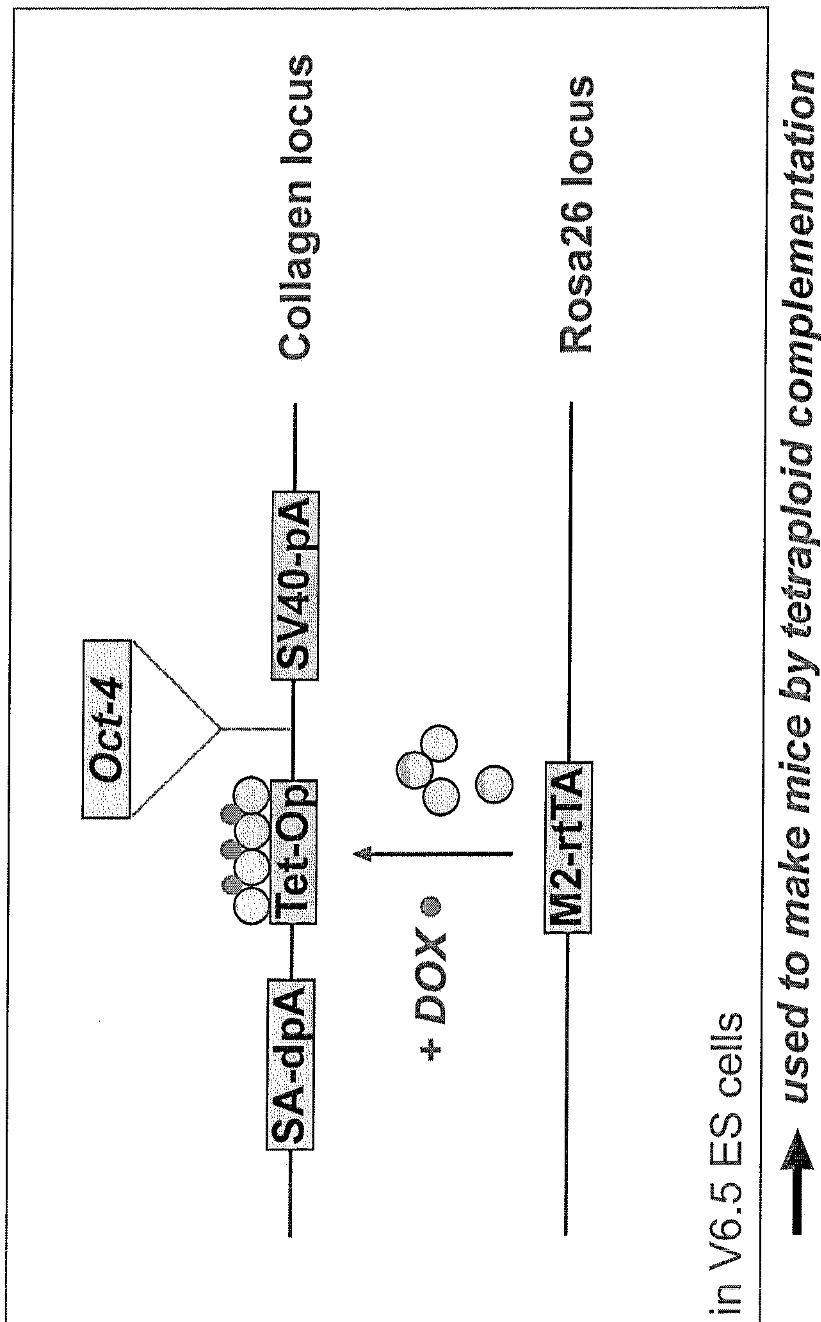
Figure 1. Inducible *Oct4* allele



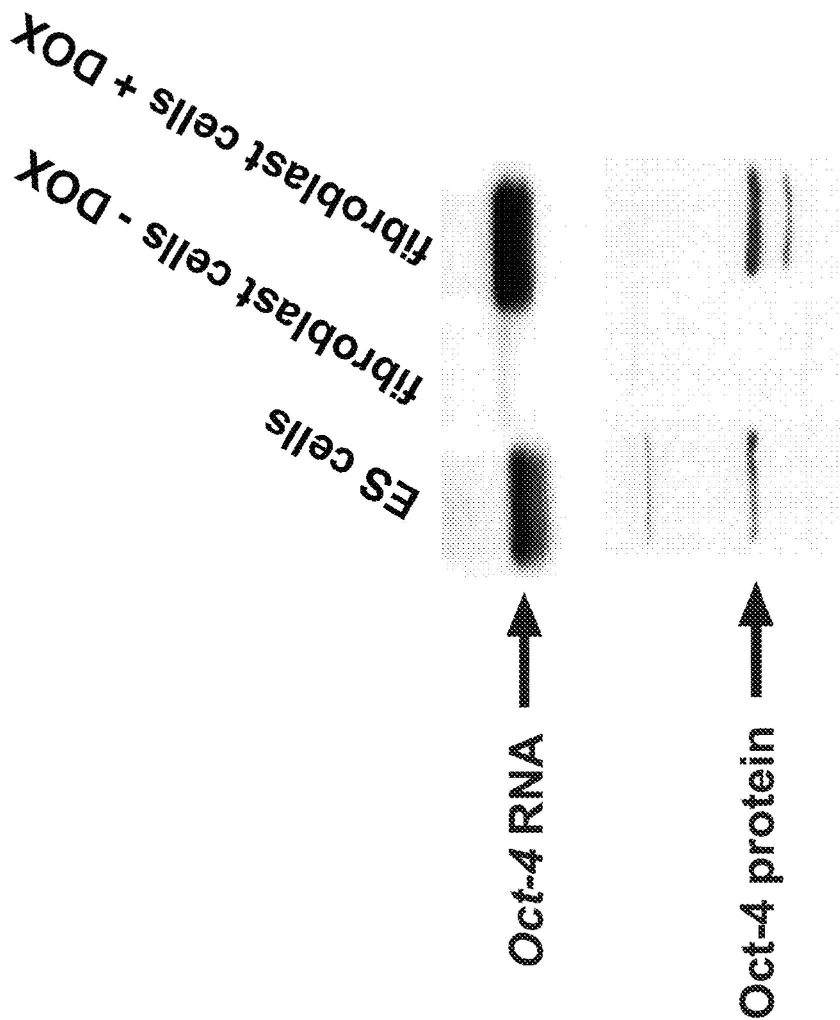


Figure 2. The system works...

COMPOSITIONS FOR IDENTIFYING REPROGRAMMING FACTORS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 12/703,061, filed Feb. 9, 2010, which is a divisional of U.S. application Ser. No. 10/997,146, filed Nov. 24, 2004 (U.S. Pat. No. 7,682,828), which claims the benefit of U.S. Provisional Application No. 60/525,612, filed Nov. 26, 2003, and U.S. Provisional Application No. 60/530,042, filed Dec. 15, 2003, the specifications of which are incorporated herein by reference in their entirety.

GOVERNMENTAL FUNDING

This invention was made with government support under R37 CA84198 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Pluripotent stem cells have the potential to differentiate into the full range of daughter cells having distinctly different morphological, cytological or functional phenotypes unique to a specific tissue. By contrast, descendants of pluripotent cells are restricted progressively in their differentiation potential, with some cells having only one fate. Pluripotent cells have extraordinary scientific and therapeutic potential, as they can be differentiated along the desired differentiation pathway in a precisely controlled manner and used in cell-based therapy.

Two categories of pluripotent stem cells are known to date: embryonic stem cells and embryonic germ cells. Embryonic stem cells are pluripotent stem cells that are derived directly from an embryo. Embryonic germ cells are pluripotent stem cells that are derived directly from the fetal tissue of aborted fetuses. For purposes of simplicity, embryonic stem cells and embryonic germ cells will be collectively referred to as "ES" cells herein.

ES cells are presently obtained via several methods. In a first method, an ES cell line is derived from the inner cell mass of a normal embryos in the blastocyst stage (See U.S. Pat. No. 6,200,806, Thompson, J. A. et al. Science, 282:1145-7, 1998 and Hogan et al., 2003). A second method for creating pluripotent ES cells utilizes the technique of somatic cell nuclear transfer (SCNT). In this technique, the nucleus is removed from a normal egg, thus removing the genetic material. Next, a donor diploid somatic cell is placed next to the enucleated egg and the two cells are fused, or the nucleus is introduced directly into the oocyte by micromanipulation. The fused cell has the potential to develop into a viable embryo, which may then be sacrificed to remove that portion of the embryo containing the stem cell producing inner cell mass.

In a third method, the nucleus of a human cell is transplanted into an entirely enucleated animal oocyte of a species different from the donor cell (referred to herein as animal stem cell nuclear transfer, or "ASCNT"). See U.S. Pat. application Ser. No. 20010012513 (2001). The resultant chimeric cells are used for the production of pluripotent ES cells, in particular human-like pluripotent ES cells. One disadvantage of this technique is that these chimeric cells may contain unknown non-human viruses and still contain the mitochondria of the animal species. Thus, there would be substantial risks of immune rejection if such cells were used in cell transplantation therapies.

In a fourth method, ES cells can be isolated from the primordial germ cells found in the genital ridges of post-implanted embryos.

As described above, all presently available methods depend on controversial sources—embryos (either created naturally or via cloning), fetal tissue and via the mixing of materials of multiple species. The controversy surrounding the sources for such cells, according to many leading scientists and public and private organizations including the NIH, has greatly compromised and slowed the study of such cells and their application.

There is thus a great demand for alternative methods of generating pluripotent cells.

SUMMARY OF THE INVENTION

The present invention provides engineered somatic cells, in which one or more endogenous pluripotency gene(s) is operably linked to a selectable marker in such a manner that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene to which the marker is linked. The invention also provides transgenic mice containing these engineered somatic cells.

The present invention also provides methods for reprogramming somatic cells to a less differentiated state. In the methods, engineered somatic cells of the invention are treated with an agent. Cells that express the selectable marker are then selected, and assessed for pluripotency characteristics. The treatment with an agent may be contacting the cells with an agent which alters chromatin structure, or may be transfecting the cells with at least one pluripotency gene, or both.

The present invention further provides methods for identifying an agent that reprograms somatic cells to a less differentiated state. In the methods, the engineered somatic cells described above are contacted with a candidate agent. Cells that express the selectable marker are then selected, and assessed for pluripotency characteristics. The presence of at least a subset of pluripotency characteristics indicates that the agent is capable of reprogramming somatic cells to a less-differentiated state. The agents identified by the present invention can then by used to reprogram somatic cells by contacting somatic cells with the agents.

The present invention also provides methods for identifying a gene that causes the expression of at least one endogenous pluripotency gene in somatic cells. In the methods, the engineered somatic cells are transfected with a cDNA library prepared from a pluripotent cell, such as an ES cell. The cells that express the appropriate selectable marker are then selected, and the expression of the appropriate endogenous pluripotency gene is examined. The expression of an endogenous pluripotency gene indicates that the cDNA encodes a protein whose expression in the cell results in, directly or indirectly, expression of the endogenous pluripotency gene.

The present invention further provides methods for treating a condition in an individual in need of such treatment. In certain embodiments, somatic cells are obtained from the individual and reprogrammed by the methods of the invention under conditions suitable for the cells to develop into cells of a desired cell type. The reprogrammed cells of a desired cell type are then harvested and introduced into the individual to treat the condition. In certain further embodiments, the somatic cells obtained from the individual contains a mutation in one or more genes. In these instances, the methods are modified so that the somatic cells obtained from the individual are first treated to restore the one or more normal gene(s) to the cells such that the resulting cells carry the normal endogenous gene, which are then introduced into the

individual. In certain other embodiments, methods of the invention can be used to treat individuals in need of a functional organ. In the methods, somatic cells are obtained from an individual in need of a functional organ, and reprogrammed by the methods of the invention to produce reprogrammed somatic cells. Such reprogrammed somatic cells are then cultured under conditions suitable for development of the reprogrammed somatic cells into a desired organ, which is then introduced into the individual. The methods are useful for treating any one of the following conditions: a neurological, endocrine, structural, skeletal, vascular, urinary, digestive, integumentary, blood, autoimmune, inflammatory, or muscular condition.

The present invention also provides methods for producing a cloned animal. In the methods, a somatic cell is isolated from an animal having desired characteristics, and reprogrammed using the methods of the invention to produce one or more reprogrammed pluripotent somatic cell ("RPSC"). The RPSCs are then inserted into a recipient embryo, and the resulting embryo is cultured to produce an embryo of suitable size for implantation into a recipient female, which is then transferred into a recipient female to produce a pregnant female. The pregnant female is maintained under conditions appropriate for carrying the embryo to term to produce chimeric animal progeny, which is then bred with a wild type animal to produce a cloned animal.

In certain embodiments, the RPSCs may alternatively be cryopreserved for future cloning uses. In certain other embodiments, genetic modification, such as a targeted mutation, may be introduced into the RPSCs prior to its insertion into a recipient embryo.

The present invention also provides methods for producing a cloned avian. In the methods, a somatic cell is isolated from an avian having desired characteristics, and reprogrammed using the methods of the invention to produce one or more reprogrammed pluripotent somatic cell ("RPSC"). The RPSCs are then inserted into eggs that are unable to develop into an embryo, and the resulting eggs are then incubated to produce avian offspring having the genotype of the RPSC, thereby producing a cloned avian.

It is contemplated that all embodiments described above are applicable to all different aspects of the invention. It is also contemplated that any of the above embodiments can be freely combined with one or more other such embodiments whenever appropriate.

Specific embodiments of the invention are described in more detail below. However, these are illustrative embodiments, and should not be construed as limiting in any respect.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts an inducible Oct4 allele.

FIG. **2** shows the expression of the inducible Oct4 transgene by Northern blot and Western blot analysis.

DETAILED DESCRIPTION OF THE INVENTION

Overview

Presently, human ES cells or ES-like cells can only be generated from controversial sources. It would be useful to reprogram somatic cells directly into pluripotent cells. Nuclei from somatic cells retain the totopotency potential to direct development of an animal, as demonstrated by nuclear transfer technology. It would be useful to reprogram somatic cells directly into ES cells without the use of oocytes and nuclear transfer technology.

Applicants have devised novel methods of reprogramming somatic cells to generate pluripotent cells or multipotent cells. Applicants have also devised novel methods to identify agents that reprogram somatic cells. The methods take advantage of the engineered somatic cells designed by Applicants, in which an endogenous gene typically associated with pluripotency ("pluripotency gene") is engineered to be operably linked to a selectable marker in a manner that the expression the endogenous pluripotency gene substantially matches the expression of the selectable marker. Because pluripotency genes are generally expressed only in pluripotent cells and not in somatic cells, the expression of an endogenous pluripotent gene(s) is an indication of successful reprogramming. Having a selectable marker operably linked to an endogenous pluripotency gene gives one a powerful mechanism to select for potentially reprogrammed somatic cells, which likely is a rare occurrence. The resulting cells may be further assessed for pluripotency characteristics to confirm whether a somatic cell has been successfully reprogrammed to pluripotency.

Generating pluripotent or multipotent cells by somatic cell reprogramming using the methods of the present invention has at least two advantages. First, the methods of the present invention allow one to generate autologous pluripotent cells, which are cells specific to a patient. The use of autologous cells in cell therapy offers a major advantage over the use of non-autologous cells, which are likely to be subject to immunological rejection. In contrast, autologous cells are unlikely to elicit significant immunological responses (See Munsie et al, 2000). Second, the methods of the present invention allow one to generate pluipotent without using embryos, oocytes and/or nuclear transfer technology.

A pluripotent cell is a cell that has the potential to divide in vitro for a long period of time (greater than one year) and has the unique ability to differentiate into cells derived from all three embryonic germ layers—endoderm, mesoderm and ectoderm.

A multipotent cell is a cell that is able to differentiate into some but not all of the cells derived from all three germ layers. Thus, a multipotent cell is a partially differentiated cell. Adult stem cells are multipotent cells. Known adult stem cells include, for example, hematopoietic stem cells and neural stem cells. A hematopoietic stem cell is multipotent because it has the ability to differentiate into all types of specific blood cells, but it is unlikely that they can differentiate into all cells of a given animal or human. Multipotent/adult stem cells have a great deal of promise in research and in the area of therapeutic applications. For example, multipotent/adult stem cells have already been used in humans in attempts to treat certain blood, neural and cancer diseases.

The term "pluripotency gene", as used herein, refers to a gene that is associated with pluripotency. The expression of a pluripotency gene is typically restricted to pluripotent stem cells, and is crucial for the functional identity of pluripotent stem cells. The transcription factor Oct-4 (also called Pou5fl, Oct-3, Oct3/4) is an example of a pluripotency gene. Oct-4 has been shown to be required for establishing and maintaining the undifferentiated phenotype of ES cells and plays a major role in determining early events in embryogenesis and cellular-differentiation (Nichols et al., 1998, Cell 95:379-391; Niwa et al., 2000, Nature Genet. 24:372-376). Oct-4 is down-regulated as stem cells differentiate into specialised cells. Other exemplary pluripotency genes include Nanog, and Stella (See Chambers et al., 2003, Cell 113: 643-655; Mitsui et al., Cell. 2003, 113(5):631-42; Bortvin et al. Development. 2003, 130(8):1673-80; Saitou et al., Nature. 2002, 418 (6895):293-300.

Engineered Somatic Cells and Transgenic Mice Comprising Such Cells

The present invention provides somatic cells comprising an endogenous pluripotency gene linked to DNA encoding a selectable marker in such a manner that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene. In one embodiment, the somatic cells of the present invention comprise a first endogenous pluripotency gene linked to DNA encoding a first selectable marker in such a manner that the expression of the first selectable marker substantially matches the expression of the first endogenous pluripotency gene. The somatic cells may also be engineered to comprise any number of endogenous pluripotency genes respectively linked to a distinct selectable marker. Thus, in another embodiment, the somatic cells of the present invention comprise two endogenous pluripotency genes, each of which is linked to DNA encoding a distinct selectable marker. In a further embodiment, the somatic cells of the present invention comprise three endogenous pluripotency genes, each of which is linked to DNA encoding a distinct selectable marker. The somatic cells described above will be collectively referred in this application as "engineered somatic cells." The engineered somatic cells may be further engineered to have one or more pluripotency gene expressed as a transgene under an inducible promoter.

The selectable marker is linked to an appropriate endogenous pluripotency gene such that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene. By "substantially match", it is meant that the expression of the selectable marker substantially reflects the expression pattern of the endogenous pluripotency gene. In other words, the selectable marker and the endogenous pluripotency gene are co-expressed. For purpose of the present invention, it is not necessary that the expression level of the endogenous gene and the selectable marker is the same or even similar. It is only necessary that the cells in which an endogenous pluripotency gene is activated will also express the selectable marker at a level sufficient to confer a selectable phenotype on the reprogrammed cells. For example, when the selectable marker is a marker that confers resistance to a lethal drug (a "drug resistance marker"), the cells are engineered in a way that allows cells in which an endogeneous pluripotency gene is activated to also express the drug resistance marker at a sufficient level to confer on reprogrammed cells resistance to lethal drugs. Thus, reprogrammed cells will survive and proliferate whereas non-reprogrammed cells will die.

The DNA encoding a selectable marker may be inserted downstream from the end of the open reading frame (ORF) encoding the desired endogenous pluripotency gene, anywhere between the last nucleotide of the ORF and the first nucleotide of the polyadenylation site. An internal ribosome entry site (IRES) may be placed in front of the DNA encoding the selectable marker. Alternatively, the DNA encoding a selectable marker may be inserted anywhere within the ORF of the desired endogenous pluripotency gene, downstream of the promoter, with a termination signal. An internal ribosome entry site (IRES) may be placed in front of the DNA encoding the selectable marker. The selectable marker may be inserted into only one allele, or both alleles, of the endogenous pluripotency gene.

The somatic cells in the invention may be primary cells or immortalized cells. Such cells may be primary cells (non-immortalized cells), such as those freshly isolated from an animal, or may be derived from a cell line (immortalized cells).

The somatic cells in the present invention are mammalian cells, such as, for example, human cells or mouse cells. They may be obtained by well-known methods, from different organs, e.g., skin, lung, pancreas, liver, stomach, intestine, heart, reproductive organs, bladder, kidney, urethra and other urinary organs, etc., generally from any organ or tissue containing live somatic cells. Mammalian somatic cells useful in the present invention include, by way of example, adult stem cells, sertoli cells, endothelial cells, granulosa epithelial, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc. generally any live somatic cells. The term "somatic cells", as used herein, also includes adult stem cells. An adult stem cell is a cell that is capable of giving rise to all cell types of a particular tissue. Exemplary adult stem cells include hematopoietic stem cells, neural stem cells, and mesenchymal stem cells.

In one embodiment, the engineered somatic cells are obtained from a transgenic mouse comprising such engineered somatic cells. Such transgenic mouse can be produced using standard techniques known in the art. For example, Bronson et al. describe a technique for inserting a single copy of a transgene into a chosen chromosomal site. See Bronson et al., 1996. Briefly, a vector containing the desired integration construct (for example, a construct containing a selectable marker linked to a pluripotency gene) is introduced into ES cells by standard techniques known in the art. The resulting ES cells are screened for the desired integration event, in which the knock-in vector is integrated into the desired endogenous pluripotency gene locus such that the selectable marker is integrated into the genomic locus of the pluripotency gene and is under the control of the pluripotency gene promoter. The desired ES cell is then used to produce transgenic mouse in which all cell types contain the correct integration event. Desired types of cells may be selectively obtained from the transgenic mouse and maintained in vitro. In one embodiment, two or more transgenic mice may be created, each carrying a distinct integration construct. These mice may then be bred to generate mice that carry multiple desired integration construct. For example, one type of transgenic mouse may be created to carry an endogenous pluripotency gene linked to a selectable marker, while a second type of transgenic mouse may be created to carry a pluripotency gene expressed as a transgene under an inducible promoter. These two types of mice may then be bred to generate transgenic mice that have both a selectable marker linked to an endogenous pluripotency gene and an additional pluripotency gene expressed as a transgene under an inducible promoter. These two pluripotency genes may or may not be the same. Many variables are contemplated: the identity of the endogenous pluripotency gene linked to marker, the identity of the pluripotency gene expressed as a transgene, and the number of the endogenous pluripotency gene linked to a selectable marker, and the number of pluripotency gene expressed as a transgene. The present invention encompasses all possible combinations of these variables.

Alternatively, engineered somatic cells of the present invention may be produced by direct introduction of the desired construct into somatic cells. DNA construct may be introduced into cells by any standard technique known in the art, such as viral transfection (eg. using an adenoviral system) or liposome-mediated transfection. Any means known in the art to generate somatic cells with targeted integration can be used to produce somatic cells of the invention. In mammalian

cells, homologous recombination occurs at much lower frequency compared to non-homologous recombination. To facilitate the selection of homologous recombination events over the non-homologous recombination events, at least two enrichment methods have been developed: the positive-negative selection (PNS) method and the "promoterless" selection method (Sedivy and Dutriaux, 1999). Briefly, PNS, the first method, is in genetic terms a negative selection: it selects against recombination at the incorrect (non-homologous) loci by relying on the use of a negatively selectable gene that is placed on the flanks of a targeting vector. On the other hand, the second method, the "promoterless" selection, is a positive selection in genetic terms: it selects for recombination at the correct (homologous) locus by relying on the use of a positively selectable gene whose expression is made conditional on recombination at the homologous target site. The disclosure of Sedivy and Dutriaux is incorporated herein.

A selectable marker, as used herein, is a marker that, when expressed, confers upon recipient cells a selectable phenotype, such as antibiotic resistance, resistance to a cytotoxic agent, nutritional prototrophy or expression of a surface protein. The presence of a selectable marker linked to an endogenous pluripotency gene makes it possible to identify and select reprogrammed cells in which the endogenous pluripotency gene is expressed. A variety of selectable marker genes can be used, such as neomycin resistance gene (neo), puromycin resistance gene (puro), guanine phosphoribosyl transferase (gpt), dihydrofolate reductase (DHFR), adenosine deaminase (ada), puromycin-N-acetyltransferase (PAC), hygromycin resistance gene (hyg), multidrug resistance gene (mdr), and hisD gene.

The present invention further provides transgenic mice comprising the somatic cells of the invention.

Methods for Reprogramming Somatic Cells

The present invention further provides methods for reprogramming somatic cells to a less differentiated state. The resulting cells are termed "reprogrammed somatic cells" ("RSC") herein. A RSC may be a reprogrammed pluripotent somatic cell ("RPSC"), a reprogrammed multipotent somatic cell ("RMSC"), or a reprogrammed somatic cell of varying differentiation status.

In general, the methods comprise treating the engineered somatic cells with an agent. The treatment with an agent may be contacting the cells with an agent which alters chromatin structure, or may be transfecting the cells with one or more pluripotency gene, or both. The above two treatments may be concurrent, or may be sequential, with no particular preference for order. In a further embodiment, reprogrammed somatic cells are identified by selecting for cells that express the appropriate selectable marker. In still a further embodiment, reprogrammed somatic cells are further assessed for pluripotency characteristics. The presence of pluripotency characteristics indicates that the somatic cells have been reprogrammed to a pluripotent state.

Differentiation status of cells is a continuous spectrum, with terminally differentiated state at one end of this spectrum and de-differentiated state (pluripotent state) at the other end. Reprogramming, as used herein, refers to a process that alters or reverses the differentiation status of a somatic cell, which can be either partially or terminally differentiated. Reprogramming includes complete reversion, as well as partial reversion, of the differentiation status of a somatic cell. In other words, the term "reprogramming", as used herein, encompasses any movement of the differentiation status of a cell along the spectrum toward a less-differentiated state. For example, reprogramming includes reversing a multipotent cell back to a pluripotent cell, reversing a terminally differentiated cell back to either a multipotent cell or a pluripotent cell. In one embodiment, reprogramming of a somatic cell turns the somatic cell all the way back to a pluripotent state. In another embodiment, reprogramming of a somatic cell turns the somatic cell back to a multipotent state. The term "less-differentiated state", as used herein, is thus a relative term and includes a completely de-differentiated state and a partially differentiated state.

The term "pluripotency characteristics", as used herein, refers to many characteristics associated with pluripotency, including, for example, the ability to differentiate into all types of cells and an expression pattern distinct for a pluripotent cell, including expression of pluripotency genes, expression of other ES cell markers, and on a global level, a distinct expression profile known as "stem cell molecular signature" or "stemness."

Thus, to assess reprogrammed somatic cells for pluripotency characteristics, one may analyze such cells for different growth characteristics and ES cell-like morphology. Cells may be injected subcutaneously into immunocompromised SCID mice to induce teratomas (a standard assay for ES cells). ES-like cells can be differentiated into embryoid bodies (another ES specific feature). Moreover, ES-like cells can be differentiated in vitro by adding certain growth factors known to drive differentiation into specific cell types. Self-renewing capacity, marked by induction of telomerase activity, is another pluripotency characteristics that can be monitored. One may carry out functional assays of the reprogrammed somatic cells by introducing them into blastocysts and determine whether the cells are capable of giving rise to all cell types. See Hogan et al., 2003. If the reprogrammed cells are capable of forming a few cell types of the body, they are multipotent; if the reprogrammed cells are capable of forming all cell types of the body including germ cells, they are pluripotent.

One may also examine the expression of an individual pluripotency gene in the reprogrammed somatic cells to assess their pluripotency characteristics. Additionally, one may assess the expression of other ES cell markers. Stage-specific embryonic 15 antigens-1, -3, and -4 (SSEA-1, SSEA-3, SSEA-4) are glycoproteins specifically expressed in early embryonic development and are markers for ES cells (Solter and Knowles, 1978, Proc. Natl. Acad. Sci. USA 75:5565-5569; Kannagi et al., 1983, EMBO J. 2:2355-2361). Elevated expression of the enzyme Alkaline Phosphatase (AP) is another marker associated with undifferentiated embryonic stem cells (Wobus et al., 1984, Exp. Cell 152:212-219; Pease et al., 1990, Dev. Biol. 141:322-352). Other stem/progenitor cells markers include the intermediate neurofilament nestin (Lendahl et al., 1990, Cell 60:585-595; Dah-Istrand et al., 1992, J. Cell Sci. 103:589-597), the membrane glycoprotein prominin/AC133 (Weigmann et al., 1997, Proc. Natl. Acad. USA 94:12425-12430; Corbeil et al., 1998, Blood 91:2625-22626), the transcription factor Tcf-4 (Korinek et al, 1998, Nat. Genet. 19: 379-383; Lee et al., 1999, J. Biol. Chem. 274.1566-1572), and the transcription factor Cdx1 (Duprey et al., 1988, Genes Dev. 2:1647-1654; Subramania'n et al., 1998, Differentiation 64:11-18).

One may additionally conduct expression profiling of the reprogrammed somatic cells to assess their pluripotency characteristics. Pluripotent cells, such as embryonic stem cells, and multipotent cells, such as adult stem cells, are known to have a distinct pattern of global gene expression profile. This distinct pattern is termed "stem cell molecular signature", or "stemness". See, for example, Ramalho-Santos et al., Science 298: 597-600 (2002); Ivanova et al., Science 298: 601-604.

Somatic cells may be reprogrammed to gain either a complete set of the pluripotency characteristics and are thus pluripotent. Alternatively, somatic cells may be reprogrammed to gain only a subset of the pluripotency characteristics. In another alternative, somatic cells may be reprogrammed to be multipotent.

In a further embodiment, in conjunction with contacting the somatic cells of the invention with an agent which alters chromatin structure, at least one gene that affects pluripotent state of a cell may be further introduced into the same cells. This may be carried out sequentially. For example, the somatic cells of the invention may be first contacted with an agent which alters chromatin structure. Then at least one pluripotency gene can be introduced into the same cells, or vice versa. Alternatively, the two steps may be carried out simultaneously.

Genes that affect pluripotent state of a cell includes pluripotency genes, genes involved in chromatin remodeling, and genes that are important for maintaining pluripotency, such as LIF, BMP, and PD098059 (See Cell, 115: 281-292 (2003); Philos Trans R Soc Lond B Biol Sci. 2003 Aug. 29; 358 (1436):1397-402).

The exogenously introduced pluripotency gene may be carried out in several ways. In one embodiment, the exogenously introduced pluripotency gene may be expressed from a chromosomal locus different from the endogenous chromosomal locus of the pluripotency gene. Such chromosomal locus may be a locus with open chromatin structure, and contain gene(s) dispensible for a somatic cell. In other words, the desirable chromosomal locus contains gene(s) whose disruption will not cause cells to die. Exemplary chromosomal loci include, for example, the mouse ROSA 26 locus and type II collagen (Col2a1) locus (See Zambrowicz et al., 1997) The exogenously introduced pluripotency gene may be expressed from an inducible promoter such that their expression can be regulated as desired.

In an alternative embodiment, the exogenously introduced pluripotency gene may be transiently transfected into cells, either individually or as part of a cDNA expression library, prepared from pluripotent cells. Such pluripotent cells may be embryonic stem cells, oocytes, blastomeres, inner cell mass cells, embryonic germ cells, embryoid body (embryonic) cells, morula-derived cells, teratoma (teratocarcinoma) cells, and multipotent partially differentiated embryonic stem cells taken from later in the embryonic development process.

The cDNA library is prepared by conventional techniques. Briefly, mRNA is isolated from an organism of interest. An RNA-directed DNA polymerase is employed for first, strand synthesis using the mRNA as template. Second strand synthesis is carried out using a DNA-directed DNA polymerase which results in the cDNA product. Following conventional processing to facilitate cloning of the cDNA, the cDNA is inserted into an expression vector such that the cDNA is operably linked to at least one regulatory sequence. The choice of expression vectors for use in connection with the cDNA library is not limited to a particular vector. Any expression vector suitable for use in mouse cells is appropriate. In one embodiment, the promoter which drives expression from the cDNA expression construct is an inducible promoter. The term regulatory sequence includes promoters, enhancers and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology*, Academic Press, San Diego, Calif. (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express cDNAs. Such useful expression control sequences, include, for example, the early and late promoters of SV40, tet promoter, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

The exogenously introduced pluripotency gene may be expressed from an inducible promoter. The term "inducible promoter", as used herein, refers to a promoter that, in the absence of an inducer (such as a chemical and/or biological agent), does not direct expression, or directs low levels of expression of an operably linked gene (including cDNA), and, in response to an inducer, its ability to direct expression is enhanced. Exemplary inducible promoters include, for example, promoters that respond to heavy metals (CRC Boca Raton, Fla. (1991), 167-220; Brinster et al. Nature (1982), 296, 39-42), to thermal shocks, to hormones (Lee et al. P.N.A.S. USA (1988), 85, 1204-1208; (1981), 294, 228-232; Klock et al. Nature (1987), 329, 734-736; Israel and Kaufman, Nucleic Acids Res. (1989), 17, 2589-2604), promoters that respond to chemical agents, such as glucose, lactose, galactose or antibiotic.

A tetracycline-inducible promoter is an example of an inducible promoter that responds to an antibiotics. See Gossen et al., 2003. The tetracycline-inducible promoter comprises a minimal promoter linked operably to one or more tetracycline operator(s). The presence of tetracycline or one of its analogues leads to the binding of a transcription activator to the tetracycline operator sequences, which activates the minimal promoter and hence the transcription of the associated cDNA. Tetracycline analogue includes any compound that displays structural homologies with tetracycline and is capable of activating a tetracycline-inducible promoter. Exemplary tetracycline analogues includes, for example, doxycycline, chlorotetracycline and anhydrotetracycline.

Thus, in one embodiment, the present invention provides mice and somatic cells carrying at least one pluripotency gene expressed as a transgene under an inducible promoter. It is possible that somatic cells with such inducible pluripotency transgene(s) are more prone to be reprogrammed.

Any of the engineered somatic cells of the present invention may be used in the methods. In one embodiment, somatic cells used in the methods comprise only one endogenous pluripotency gene linked to a first selectable marker, and the selection step is carried out to select for the expression of the first selectable marker. In an alternative embodiment, the somatic cells used in the methods comprise any number of endogenous pluripotency genes, each of which is linked to a distinct selectable marker respectively, and the selection step is carried out to select for at least a subset of the selectable markers. For example, the selection step may be carried out to select for all the selectable markers linked to the various endogenous pluripotency genes.

In an alternative embodiment, somatic cells used in the method comprise a selectable marker linked to an endogenous pluripotency gene and an additional pluripotency gene expressed as a transgene under an inducible promoter. For these cells, the method of reprogramming may comprises induce the expression of the pluripotency transgene and select for the expression of the selectable marker. The method may further comprise contacting the somatic cells with an agent that alter chromatic structure.

Without wishing to be bound by theory, the agents used in the method may cause chromatin to take on a more open structure, which is more permissive for gene expression. DNA methylation and histone acetylation are two known events that alter chromatin toward a more closed structure. For example, loss of methylation by genetic deletion of DNA methylation enzyme Dnmt1 in fibroblasts results in reactivation of endogenous Oct4 gene. See J. Biol. Chem. 277: 34521-30, 2002; and Bergman and Mostoslaysky, Biol. Chem. 1990. Thus, DNA methylation inhibitors and histone deacetyation inhibitors are two classes of agents that may be used in the methods of the invention. Exemplary agents include 5-aza-cytidine, TSA and valproic acid.

In another embodiment, methods of the invention may further include repeating the steps of treating the cells with an agent. The agent used in the repeating treatment may be the same as, or different from, the one used during the first treatment.

Methods for Screening for an Agent that Reprograms Somatic Cells

The present invention also provides methods for identifying an agent that reprograms somatic cells to a less-differentiated state, as well as the agents thus identified. In one embodiment, the methods comprise contacting the engineered somatic cells of the invention with a candidate agent, selecting for cells that express the appropriate selectable marker. The presence of cells that express the appropriate selectable marker indicates that the agent reprograms somatic cells. Such an agent is referred as a "reprogramming agent" for purpose of this application.

In a further embodiment, the methods comprise contacting the engineered somatic cells of the invention with a candidate agent, selecting for cells that express the appropriate selectable marker, and assessing the cells so selected for pluripotency characteristics. The presence of a complete set of pluripotency characteristics indicates that the agent reprograms somatic cells to become pluripotent.

Candidate agents used in the invention encompass numerous chemical classes, though typically they are organic molecules, including small organic compounds. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, nucleic acids and derivatives, structural analogs or combinations thereof.

Candidate gents may be naturally arising, recombinant or designed in the laboratory. The candidate agents may be isolated from microorganisms, animals, or plants, or may be produced recombinantly, or synthesized by chemical methods known in the art. In some embodiments, candidate agents are isolated from libraries of synthetic or natural compounds using the methods of the present invention. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, including acylation, alkylation, esterification, amidification, to produce structural analogs.

There are numerous commercially available compound libraries, including, for example, the Chembridge DIVERSet. Libraries are also available from academic investigators, such as the Diversity set from the NCI developmental therapeutics program.

The screening methods mentioned above are based on assays performed on cells. These cell-based assays may be performed in a high throughput screening (HTS) format, which has been described in the art. For example, Stockwell et al. described a high-throughput screening of small molecules in miniaturized mammalian cell-based assays involving post-translational modifications (Stockwell et al., 1999). Likewise, Qian et al. described a leukemia cell-based assay for high-throughtput screening for anti-cancer agents (Qian et al., 2001). Both references are incorporated herein in their entirety.

A reprogramming agent may belong to any one of many different categories. For example, a reprogramming agent may be a chromatin remodeling agent. A chromatin remodeling agent may be a protein involved in chromatin remodeling or an agent known to alter chromatin toward a more open structure, such as a DNA methylation inhibitor or a histone deacetyation inhibitor. Exemplary compounds include 5-aza-cytidine, TSA and valproic acid. For another example, such an agent may be a pluripotency protein, including, for example, Nanog, Oct-4 and Stella. Such an agent may also be a gene essential for pluripotency, including, for example, Sox2, FoxD3, and LIF, and Stat3. See Smith et al. 1988, William et al., 1988, Ihle, 1996, Avilion et al., 2003, and Hanna et al., 2002)

Methods for Reprogramming Somatic Cells with a Reprogramming Agent

The reprogramming agent identified by the methods of the present invention is useful for reprogramming somatic cells into pluripotent or multipotent cells. Accordingly, the present invention provides methods for reprogramming somatic cells to a less differentiated state, comprising contacting somatic cells with a reprogramming agent. The somatic cells used may be native somatic cells, or engineered somatic cells. It is not necessary for these cells to carry a selectable marker integrated into the endogenous locus of a pluripotency gene.

Reprogrammed Somatic Cells and These Uses

The present invention also provides reprogrammed somatic cells (RSCs), including reprogrammed pluripotent somatic cells (RPSCs), produced by the methods of the invention. These methods, useful for the generation of cells of a desired cell type, have wide range of applications. For one example, these methods have applications in livestock management, involving the precise genetic manipulation of animals for economic or health purposes. For another example, these methods have medical application in treating or preventing a condition.

Accordingly, the invention provides methods for the treatment or prevention of a condition in a mammal. In one embodiment, the methods start with obtaining somatic cells from the individual, reprogramming the somatic cells so obtained by methods of the present invention to obtain RPSCs. The RPSCs are then cultured under conditions suitable for development of the RPSCs into cells of a desired cell type. The developed cells of the desired cell type are harvested and introduced into the individual to treat the condi-

tion. In an alternative embodiment, the methods start with obtaining somatic cells from the individual, reprogramming the somatic cells so obtained by methods of the present invention. The RPSCs are then cultured under conditions suitable for development of the RPSCs into a desired organ, which is harvested and introduced into the individual to treat the condition.

The RPSCs of the present invention are ES-like cells, and thus may be induced to differentiate to obtain the desired cell types according to known methods to differentiate ES cells. For example, the RPSCs may be induced to differentiate into hematopoietic stem cells, muscle cells, cardiac muscle cells, liver cells, cartilage cells, epithelial cells, urinary tract cells, etc., by culturing such cells in differentiation medium and under conditions which provide for cell differentiation. Medium and methods which result in the differentiation of embryonic stem cells are known in the art as are suitable culturing conditions.

For example, Palacios et al., Proc. Natl. Acad. Sci., USA, 92: 7530-37 (1995) teaches the production of hematopoietic stem cells from an embryonic cell line by subjecting stem cells to an induction procedure comprising initially culturing aggregates of such cells in a suspension culture medium lacking retinoic acid followed by culturing in the same medium containing retinoic acid, followed by transferral of cell aggregates to a substrate which provides for cell attachment.

Moreover, Pedersen, J. Reprod. Fertil. Dev., 6: 543-52 (1994) is a review article which references numerous articles disclosing methods for in vitro differentiation of embryonic stem cells to produce various differentiated cell types including hematopoietic cells, muscle, cardiac muscle, nerve cells, among others.

Further, Bain et al., Dev. Biol., 168:342-357 (1995) teaches in vitro differentiation of embryonic stem cells to produce neural cells which possess neuronal properties. These references are exemplary of reported methods for obtaining differentiated cells from embryonic or stem-like cells. These references and in particular the disclosures therein relating to methods for differentiating embryonic stem cells are incorporated by reference in their entirety herein.

Thus, using known methods and culture medium, one skilled in the art may culture the subject embryonic or stem-like cells to obtain desired differentiated cell types, e.g., neural cells, muscle cells, hematopoietic cells, etc. In addition, the use of inducible Bcl-2 or Bcl-x1 might be useful for enhancing in vitro development of specific cell lineages. In vivo, BcI-2 prevents many, but not all, forms of apoptotic cell death that occur during lymphoid and neural development. A thorough discussion of how Bcl-2 expression might be used to inhibit apoptosis of relevant cell lineages following transfection of donor cells is disclosed in U.S. Pat. No. 5,646,008, which is herein incorporated by reference.

The subject RPSCs may be used to obtain any desired differentiated cell type. Therapeutic usages of such differentiated human cells are unparalleled. For example, human hematopoietic stem cells may be used in medical treatments requiring bone marrow transplantation. Such procedures are used to treat many diseases, e.g., late stage cancers such as ovarian cancer and leukemia, as well as diseases that compromise the immune system, such as AIDS. Hematopoietic stem cells can be obtained, e.g., by fusing adult somatic cells of a cancer or AIDS patient, e.g., epithelial cells or lymphocytes with an enucleated oocyte, e.g., bovine oocyte, obtaining embryonic or stem-like cells as described above, and culturing such cells under conditions which favor differentiation, until hematopoietic stem cells are obtained. Such hematopoietic cells may be used in the treatment of diseases including cancer and AIDS.

The methods of the present invention can also be used to treat, prevent, or stabilize a neurological disease such as Alzheimer's disease, Parkinson's disease, Huntington's disease, or ALS, lysosomal storage diseases, multiple sclerosis, or a spinal cord injury. For example, somatic cells may be obtained from the individual in need of treatment, and reprogrammed to gain pluripotency, and cultured to derive neurectoderm cells that may be used to replace or assist the normal function of diseased or damaged tissue.

For the treatment or prevention of endocrine conditions, RPSCs that produce a hormone, such as a growth factor, thyroid hormone, thyroid-stimulating hormone, parathyroid hormone, steroid, serotonin, epinephrine, or norepinephrine may be administered to a mammal. Additionally, reprogrammed epithelial cells may be administered to repair damage to the lining of a body cavity or organ, such as a lung, gut, exocrine gland, or urogenital tract. It is also contemplated that RPSCs may be administered to a mammal to treat damage or deficiency of cells in an organ such as the bladder, brain, esophagus, fallopian tube, heart, intestines, gallbladder, kidney, liver, lung, ovaries, pancreas, prostate, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, ureter, urethra, or uterus.

The great advantage of the present invention is that it provides an essentially limitless supply of isogenic or synegenic human cells suitable for transplantation. Therefore, it will obviate the significant problem associated with current transplantation methods, i.e., rejection of the transplanted tissue which may occur because of host versus graft or graft versus host rejection. Conventionally, rejection is prevented or reduced by the administration of anti-rejection drugs such as cyclosporin. However, such drugs have significant adverse side-effects, e.g., immunosuppression, carcinogenic properties, as well as being very expensive. The present invention should eliminate, or at least greatly reduce, the need for anti-rejection drugs, such as cyclosporine, imulan, FK-506, glucocorticoids, and rapamycin, and derivatives thereof.

RPSCs may also be combined with a matrix to form a tissue or organ in vitro or in vivo that may be used to repair or replace a tissue or organ in a recipient mammal. For example, RPSCs may be cultured in vitro in the presence of a matrix to produce a tissue or organ of the urogenital system, such as the bladder, clitoris, corpus cavermosum, kidney, testis, ureter, uretal valve, or urethra, which may then be transplanted into a mammal (Attila, Curr. Opin. Urol. 9(6):517-526, 1999). In another transplant application, synthetic blood vessels are formed in vitro by culturing reprogrammed cells in the presence of an appropriate matrix, and then the vessels are transplanted into a mammal for the treatment or prevention of a cardiovascular or circulatory condition. For the generation of donor cartilage or bone tissue, RPSCs such as chondrocytes or osteocytes are cultured in vitro in the presence of a matrix under conditions that allow the formation of cartilage or bone, and then the matrix containing the donor tissue is administered to a mammal. Alternatively, a mixture of the cells and a matrix may be administered to a mammal for the formation of the desired tissue in vivo. Preferably, the cells are attached to the surface of the matrix or encapsulated by the matrix. Examples of matrices that may be used for the formation of donor tissues or organs include collagen matrices, carbon fibers, polyvinyl alcohol sponges, acrylateamide sponges, fibrin-thrombin gels, hyaluronic acid-based polymers, and synthetic polymer matrices containing polyanhydride, poly-

orthoester, polyglycolic acid, or a combination thereof (see, for example, U.S. Pat. Nos. 4,846,835; 4,642,120; 5,786,217; and 5,041,138).

The RPSCs produced according to the invention may be used to produce genetically engineered or transgenic differentiated cells. Essentially, this will be effected by introducing a desired gene or genes, or removing all or part of an endogenous gene or genes of RPSCs produced according to the invention, and allowing such cells to differentiate into the desired cell type. A preferred method for achieving such modification is by homologous recombination because such technique can be used to insert, delete or modify a gene or genes at a specific site or sites in the stem-like cell genome.

This methodology can be used to replace defective genes, e.g., defective immune system genes, cystic fibrosis genes, or to introduce genes which result in the expression of therapeutically beneficial proteins such as growth factors, lymphokines, cytokines, enzymes, etc. For example, the gene encoding brain derived growth factor maybe introduced into human embryonic or stem-like cells, the cells differentiated into neural cells and the cells transplanted into a Parkinson's patient to retard the loss of neural cells during such disease. Examples of mutations that may be rescued using these methods include mutations in the cystic fibrosis gene; mutations associated with Dunningan's disease such as the R482W, R482Q, and R584H mutations in the lamin A gene; and mutations associated with the autosomal-dominant form of Emery Deyfuss muscular dystrophy such as the R249Q, R453W, and Q6STOP mutations in the lamin A gene. In the Q6STOP mutation, the codon for Gln6 is mutated to a stop codon.

Previously, cell types transfected with BDNF varied from primary cells to immortalized cell lines, either neural or non-neural (myoblast and fibroblast) derived cells. For example, astrocytes have been transfected with BDNF gene using retroviral vectors, and the cells grafted into a rat model of Parkinson's disease (Yoshimoto et al., Brain Research, 691:25-36, (1995)). This ex vivo therapy reduced Parkinson's-like symptoms in the rats up to 45% 32 days after transfer. Also, the tyrosine hydroxylase gene has been placed into astrocytes with similar results (Lundberg et al., Develop. Neurol., 139: 39-53 (1996) and references cited therein).

However, such ex vivo systems have problems. In particular, retroviral vectors currently used are down-regulated in vivo and the transgene is only transiently expressed (review by Mulligan, Science, 260: 926-932 (1993)). Also, such studies used primary cells, astrocytes, which have finite life span and replicate slowly. Such properties adversely affect the rate of transfection and impede selection of stably transfected cells. Moreover, it is almost impossible to propagate a large population of gene targeted primary cells to be used in homologous recombination techniques.

By contrast, the difficulties associated with retroviral systems should be eliminated by the use of RPSCs of the present invention, which are ES-like cells. Using known methods to introduced desired genes/mutations into ES cells, RPSCs may be genetically engineered, and the resulting engineered cells differentiated into desired cell types, e.g., heniatopoietic cells, neural cells, pancreatic cells, cartilage cells, etc. Genes which may be introduced into the RPSCs include, for example, epidermal growth factor, basic fibroblast growth factor, glial derived neurotrophic growth factor, insulin-like growth factor (I and II), neurotrophin3, neurotrophin-4/5, ciliary neurotrophic factor, AFT-1, cytokine genes (interleukins, interferons, colony stimulating factors, tumor necrosis factors (alpha and beta), etc.), genes encoding therapeutic enzymes, collagen, human serum albumin, etc.

In addition, it is also possible to use one of the negative selection systems now known in the art for eliminating therapeutic cells from a patient if necessary. For example, donor cells transfected with the thymidine kinase (TK) gene will lead to the production of embryonic cells containing the 1K gene. Differentiation of these cells will lead to the isolation of therapeutic cells of interest which also express the TK gene. Such cells may be selectively eliminated at any time from a patient upon gancyclovir administration. Such a negative selection system is described in U.S. Pat. No. 5,698,446, and is herein incorporated by reference.

Examples of diseases, disorders, or conditions that may be treated or prevented include neurological, endocrine, structural, skeletal, vascular, urinary, digestive, integumentary, blood, immune, auto-immune, inflammatory, endocrine, kidney, bladder, cardiovascular, cancer, circulatory, digestive, hematopoeitic, and muscular diseases, disorders, and conditions. In addition, reprogrammed cells may be used for reconstructive applications, such as for repairing or replacing tissues or organs.

With respect to the therapeutic methods of the invention, it is not intended that the administration of RPSCs to a mammal be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration, including intramuscular, intravenous, intraarticular, intralesional, subcutaneous, or any other route sufficient to provide a dose adequate to prevent or treat a disease. The RPSCs may be administered to the mammal in a single dose or multiple doses. When multiple doses are administered, the doses may be separated from one another by, for example, one week, one month, one year, or ten years. One or more growth factors, hormones, interleukins, cytokines, or other cells may also be administered before, during, or after administration of the cells to further bias them towards a particular cell type.

The RPSCs of the present invention may be used as an in vitro model of differentiation, in particular for the study of genes which are involved in the regulation of early development. Differentiated cell tissues and organs using the RPSCs may be used in drug studies.

Furthermore, the RPSCs produced according to the invention maybe introduced into animals, e.g., SCID mice, cows, pigs, e.g., under the renal capsule or intramuscularly and used to produce a teratoma therein. This teratoma can be used to derive different tissue types. Also, the inner cell mass produced by X-species nuclear transfer may be introduced together with a biodegradable, biocompatible polymer matrix that provides for the formation of 3-dimensional tissues. After tissue formation, the polymer degrades, ideally just leaving the donor tissue, e.g., cardiac, pancreatic, neural, lung, liver. In some instances, it may be advantageous to include growth factors and proteins that promote angiogenesis. Alternatively, the formation of tissues can be effected totally in vitro, with appropriate culture media and conditions, growth factors, and biodegradable polymer matrices.

Applications of the Somatic Cell Reprogramming Methods and RPSCs in Animals

The reprogramming methods disclosed herein may be used to generate RPSCs for a variety of animal species. The RPSCs generated can be useful to produce desired animals. Animals include, for example, avians and mammals as well as any animal that is an endangered species. Exemplary birds include domesticated birds (e.g., quail, chickens, ducks, geese, turkeys, and guinea hens) as well as other birds such as birds of prey (e.g., hawks, falcons, ospreys, condors, etc.), endangered birds (e.g., parrots, California condor, etc.), ostriches etc. Exemplary mammals include murine, caprine,

ovine, bovine, porcine, canine, feline and primate. Of these, preferred members include domesticated animals, including, for examples, cattle, buffalo, pigs, horses, cows, rabbits, guinea pigs, sheep, and goats.

RPSCs generated by the reprogramming methods of the present invention allows one, for the first time, to genetically engineer animals other than mouse and human. RPSCs are ES-like cells, and are thus amenable to genetic manipulation. To date, no ES cells are available for animals other than mouse and human. As a result, for these animals, it is currently practically impossible to create genetically modified animals having targeted mutations. The ES-cell like RPSCs can be manipulated to introduce desired targeted genetic modifications. The resulting engineered RPSCs can then be used to generate a cloned animal with the desired genetic modifications in its germ line, using methods described for ES cells in mouse. See Capecchi and Thomas, U.S. Pat. Nos. 5,487,992, 5,627,059, 5,631,153, and 6,204,061. Genetic engineering in animals has potentially great applications in a variety of animals, especially farm animals.

The somatic cell reprogramming methods of the present invention provides at least two methods for delivering optimized farm animals. In the first, somatic cell reprogramming can be used to capture the best available phenotype for a farm animal stock. The current technologies used to deliver optimized farm animals are based on selective breeding, and expansion from preferred breeding stocks. Animals that have been selected on the basis of superior characteristics, including, for example, meat content, egg production (in the case of poultry), feed conversion ratio, are used to breed large numbers of animals that are in turn used in the human food supply. This traditional process has profound inherent inefficiencies. The phenotype observed in an individual animal is often only partially transmitted in the progeny of that animal. Therefore, traditional breeding schemes are inefficient in capturing the very best phenotype in all of the progeny animals. In contrast, the reprogramming methods of the present invention provides a controlled and efficient way to achieve the same goal, by generating RPSCs from somatic cells of an animal with the desired characteristics. The RPSCs generated may be used immediately to generate cloned animals derived from the RPSCs. Known methods for generating mice from ES cells can be used for this procedure. Alternatively, the RPSCs generated may be cryopreserved and thawed in response to a grower's needs.

In the second method, somatic cells from an animal with the desired characteristics are reprogrammed to produce RPSCs. The RPSCs are further genetically engineered to introduce desired genetic modification(s), before being placed into a recipient embryo to produce desired progeny.

The reprogramming methods can also be used to rescue endangered species. Somatic cell reprogramming provides an efficient method to generate RPSCs from somatic cells of an endangered animal. The resulting RPSCs can be used immediately to expand the numbers of the endangered animal. Alternatively, the RPSCs can be cryopreserved to generate a RPSC stock for the endangered species, as a safeguard measure against extinction of the endangered species.

Methods for Gene Identification

The present invention provides methods for identifying a gene that activates the expression of an endogenous pluripotency gene in somatic cells. The methods comprise: transfecting the somatic cells of the present invention with a cDNA library prepared from ES cells or oocytes, selecting for cells that express the first selectable marker, and assessing the expression of the first endogenous pluripotency gene in the transfected cells that express the first selectable marker. The expression of the first endogenous pluripotency gene indicates that the cDNA encodes a gene that activates the expression of an endogenous pluripotency gene in somatic cells.

The methods are applicable for identifying a gene that activates the expression of at least two endogenous pluripotency genes in somatic cells. The somatic cells used in the methods further comprise a second endogenous pluripotency gene linked to a second selectable marker. The methods are modified to select for transfected cells that express both selectable markers, among which the expression of the first and the second endogenous pluripotency genes are assessed. The expression of both the first and the second endogenous pluripotency genes indicates that the cDNA encodes a gene that activates the expression of at least two pluripotency genes in somatic cells.

The methods are further applicable for identifying a gene that activates the expression of at least three endogenous pluripotency genes in somatic cells. The somatic cells used in the methods further comprise a third endogenous pluripotency gene linked to a third selectable marker. The methods are modified to select for transfected cells that express all three selectable markers, among which the expression of all three endogenous pluripotency genes are assessed. The expression of all three endogenous pluripotency genes indicates that the cDNA encodes a gene that activates the expression of at least three pluripotency genes in somatic cells.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of mouse genetics, developmental biology, cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, Current Protocols in Cell Biology, ed. by Bonifacino, Dasso, Lippincott-Schwartz, Harford, and Yamada, John Wiley and Sons, Inc., New York, 1999; Manipulating the Mouse Embryos, A Laboratory Manual, 3rd Ed., by Hogan et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2003; Gene Targeting: A Practical Approach, IRL Press at Oxford University Press, Oxford, 1993; and Gene Targeting Protocols, Human Press, Totowa, N.J., 2000. All patents, patent applications and references cited herein are incorporated in their entirety by reference.

EXEMPLIFICATION

The invention now being generally described, it will be more readily understood by reference to the following example, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example

Oct4-Induced Fibroblasts are More Susceptible to Reprogramming than Unduced Fibroblasts as Demonstrated by Nuclear Transfer Experiment

A. Generation of Transgenic Mouse Carrying an Inducible Oct4 Transgene

An inducible Oct4 allele is constructed as the following: first, two integration vectors are constructed. The first integration vector, inducible Oct4 integration vector, contains an Oct4 gene driven by a tetracycline-inducible promoter (Tet-Op). The Tet-Op-Oct4 cassette is flanked by a splice-acceptor double poly-A signal (SA-dpA) at its 5' end and a SV40 polyA tail (SV40-pA) at its 3' end. The second integration vector, tetracycline activator integration vector, contains a

mutant form of tetracycline activator, M2-rtTA, which is more responsive to doxycycline (Dox) induction than the wild type activator. (Urlinger S. et al., 2000)

The two integration vectors are introduced into V6.5 ES cells: the inducible Oct4 integration vector and the tetracycline activator integration vector are introduced into the Collagen locus and the Rosa26 locus respectively via site-specific integration, as shown in FIG. **1**. The resulting ES cells are used to make Oct4-inducible mice by tetraploid complementation.

B. Expression of the Inducible Oct4 Transgene

Fibroblasts derived from tail biopsies of the Oct4-inducible mice were cultured. A fraction of the cultured fibroblasts were induced with doxycycline for 3 days (at 2 microgram/ml), and Oct4 expression was detected by Northern blot and Western blot analysis. As shown in FIG. **2**, the Oct4 expression level in fibroblasts treated with doxycycline is comparable to the Oct4 expression level in ES cells, and undetectable in fibroblasts not treated with doxycycline. The expression results demonstrate that the inducible Oct4 transgene is expressed as planned.

C. Nuclear Transfer Experiment

Nuclear transfer was performed on fibroblasts derived from tail biopsies of mice that carry the inducible Oct4 transgene. Dox induction was for 24 hours prior to nuclear transfer. Cloned embryos were then activated and cultured to the blastocyst stage to derive ES cells as described previously (Hochedlinger and Jaenisch, 2002), As shown in Table 1, on average, blastocyst formation and ES cell derivation (as measured as a fraction of eggs with pronucleus formation) is more efficient from Oct4 induced fibroblast than from uninduced fibroblasts. This result demonstrated that induced Oct4 expression in somatic cells such as fibroblasts make these cells more susceptible to reprogramming.

One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods, systems and kits are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention and are defined by the scope of the claims. It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

TABLE 1

In vitro development of clones derived from Oct4-induced fibroblasts

Expt.	Oct4	eggs w/PN	Blastocysts (% PN)		ES lines (% PN)	
#1	–	22	5 (23%)	19%	0 (0%)	3%
#2	–	35	5 (14%)		2 (6%)	
#3	+	37	10 (27%)	24%	2 (5%)	7%
#4	+	47	10 (21%)		4 (9%)	

PN . . . ProNucleus formation

Nuclear transfer was performed on fibroblasts derived from tail biopsies of mice that carry the inducible Oct4 transgene. Dox induction was for 24 hours prior to nuclear transfer. Cloned embryos were then activated and cultured to the blastocyst stage to derive ES cells as described previously (Hochedlinger and Jaenisch, Nature, 2002). These preliminary results show that on average blastocyst formation and ES cell derivation is more efficient from Oct4 induced than from uninduced fibroblasts.

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Yamada, Y., Miyashita, T., Savagner, P., Horton, W., Brown, K. S., Abramczuk, J., Xie, H. X., Kohno, K., Bolander, M. and Bruggeman, L. (1990). Regulation of the collagen II gene in vitro and in transgenic mice. *Ann. New York Acad. Sci.* 580, 81-87

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What is claimed is:

1. A composition comprising an isolated primary somatic cell that comprises an exogenously introduced nucleic acid encoding Oct 4, wherein the exogenously introduced nucleic acid increases Oct4 expression in the cell.

2. The composition of claim **1**, wherein the isolated primary somatic cell is a mammalian cell.

3. The composition of claim **1**, wherein the isolated primary somatic cell is a human cell or a mouse cell.

4. The composition of claim **1**, wherein the isolated primary somatic cell is an adult stem cell.

5. The composition of claim **4**, wherein the adult stem cell is selected from the group consisting of: a hematopoietic stem cell, a neural stem cell, and a mesenchymal stem cell.

6. The composition of claim **1**, further comprising a candidate agent of interest with respect to its potential to reprogram a somatic cell.

7. The composition of claim **6**, wherein the candidate agent of interest is a DNA methylation inhibitor, a histone deacetylase inhibitor or PD098059.

8. The composition of claim **6**, wherein the candidate agent of interest is an exogenous nucleic acid encoding a pluripotency protein selected from the group consisting of: Nanog and Sox-2.

9. A composition comprising a cDNA encoding an Oct4 protein and a cDNA encoding a Sox2 protein, wherein the composition further comprises a DNA methylation inhibitor, a histone deacetylase inhibitor or PD098059.

10. The composition of claim **9**, further comprising a cDNA encoding an Oct4 protein and an isolated nucleic acid encoding a Nanog protein.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

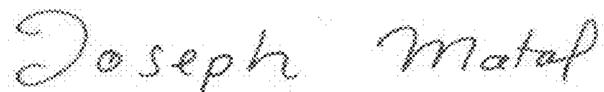
PATENT NO. : 8,951,797 B2
APPLICATION NO. : 13/646411
DATED : February 10, 2015
INVENTOR(S) : Jaenisch et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims

Column 21, Claim 10, Lines 2-3, the phrase "encoding an Oct4 protein and an isolated nucleic acid" should be removed.

Signed and Sealed this
Twenty-seventh Day of June, 2017



Joseph Matal
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Under Secretary of Commerce for Intellectual Property and
Director of the United States Patent and Trademark Office

EXHIBIT D

US008940536B2

(12) **United States Patent**
Jaenisch et al.

(10) **Patent No.: US 8,940,536 B2**
(45) **Date of Patent: *Jan. 27, 2015**

(54) **METHODS FOR MAKING SOMATIC CELLS MORE SUSCEPTIBLE TO REPROGRAMMING**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **12/703,061**

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Related U.S. Application Data

(62) Division of application No. 10/997,146, filed on Nov. 24, 2004, now Pat. No. 7,682,828.

(60) Provisional application No. 60/525,612, filed on Nov. 26, 2003, provisional application No. 60/530,042, filed on Dec. 15, 2003.

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C07K 14/47	(2006.01)
C12N 15/877	(2010.01)
C12N 5/074	(2010.01)

(52) **U.S. Cl.**

CPC ***C12N 15/85*** (2013.01); ***A01K 67/0273*** (2013.01); ***A01K 67/0275*** (2013.01); ***C07K 14/4702*** (2013.01); ***C12N 15/8509*** (2013.01); ***C12N 15/8775*** (2013.01); ***C12N 5/0696*** (2013.01); *A01K 2217/05* (2013.01); *A01K 2227/105* (2013.01); *C12N 2830/003* (2013.01); *C12N 2830/006* (2013.01)

USPC **435/377**; 435/325

(58) **Field of Classification Search**

USPC 435/377, 325

See application file for complete search history.

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(57) **ABSTRACT**

The invention provides methods for reprogramming somatic cells to generate multipotent or pluripotent cells. Such methods are useful for a variety of purposes, including treating or preventing a medical condition in an individual. The invention further provides methods for identifying an agent that reprograms somatic cells to a less differentiated state.

17 Claims, 2 Drawing Sheets

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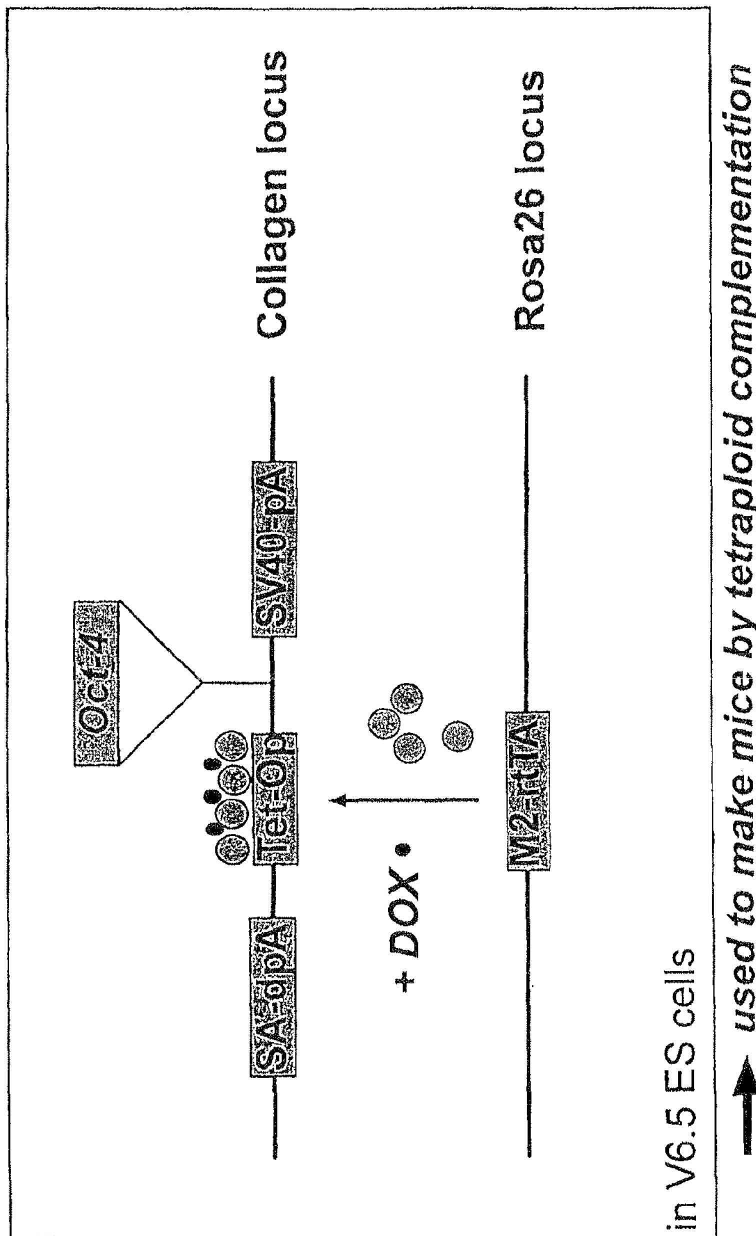
Figure 1. Inducible *Oct4* allele



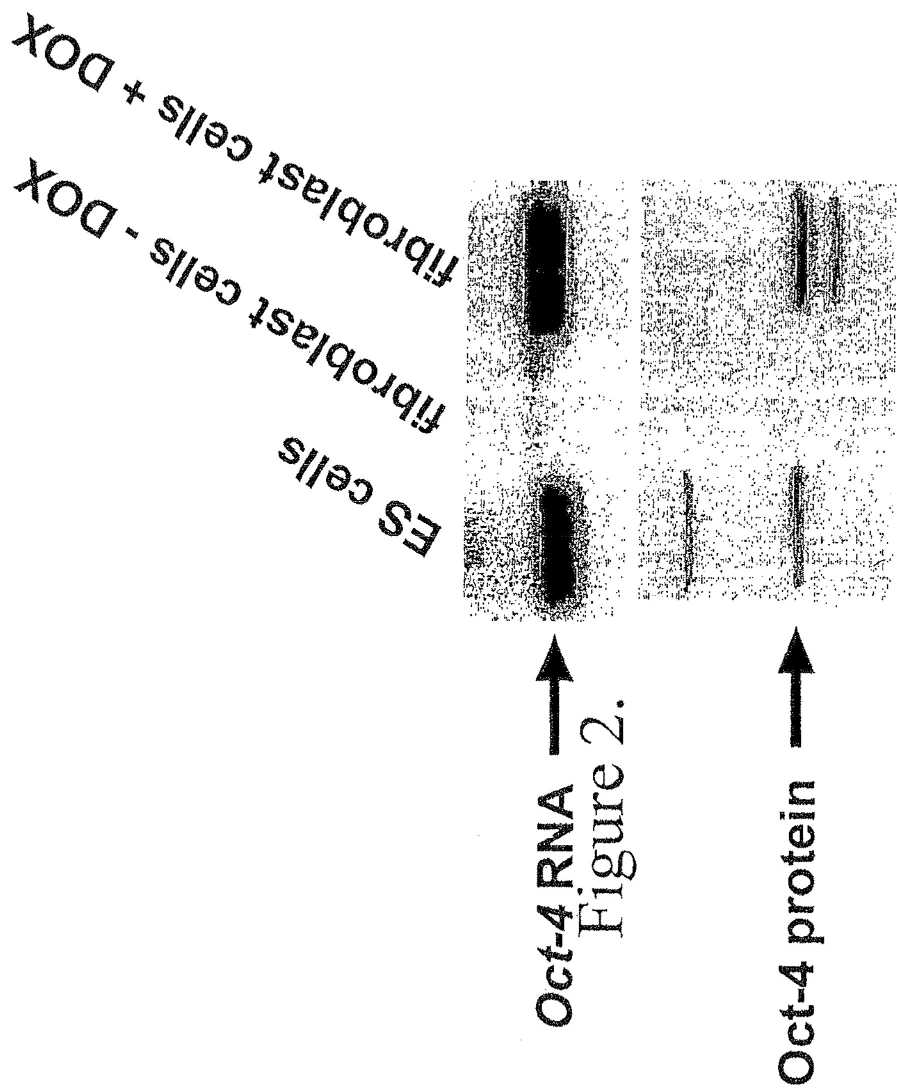


Figure 2.

METHODS FOR MAKING SOMATIC CELLS MORE SUSCEPTIBLE TO REPROGRAMMING

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional of U.S. application Ser. No. 10/997,146, filed Nov. 24, 2004, now U.S. Pat. No. 7,682,828 which claims the benefit of U.S. Provisional Application No. 60/525,612, filed Nov. 26, 2003, and U.S. Provisional Application No. 60/530,042, filed Dec. 15, 2003, the specifications of which are incorporated herein by reference in their entirety.

GOVERNMENTAL FUNDING

The invention described herein was supported, in whole or in part, by Grant R37 CA84198 from the National Institutes of Health. The United States government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Pluripotent stem cells have the potential to differentiate into the full range of daughter cells having distinctly different morphological, cytological or functional phenotypes unique to a specific tissue. By contrast, descendants of pluripotent cells are restricted progressively in their differentiation potential, with some cells having only one fate. Pluripotent cells have extraordinary scientific and therapeutic potential, as they can be differentiated along the desired differentiation pathway in a precisely controlled manner and used in cell-based therapy.

Two categories of pluripotent stem cells are known to date: embryonic stem cells and embryonic germ cells. Embryonic stem cells are pluripotent stem cells that are derived directly from an embryo. Embryonic germ cells are pluripotent stem cells that are derived directly from the fetal tissue of aborted fetuses. For purposes of simplicity, embryonic stem cells and embryonic germ cells will be collectively referred to as "ES" cells herein.

ES cells are presently obtained via several methods. In a first method, an ES cell line is derived from the inner cell mass of a normal embryos in the blastocyst stage (See U.S. Pat. No. 6,200,806, Thompson, J. A. et al. Science, 282:1145-7, 1998 and Hogan et al., 2003). A second method for creating pluripotent ES cells utilizes the technique of somatic cell nuclear transfer (SCNT). In this technique, the nucleus is removed from a normal egg, thus removing the genetic material. Next, a donor diploid somatic cell is placed next to the enucleated egg and the two cells are fused, or the nucleus is introduced directly into the oocyte by micromanipulation. The fused cell has the potential to develop into a viable embryo, which may then be sacrificed to remove that portion of the embryo containing the stem cell producing inner cell mass.

In a third method, the nucleus of a human cell is transplanted into an entirely enucleated animal oocyte of a species different from the donor cell (referred to herein as animal stem cell nuclear transfer, or "ASCNT"). See U.S. Pat. application Ser. No. 20010012513 (2001). The resultant chimeric cells are used for the production of pluripotent ES cells, in particular human-like pluripotent ES cells. One disadvantage of this technique is that these chimeric cells may contain unknown non-human viruses and still contain the mitochondria of the animal species. Thus, there would be substantial risks of immune rejection if such cells were used in cell transplantation therapies.

In a fourth method, ES cells can be isolated from the primordial germ cells found in the genital ridges of post-implanted embryos.

As described above, all presently available methods depend on controversial sources—embryos (either created naturally or via cloning), fetal tissue and via the mixing of materials of multiple species. The controversy surrounding the sources for such cells, according to many leading scientists and public and private organizations including the NIH, has greatly compromised and slowed the study of such cells and their application.

There is thus a great demand for alternative methods of generating pluripotent cells.

SUMMARY OF THE INVENTION

The present invention provides engineered somatic cells, in which one or more endogenous pluripotency gene(s) is operably linked to a selectable marker in such a manner that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene to which the marker is linked. The invention also provides transgenic mice containing these engineered somatic cells.

The present invention also provides methods for reprogramming somatic cells to a less differentiated state. In the methods, engineered somatic cells of the invention are treated with an agent. Cells that express the selectable marker are then selected, and assessed for pluripotency characteristics. The treatment with an agent may be contacting the cells with an agent which alters chromatin structure, or may be transfecting the cells with at least one pluripotency gene, or both.

The present invention further provides methods for identifying an agent that reprograms somatic cells to a less differentiated state. In the methods, the engineered somatic cells described above are contacted with a candidate agent. Cells that express the selectable marker are then selected, and assessed for pluripotency characteristics. The presence of at least a subset of pluripotency characteristics indicates that the agent is capable of reprogramming somatic cells to a less-differentiated state. The agents identified by the present invention can then by used to reprogram somatic cells by contacting somatic cells with the agents.

The present invention also provides methods for identifying a gene that causes the expression of at least one endogenous pluripotency gene in somatic cells. In the methods, the engineered somatic cells are transfected with a cDNA library prepared from a pluripotent cell, such as an ES cell. The cells that express the appropriate selectable marker are then selected, and the expression of the appropriate endogenous pluripotency gene is examined. The expression of an endogenous pluripotency gene indicates that the cDNA encodes a protein whose expression in the cell results in, directly or indirectly, expression of the endogenous pluripotency gene.

The present invention further provides methods for treating a condition in an individual in need of such treatment. In certain embodiments, somatic cells are obtained from the individual and reprogrammed by the methods of the invention under conditions suitable for the cells to develop into cells of a desired cell type. The reprogrammed cells of a desired cell type are then harvested and introduced into the individual to treat the condition. In certain further embodiments, the somatic cells obtained from the individual contains a mutation in one or more genes. In these instances, the methods are modified so that the somatic cells obtained from the individual are first treated to restore the one or more normal gene(s) to the cells such that the resulting cells carry the normal endogenous gene, which are then introduced into the

individual. In certain other embodiments, methods of the invention can be used to treat individuals in need of a functional organ. In the methods, somatic cells are obtained from an individual in need of a functional organ, and reprogrammed by the methods of the invention to produce reprogrammed somatic cells. Such reprogrammed somatic cells are then cultured under conditions suitable for development of the reprogrammed somatic cells into a desired organ, which is then introduced into the individual. The methods are useful for treating any one of the following conditions: a neurological, endocrine, structural, skeletal, vascular, urinary, digestive, integumentary, blood, autoimmune, inflammatory, or muscular condition.

The present invention also provides methods for producing a cloned animal. In the methods, a somatic cell is isolated from an animal having desired characteristics, and reprogrammed using the methods of the invention to produce one or more reprogrammed pluripotent somatic cell ("RPSC"). The RPSCs are then inserted into a recipient embryo, and the resulting embryo is cultured to produce an embryo of suitable size for implantation into a recipient female, which is then transferred into a recipient female to produce a pregnant female. The pregnant female is maintained under conditions appropriate for carrying the embryo to term to produce chimeric animal progeny, which is then bred with a wild type animal to produce a cloned animal.

In certain embodiments, the RPSCs may alternatively be cryopreserved for future cloning uses. In certain other embodiments, genetic modification, such as a targeted mutation, may be introduced into the RPSCs prior to its insertion into a recipient embryo.

The present invention also provides methods for producing a cloned avian. In the methods, a somatic cell is isolated from an avian having desired characteristics, and reprogrammed using the methods of the invention to produce one or more reprogrammed pluripotent somatic cell ("RPSC"). The RPSCs are then inserted into eggs that are unable to develop into an embryo, and the resulting eggs are then incubated to produce avian offspring having the genotype of the RPSC, thereby producing a cloned avian.

It is contemplated that all embodiments described above are applicable to all different aspects of the invention. It is also contemplated that any of the above embodiments can be freely combined with one or more other such embodiments whenever appropriate.

Specific embodiments of the invention are described in more detail below. However, these are illustrative embodiments, and should not be construed as limiting in any respect.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts an inducible Oct4 allele.

FIG. **2** shows the expression of the inducible Oct4 transgene by Northern blot and Western blot analysis.

DETAILED DESCRIPTION OF THE INVENTION

Overview

Presently, human ES cells or ES-like cells can only be generated from controversial sources. It would be useful to reprogram somatic cells directly into pluripotent cells. Nuclei from somatic cells retain the totopotency potential to direct development of an animal, as demonstrated by nuclear transfer technology. It would be useful to reprogram somatic cells directly into ES cells without the use of oocytes and nuclear transfer technology.

Applicants have devised novel methods of reprogramming somatic cells to generate pluripotent cells or multipotent cells. Applicants have also devised novel methods to identify agents that reprogram somatic cells. The methods take advantage of the engineered somatic cells designed by Applicants, in which an endogenous gene typically associated with pluripotency ("pluripotency gene") is engineered to be operably linked to a selectable marker in a manner that the expression the endogenous pluripotency gene substantially matches the expression of the selectable marker. Because pluripotency genes are generally expressed only in pluripotent cells and not in somatic cells, the expression of an endogenous pluripotent gene(s) is an indication of successful reprogramming. Having a selectable marker operably linked to an endogenous pluripotency gene gives one a powerful mechanism to select for potentially reprogrammed somatic cells, which likely is a rare occurrence. The resulting cells may be further assessed for pluripotency characteristics to confirm whether a somatic cell has been successfully reprogrammed to pluripotency.

Generating pluripotent or multipotent cells by somatic cell reprogramming using the methods of the present invention has at least two advantages. First, the methods of the present invention allow one to generate autologous pluripotent cells, which are cells specific to a patient. The use of autologous cells in cell therapy offers a major advantage over the use of non-autologous cells, which are likely to be subject to immunological rejection. In contrast, autologous cells are unlikely to elicit significant immunological responses (See Munsie et al, 2000). Second, the methods of the present invention allow one to generate pluripotent without using embryos, oocytes and/or nuclear transfer technology.

A pluripotent cell is a cell that has the potential to divide in vitro for a long period of time (greater than one year) and has the unique ability to differentiate into cells derived from all three embryonic germ layers—endoderm, mesoderm and ectoderm.

A multipotent cell is a cell that is able to differentiate into some but not all of the cells derived from all three germ layers. Thus, a multipotent cell is a partially differentiated cell. Adult stem cells are multipotent cells. Known adult stem cells include, for example, hematopoietic stem cells and neural stem cells. A hematopoietic stem cell is multipotent because it has the ability to differentiate into all types of specific blood cells, but it is unlikely that they can differentiate into all cells of a given animal or human. Multipotent/adult stem cells have a great deal of promise in research and in the area of therapeutic applications. For example, multipotent/adult stem cells have already been used in humans in attempts to treat certain blood, neural and cancer diseases.

The term "pluripotency gene", as used herein, refers to a gene that is associated with pluripotency. The expression of a pluripotency gene is typically restricted to pluripotent stem cells, and is crucial for the functional identity of pluripotent stem cells. The transcription factor Oct-4 (also called Pou5fl, Oct-3, Oct3/4) is an example of a pluripotency gene. Oct-4 has been shown to be required for establishing and maintaining the undifferentiated phenotype of ES cells and plays a major role in determining early events in embryogenesis and cellular-differentiation (Nichols et al., 1998, Cell 95:379-391; Niwa et al., 2000, Nature Genet. 24:372-376). Oct-4 is down-regulated as stem cells differentiate into specialised cells. Other exemplary pluripotency genes include Nanog, and Stella (See Chambers et al., 2003, Cell 113: 643-655; Mitsui et al., Cell. 2003, 113(5):631-42; Bortvin et al. Development. 2003, 130(8):1673-80; Saitou et al., Nature. 2002, 418 (6895):293-300.

Engineered Somatic Cells and Transgenic Mice Comprising Such Cells

The present invention provides somatic cells comprising an endogenous pluripotency gene linked to DNA encoding a selectable marker in such a manner that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene. In one embodiment, the somatic cells of the present invention comprise a first endogenous pluripotency gene linked to DNA encoding a first selectable marker in such a manner that the expression of the first selectable marker substantially matches the expression of the first endogenous pluripotency gene. The somatic cells may also be engineered to comprise any number of endogenous pluripotency genes respectively linked to a distinct selectable marker. Thus, in another embodiment, the somatic cells of the present invention comprise two endogenous pluripotency genes, each of which is linked to DNA encoding a distinct selectable marker. In a further embodiment, the somatic cells of the present invention comprise three endogenous pluripotency genes, each of which is linked to DNA encoding a distinct selectable marker. The somatic cells described above will be collectively referred in this application as "engineered somatic cells." The engineered somatic cells may be further engineered to have one or more pluripotency gene expressed as a transgene under an inducible promoter.

The selectable marker is linked to an appropriate endogenous pluripotency gene such that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene. By "substantially match", it is meant that the expression of the selectable marker substantially reflects the expression pattern of the endogenous pluripotency gene. In other words, the selectable marker and the endogenous pluripotency gene are co-expressed. For purpose of the present invention, it is not necessary that the expression level of the endogenous gene and the selectable marker is the same or even similar. It is only necessary that the cells in which an endogenous pluripotency gene is activated will also express the selectable marker at a level sufficient to confer a selectable phenotype on the reprogrammed cells. For example, when the selectable marker is a marker that confers resistance to a lethal drug (a "drug resistance marker"), the cells are engineered in a way that allows cells in which an endogeneous pluripotency gene is activated to also express the drug resistance marker at a sufficient level to confer on reprogrammed cells resistance to lethal drugs. Thus, reprogrammed cells will survive and proliferate whereas non-reprogrammed cells will die.

The DNA encoding a selectable marker may be inserted downstream from the end of the open reading frame (ORF) encoding the desired endogenous pluripotency gene, anywhere between the last nucleotide of the ORF and the first nucleotide of the polyadenylation site. An internal ribosome entry site (IRES) may be placed in front of the DNA encoding the selectable marker. Alternatively, the DNA encoding a selectable marker may be inserted anywhere within the ORF of the desired endogenous pluripotency gene, downstream of the promoter, with a termination signal. An internal ribosome entry site (IRES) may be placed in front of the DNA encoding the selectable marker. The selectable marker may be inserted into only one allele, or both alleles, of the endogenous pluripotency gene.

The somatic cells in the invention may be primary cells or immortalized cells. Such cells may be primary cells (non-immortalized cells), such as those freshly isolated from an animal, or may be derived from a cell line (immortalized cells).

The somatic cells in the present invention are mammalian cells, such as, for example, human cells or mouse cells. They may be obtained by well-known methods, from different organs, e.g., skin, lung, pancreas, liver, stomach, intestine, heart, reproductive organs, bladder, kidney, urethra and other urinary organs, etc., generally from any organ or tissue containing live somatic cells. Mammalian somatic cells useful in the present invention include, by way of example, adult stem cells, sertoli cells, endothelial cells, granulosa epithelial, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc. generally any live somatic cells. The term "somatic cells", as used herein, also includes adult stem cells. An adult stem cell is a cell that is capable of giving rise to all cell types of a particular tissue. Exemplary adult stem cells include hematopoietic stem cells, neural stem cells, and mesenchymal stem cells.

In one embodiment, the engineered somatic cells are obtained from a transgenic mouse comprising such engineered somatic cells. Such transgenic mouse can be produced using standard techniques known in the art. For example, Bronson et al. describe a technique for inserting a single copy of a transgene into a chosen chromosomal site. See Bronson et al., 1996. Briefly, a vector containing the desired integration construct (for example, a construct containing a selectable marker linked to a pluripotency gene) is introduced into ES cells by standard techniques known in the art. The resulting ES cells are screened for the desired integration event, in which the knock-in vector is integrated into the desired endogenous pluripotency gene locus such that the selectable marker is integrated into the genomic locus of the pluripotency gene and is under the control of the pluripotency gene promoter. The desired ES cell is then used to produce transgenic mouse in which all cell types contain the correct integration event. Desired types of cells may be selectively obtained from the transgenic mouse and maintained in vitro. In one embodiment, two or more transgenic mice may be created, each carrying a distinct integration construct. These mice may then be bred to generate mice that carry multiple desired integration construct. For example, one type of transgenic mouse may be created to carry an endogenous pluripotency gene linked to a selectable marker, while a second type of transgenic mouse may be created to carry a pluripotency gene expressed as a transgene under an inducible promoter. These two types of mice may then be bred to generate transgenic mice that have both a selectable marker linked to an endogenous pluripotency gene and an additional pluripotency gene expressed as a transgene under an inducible promoter. These two pluripotency genes may or may not be the same. Many variables are contemplated: the identity of the endogenous pluripotency gene linked to marker, the identity of the pluripotency gene expressed as a transgene, and the number of the endogenous pluripotency gene linked to a selectable marker, and the number of pluripotency gene expressed as a transgene. The present invention encompasses all possible combinations of these variables.

Alternatively, engineered somatic cells of the present invention may be produced by direct introduction of the desired construct into somatic cells. DNA construct may be introduced into cells by any standard technique known in the art, such as viral transfection (eg. using an adenoviral system) or liposome-mediated transfection. Any means known in the art to generate somatic cells with targeted integration can be used to produce somatic cells of the invention. In mammalian

cells, homologous recombination occurs at much lower frequency compared to non-homologous recombination. To facilitate the selection of homologous recombination events over the non-homologous recombination events, at least two enrichment methods have been developed: the positive-negative selection (PNS) method and the "promoterless" selection method (Sedivy and Dutriaux, 1999). Briefly, PNS, the first method, is in genetic terms a negative selection: it selects against recombination at the incorrect (non-homologous) loci by relying on the use of a negatively selectable gene that is placed on the flanks of a targeting vector. On the other hand, the second method, the "promoterless" selection, is a positive selection in genetic terms: it selects for recombination at the correct (homologous) locus by relying on the use of a positively selectable gene whose expression is made conditional on recombination at the homologous target site. The disclosure of Sedivy and Dutriaux is incorporated herein.

A selectable marker, as used herein, is a marker that, when expressed, confers upon recipient cells a selectable phenotype, such as antibiotic resistance, resistance to a cytotoxic agent, nutritional prototrophy or expression of a surface protein. The presence of a selectable marker linked to an endogenous pluripotency gene makes it possible to identify and select reprogrammed cells in which the endogenous pluripotency gene is expressed. A variety of selectable marker genes can be used, such as neomycin resistance gene (neo), puromycin resistance gene (puro), guanine phosphoribosyl transferase (gpt), dihydrofolate reductase (DHFR), adenosine deaminase (ada), puromycin-N-acetyltransferase (PAC), hygromycin resistance gene (hyg), multidrug resistance gene (mdr), and hisD gene.

The present invention further provides transgenic mice comprising the somatic cells of the invention.

Methods for Reprogramming Somatic Cells

The present invention further provides methods for reprogramming somatic cells to a less differentiated state. The resulting cells are termed "reprogrammed somatic cells" ("RSC") herein. A RSC may be a reprogrammed pluripotent somatic cell ("RPSC"), a reprogrammed multipotent somatic cell ("RMSC"), or a reprogrammed somatic cell of varying differentiation status.

In general, the methods comprise treating the engineered somatic cells with an agent. The treatment with an agent may be contacting the cells with an agent which alters chromatin structure, or may be transfecting the cells with one or more pluripotency gene, or both. The above two treatments may be concurrent, or may be sequential, with no particular preference for order. In a further embodiment, reprogrammed somatic cells are identified by selecting for cells that express the appropriate selectable marker. In still a further embodiment, reprogrammed somatic cells are further assessed for pluripotency characteristics. The presence of pluripotency characteristics indicates that the somatic cells have been reprogrammed to a pluripotent state.

Differentiation status of cells is a continuous spectrum, with terminally differentiated state at one end of this spectrum and de-differentiated state (pluripotent state) at the other end. Reprogramming, as used herein, refers to a process that alters or reverses the differentiation status of a somatic cell, which can be either partially or terminally differentiated. Reprogramming includes complete reversion, as well as partial reversion, of the differentiation status of a somatic cell. In other words, the term "reprogramming", as used herein, encompasses any movement of the differentiation status of a cell along the spectrum toward a less-differentiated state. For example, reprogramming includes reversing a multipotent cell back to a pluripotent cell, reversing a terminally differentiated cell back to either a multipotent cell or a pluripotent cell. In one embodiment, reprogramming of a somatic cell turns the somatic cell all the way back to a pluripotent state. In another embodiment, reprogramming of a somatic cell turns the somatic cell back to a multipotent state. The term "less-differentiated state", as used herein, is thus a relative term and includes a completely de-differentiated state and a partially differentiated state.

The term "pluripotency characteristics", as used herein, refers to many characteristics associated with pluripotency, including, for example, the ability to differentiate into all types of cells and an expression pattern distinct for a pluripotent cell, including expression of pluripotency genes, expression of other ES cell markers, and on a global level, a distinct expression profile known as "stem cell molecular signature" or "stemness."

Thus, to assess reprogrammed somatic cells for pluripotency characteristics, one may analyze such cells for different growth characteristics and ES cell-like morphology. Cells may be injected subcutaneously into immunocompromised SCID mice to induce teratomas (a standard assay for ES cells). ES-like cells can be differentiated into embryoid bodies (another ES specific feature). Moreover, ES-like cells can be differentiated in vitro by adding certain growth factors known to drive differentiation into specific cell types. Self-renewing capacity, marked by induction of telomerase activity, is another plutipotency characteristics that can be monitored. One may carry out functional assays of the reprogrammed somatic cells by introducing them into blastocysts and determine whether the cells are capable of giving rise to all cell types. See Hogan et al., 2003. If the reprogrammed cells are capable of forming a few cell types of the body, they are multipotent; if the reprogrammed cells are capable of forming all cell types of the body including germ cells, they are pluripotent.

One may also examine the expression of an individual pluripotency gene in the reprogrammed somatic cells to assess their pluripotency characteristics. Additionally, one may assess the expression of other ES cell markers. Stage-specific embryonic 15 antigens-1, -3, and -4 (SSEA-1, SSEA-3, SSEA-4) are glycoproteins specifically expressed in early embryonic development and are markers for ES cells (Solter and Knowles, 1978, Proc. Natl. Acad. Sci. USA 75:5565-5569; Kannagi et al., 1983, EMBO J. 2:2355-2361). Elevated expression of the enzyme Alkaline Phosphatase (AP) is another marker associated with undifferentiated embryonic stem cells (Wobus et al., 1984, Exp. Cell 152:212-219; Pease et al., 1990, Dev. Biol. 141:322-352). Other stem/progenitor cells markers include the intermediate neurofilament nestin (Lendahl et al., 1990, Cell 60:585-595; Dah-Istrand et al., 1992, J. Cell Sci. 103:589-597), the membrane glycoprotein prominin/AC133 (Weigmann et al., 1997, Proc. Natl. Acad. USA 94:12425-12430; Corbeil et al., 1998, Blood 91:2625-22626), the transcription factor Tcf-4 (Korinek et al, 1998, Nat. Genet. 19: 379-383; Lee et al., 1999, J. Biol. Chem. 274.1566-1572), and the transcription factor Cdx1 (Duprey et al., 1988, Genes Dev. 2:1647-1654; Subramania'n et al., 1998, Differentiation 64:11-18).

One may additionally conduct expression profiling of the reprogrammed somatic cells to assess their pluripotency characteristics. Pluripotent cells, such as embryonic stem cells, and multipotent cells, such as adult stem cells, are known to have a distinct pattern of global gene expression profile. This distinct pattern is termed "stem cell molecular signature", or "stemness". See, for example, Ramalho-Santos et al., Science 298: 597-600 (2002); Ivanova et al., Science 298: 601-604.

Somatic cells may be reprogrammed to gain either a complete set of the pluripotency characteristics and are thus pluripotent. Alternatively, somatic cells may be reprogrammed to gain only a subset of the pluripotency characteristics. In another alternative, somatic cells may be reprogrammed to be multipotent.

In a further embodiment, in conjunction with contacting the somatic cells of the invention with an agent which alters chromatin structure, at least one gene that affects pluripotent state of a cell may be further introduced into the same cells. This may be carried out sequentially. For example, the somatic cells of the invention may be first contacted with an agent which alters chromatin structure. Then at least one pluripotency gene can be introduced into the same cells, or vice versa. Alternatively, the two steps may be carried out simultaneously.

Genes that affect pluripotent state of a cell includes pluripotency genes, genes involved in chromatin remodeling, and genes that are important for maintaining pluripotency, such as LIF, BMP, and PD098059 (See Cell, 115: 281-292 (2003); Philos Trans R Soc Lond B Biol Sci. 2003 Aug. 29; 358 (1436):1397-402).

The exogenously introduced pluripotency gene may be carried out in several ways. In one embodiment, the exogenously introduced pluripotency gene may be expressed from a chromosomal locus different from the endogenous chromosomal locus of the pluripotency gene. Such chromosomal locus may be a locus with open chromatin structure, and contain gene(s) dispensible for a somatic cell. In other words, the desirable chromosomal locus contains gene(s) whose disruption will not cause cells to die. Exemplary chromosomal loci include, for example, the mouse ROSA 26 locus and type II collagen (Col2a1) locus (See Zambrowicz et al., 1997) The exogenously introduced pluripotency gene may be expressed from an inducible promoter such that their expression can be regulated as desired.

In an alternative embodiment, the exogenously introduced pluripotency gene may be transiently transfected into cells, either individually or as part of a cDNA expression library, prepared from pluripotent cells. Such pluripotent cells may be embryonic stem cells, oocytes, blastomeres, inner cell mass cells, embryonic germ cells, embryoid body (embryonic) cells, morula-derived cells, teratoma (teratocarcinoma) cells, and multipotent partially differentiated embryonic stem cells taken from later in the embryonic development process.

The cDNA library is prepared by conventional techniques. Briefly, mRNA is isolated from an organism of interest. An RNA-directed DNA polymerase is employed for first strand synthesis using the mRNA as template. Second strand synthesis is carried out using a DNA-directed DNA polymerase which results in the cDNA product. Following conventional processing to facilitate cloning of the cDNA, the cDNA is inserted into an expression vector such that the cDNA is operably linked to at least one regulatory sequence. The choice of expression vectors for use in connection with the cDNA library is not limited to a particular vector. Any expression vector suitable for use in mouse cells is appropriate. In one embodiment, the promoter which drives expression from the cDNA expression construct is an inducible promoter. The term regulatory sequence includes promoters, enhancers and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology*, Academic Press, San Diego, Calif. (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express cDNAs. Such useful expression control sequences, include, for example, the early and late promoters of SV40, tet promoter, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

The exogenously introduced pluripotency gene may be expressed from an inducible promoter. The term "inducible promoter", as used herein, refers to a promoter that, in the absence of an inducer (such as a chemical and/or biological agent), does not direct expression, or directs low levels of expression of an operably linked gene (including cDNA), and, in response to an inducer, its ability to direct expression is enhanced. Exemplary inducible promoters include, for example, promoters that respond to heavy metals (CRC Boca Raton, Fla. (1991), 167-220; Brinster et al. Nature (1982), 296, 39-42), to thermal shocks, to hormones (Lee et al. P.N.A.S. USA (1988), 85, 1204-1208; (1981), 294, 228-232; Klock et al. Nature (1987), 329, 734-736; Israel and Kaufman, Nucleic Acids Res. (1989), 17, 2589-2604), promoters that respond to chemical agents, such as glucose, lactose, galactose or antibiotic.

A tetracycline-inducible promoter is an example of an inducible promoter that responds to an antibiotics. See Gossen et al., 2003. The tetracycline-inducible promoter comprises a minimal promoter linked operably to one or more tetracycline operator(s). The presence of tetracycline or one of its analogues leads to the binding of a transcription activator to the tetracycline operator sequences, which activates the minimal promoter and hence the transcription of the associated cDNA. Tetracycline analogue includes any compound that displays structural homologies with tetracycline and is capable of activating a tetracycline-inducible promoter. Exemplary tetracycline analogues includes, for example, doxycycline, chlorotetracycline and anhydrotetracycline.

Thus, in one embodiment, the present invention provides mice and somatic cells carrying at least one pluripotency gene expressed as a transgene under an inducible promoter. It is possible that somatic cells with such inducible pluripotency transgene(s) are more prone to be reprogrammed.

Any of the engineered somatic cells of the present invention may be used in the methods. In one embodiment, somatic cells used in the methods comprise only one endogenous pluripotency gene linked to a first selectable marker, and the selection step is carried out to select for the expression of the first selectable marker. In an alternative embodiment, the somatic cells used in the methods comprise any number of endogenous pluripotency genes, each of which is linked to a distinct selectable marker respectively, and the selection step is carried out to select for at least a subset of the selectable markers. For example, the selection step may be carried out to select for all the selectable markers linked to the various endogenous pluripotency genes.

In an alternative embodiment, somatic cells used in the method comprise a selectable marker linked to an endogenous pluripotency gene and an additional pluripotency gene expressed as a transgene under an inducible promoter. For these cells, the method of reprogramming may comprises induce the expression of the pluripotency transgene and select for the expression of the selectable marker. The method may further comprise contacting the somatic cells with an agent that alter chromatic structure.

Without wishing to be bound by theory, the agents used in the method may cause chromatin to take on a more open structure, which is more permissive for gene expression. DNA methylation and histone acetylation are two known events that alter chromatin toward a more closed structure. For example, loss of methylation by genetic deletion of DNA methylation enzyme Dnmt1 in fibroblasts results in reactivation of endogenous Oct4 gene. See J. Biol. Chem. 277: 34521-30, 2002; and Bergman and Mostoslaysky, Biol. Chem., 1990. Thus, DNA methylation inhibitors and histone deacelyation inhibitors are two classes of agents that may be used in the methods of the invention. Exemplary agents include 5-aza-cytidine, TSA and valproic acid.

In another embodiment, methods of the invention may further include repeating the steps of treating the cells with an agent. The agent used in the repeating treatment may be the same as, or different from, the one used during the first treatment.

Methods for Screening for an Agent that Reprograms Somatic Cells

The present invention also provides methods for identifying an agent that reprograms somatic cells to a less-differentiated state, as well as the agents thus identified. In one embodiment, the methods comprise contacting the engineered somatic cells of the invention with a candidate agent, selecting for cells that express the appropriate selectable marker. The presence of cells that express the appropriate selectable marker indicates that the agent reprograms somatic cells. Such an agent is referred as a “reprogramming agent” for purpose of this application.

In a further embodiment, the methods comprise contacting the engineered somatic cells of the invention with a candidate agent, selecting for cells that express the appropriate selectable marker, and assessing the cells so selected for pluripotency characteristics. The presence of a complete set of pluripotency characteristics indicates that the agent reprograms somatic cells to become pluripotent.

Candidate agents used in the invention encompass numerous chemical classes, though typically they are organic molecules, including small organic compounds. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, nucleic acids and derivatives, structural analogs or combinations thereof.

Candidate agents may be naturally arising, recombinant or designed in the laboratory. The candidate agents may be isolated from microorganisms, animals, or plants, or may be produced recombinantly, or synthesized by chemical methods known in the art. In some embodiments, candidate agents are isolated from libraries of synthetic or natural compounds using the methods of the present invention. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, including acylation, alkylation, esterification, amidification, to produce structural analogs.

There are numerous commercially available compound libraries, including, for example, the Chembridge DIVERSet. Libraries are also available from academic investigators, such as the Diversity set from the NCI developmental therapeutics program.

The screening methods mentioned above are based on assays performed on cells. These cell-based assays may be performed in a high throughput screening (HTS) format, which has been described in the art. For example, Stockwell et al. described a high-throughput screening of small molecules in miniaturized mammalian cell-based assays involving post-translational modifications (Stockwell et al., 1999). Likewise, Qian et al. described a leukemia cell-based assay for high-throughput screening for anti-cancer agents (Qian et al., 2001). Both references are incorporated herein in their entirety.

A reprogramming agent may belong to any one of many different categories. For example, a reprogramming agent may be a chromatin remodeling agent. A chromatin remodeling agent may be a protein involved in chromatin remodeling or an agent known to alter chromatin toward a more open structure, such as a DNA methylation inhibitor or a histone deacelyation inhibitor. Exemplary compounds include 5-aza-cytidine, TSA and valproic acid. For another example, such an agent may be a pluripotency protein, including, for example, Nanog, Oct-4 and Stella. Such an agent may also be a gene essential for pluripotency, including, for example, Sox2, FoxD3, and LIF, and Stat3. See Smith et al. 1988, William et al., 1988, Ihle, 1996, Avilion et al., 2003, and Hanna et al., 2002)

Methods for Reprogramming Somatic Cells with a Reprogramming Agent

The reprogramming agent identified by the methods of the present invention is useful for reprogramming somatic cells into pluripotent or multipotent cells. Accordingly, the present invention provides methods for reprogramming somatic cells to a less differentiated state, comprising contacting somatic cells with a reprogramming agent. The somatic cells used may be native somatic cells, or engineered somatic cells. It is not necessary for these cells to carry a selectable marker integrated into the endogenous locus of a pluripotency gene.

Reprogrammed Somatic Cells and these Uses

The present invention also provides reprogrammed somatic cells (RSCs), including reprogrammed pluripotent somatic cells (RPSCs), produced by the methods of the invention. These methods, useful for the generation of cells of a desired cell type, have wide range of applications. For one example, these methods have applications in livestock management, involving the precise genetic manipulation of animals for economic or health purposes. For another example, these methods have medical application in treating or preventing a condition.

Accordingly, the invention provides methods for the treatment or prevention of a condition in a mammal. In one embodiment, the methods start with obtaining somatic cells from the individual, reprogramming the somatic cells so obtained by methods of the present invention to obtain RPSCs. The RPSCs are then cultured under conditions suitable for development of the RPSCs into cells of a desired cell type. The developed cells of the desired cell type are harvested and introduced into the individual to treat the condi-

tion. In an alternative embodiment, the methods start with obtaining somatic cells from the individual, reprogramming the somatic cells so obtained by methods of the present invention. The RPSCs are then cultured under conditions suitable for development of the RPSCs into a desired organ, which is harvested and introduced into the individual to treat the condition.

The RPSCs of the present invention are ES-like cells, and thus may be induced to differentiate to obtain the desired cell types according to known methods to differentiate ES cells. For example, the RPSCs may be induced to differentiate into hematopoietic stem cells, muscle cells, cardiac muscle cells, liver cells, cartilage cells, epithelial cells, urinary tract cells, etc., by culturing such cells in differentiation medium and under conditions which provide for cell differentiation. Medium and methods which result in the differentiation of embryonic stem cells are known in the art as are suitable culturing conditions.

For example, Palacios et al., Proc. Natl. Acad. Sci., USA, 92: 7530-37 (1995) teaches the production of hematopoietic stem cells from an embryonic cell line by subjecting stem cells to an induction procedure comprising initially culturing aggregates of such cells in a suspension culture medium lacking retinoic acid followed by culturing in the same medium containing retinoic acid, followed by transferral of cell aggregates to a substrate which provides for cell attachment.

Moreover, Pedersen, J. Reprod. Fertil. Dev., 6: 543-52 (1994) is a review article which references numerous articles disclosing methods for in vitro differentiation of embryonic stem cells to produce various differentiated cell types including hematopoietic cells, muscle, cardiac muscle, nerve cells, among others.

Further, Bain et al., Dev. Biol., 168:342-357 (1995) teaches in vitro differentiation of embryonic stem cells to produce neural cells which possess neuronal properties. These references are exemplary of reported methods for obtaining differentiated cells from embryonic or stem-like cells. These references and in particular the disclosures therein relating to methods for differentiating embryonic stem cells are incorporated by reference in their entirety herein.

Thus, using known methods and culture medium, one skilled in the art may culture the subject embryonic or stem-like cells to obtain desired differentiated cell

types, e.g., neural cells, muscle cells, hematopoietic cells, etc. In addition, the use of inducible Bcl-2 or Bcl-xl might be useful for enhancing in vitro development of specific cell lineages. In vivo, Bcl-2 prevents many, but not all, forms of apoptotic cell death that occur during lymphoid and neural development. A thorough discussion of how Bcl-2 expression might be used to inhibit apoptosis of relevant cell lineages following transfection of donor cells is disclosed in U.S. Pat. No. 5,646,008, which is herein incorporated by reference.

The subject RPSCs may be used to obtain any desired differentiated cell type. Therapeutic usages of such differentiated human cells are unparalleled. For example, human hematopoietic stem cells may be used in medical treatments requiring bone marrow transplantation. Such procedures are used to treat many diseases, e.g., late stage cancers such as ovarian cancer and leukemia, as well as diseases that compromise the immune system, such as AIDS. Hematopoietic stem cells can be obtained, e.g., by fusing adult somatic cells of a cancer or AIDS patient, e.g., epithelial cells or lymphocytes with an enucleated oocyte, e.g., bovine oocyte, obtaining embryonic or stem-like cells as described above, and culturing such cells under conditions which favor differentiation, until hematopoietic stem cells are obtained. Such hematopoietic cells may be used in the treatment of diseases including cancer and AIDS.

The methods of the present invention can also be used to treat, prevent, or stabilize a neurological disease such as Alzheimer's disease, Parkinson's disease, Huntington's disease, or ALS, lysosomal storage diseases, multiple sclerosis, or a spinal cord injury. For example, somatic cells may be obtained from the individual in need of treatment, and reprogrammed to gain pluripotency, and cultured to derive neurectoderm cells that may be used to replace or assist the normal function of diseased or damaged tissue.

For the treatment or prevention of endocrine conditions, RPSCs that produce a hormone, such as a growth factor, thyroid hormone, thyroid-stimulating hormone, parathyroid hormone, steroid, serotonin, epinephrine, or norepinephrine may be administered to a mammal. Additionally, reprogrammed epithelial cells may be administered to repair damage to the lining of a body cavity or organ, such as a lung, gut, exocrine gland, or urogenital tract. It is also contemplated that RPSCs may be administered to a mammal to treat damage or deficiency of cells in an organ such as the bladder, brain, esophagus, fallopian tube, heart, intestines, gallbladder, kidney, liver, lung, ovaries, pancreas, prostate, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, ureter, urethra, or uterus.

The great advantage of the present invention is that it provides an essentially limitless supply of isogenic or synegenic human cells suitable for transplantation. Therefore, it will obviate the significant problem associated with current transplantation methods, i.e., rejection of the transplanted tissue which may occur because of host versus graft or graft versus host rejection. Conventionally, rejection is prevented or reduced by the administration of anti-rejection drugs such as cyclosporin. However, such drugs have significant adverse side-effects, e.g., immunosuppression, carcinogenic properties, as well as being very expensive. The present invention should eliminate, or at least greatly reduce, the need for anti-rejection drugs, such as cyclosporine, imulan, FK-506, glucocorticoids, and rapamycin, and derivatives thereof.

RPSCs may also be combined with a matrix to form a tissue or organ in vitro or in vivo that may be used to repair or replace a tissue or organ in a recipient mammal. For example, RPSCs may be cultured in vitro in the presence of a matrix to produce a tissue or organ of the urogenital system, such as the bladder, clitoris, corpus cavermosum, kidney, testis, ureter, uretal valve, or urethra, which may then be transplanted into a mammal (Atala, Curr. Opin. Urol. 9(6):517-526, 1999). In another transplant application, synthetic blood vessels are formed in vitro by culturing reprogrammed cells in the presence of an appropriate matrix, and then the vessels are transplanted into a mammal for the treatment or prevention of a cardiovascular or circulatory condition. For the generation of donor cartilage or bone tissue, RPSCs such as chondrocytes or osteocytes are cultured in vitro in the presence of a matrix under conditions that allow the formation of cartilage or bone, and then the matrix containing the donor tissue is administered to a mammal. Alternatively, a mixture of the cells and a matrix may be administered to a mammal for the formation of the desired tissue in vivo. Preferably, the cells are attached to the surface of the matrix or encapsulated by the matrix. Examples of matrices that may be used for the formation of donor tissues or organs include collagen matrices, carbon fibers, polyvinyl alcohol sponges, acrylateamide sponges, fibrin-thrombin gels, hyaluronic acid-based polymers, and synthetic polymer matrices containing polyanhydride, poly-

orthoester, polyglycolic acid, or a combination thereof (see, for example, U.S. Pat. Nos. 4,846,835; 4,642,120; 5,786,217; and 5,041,138).

The RPSCs produced according to the invention may be used to produce genetically engineered or transgenic differentiated cells. Essentially, this will be effected by introducing a desired gene or genes, or removing all or part of an endogenous gene or genes of RPSCs produced according to the invention, and allowing such cells to differentiate into the desired cell type. A preferred method for achieving such modification is by homologous recombination because such technique can be used to insert, delete or modify a gene or genes at a specific site or sites in the stem-like cell genome.

This methodology can be used to replace defective genes, e.g., defective immune system genes, cystic fibrosis genes, or to introduce genes which result in the expression of therapeutically beneficial proteins such as growth factors, lymphokines, cytokines, enzymes, etc. For example, the gene encoding brain derived growth factor maybe introduced into human embryonic or stem-like cells, the cells differentiated into neural cells and the cells transplanted into a Parkinson's patient to retard the loss of neural cells during such disease. Examples of mutations that may be rescued using these methods include mutations in the cystic fibrosis gene; mutations associated with Dunningan's disease such as the R482W, R482Q, and R584H mutations in the lamin A gene; and mutations associated with the autosomal-dominant form of Emery Deyfuss muscular dystrophy such as the R249Q, R453W, and Q6STOP mutations in the lamin A gene. In the Q6STOP mutation, the codon for Gln6 is mutated to a stop codon.

Previously, cell types transfected with BDNF varied from primary cells to immortalized cell lines, either neural or non-neural (myoblast and fibroblast) derived cells. For example, astrocytes have been transfected with BDNF gene using retroviral vectors, and the cells grafted into a rat model of Parkinson's disease (Yoshimoto et al., Brain Research, 691:25-36, (1995)). This ex vivo therapy reduced Parkinson's-like symptoms in the rats up to 45% 32 days after transfer. Also, the tyrosine hydroxylase gene has been placed into astrocytes with similar results (Lundberg et al., Develop. Neurol., 139: 39-53 (1996) and references cited therein).

However, such ex vivo systems have problems. In particular, retroviral vectors currently used are down-regulated in vivo and the transgene is only transiently expressed (review by Mulligan, Science, 260: 926-932 (1993)). Also, such studies used primary cells, astrocytes, which have finite life span and replicate slowly. Such properties adversely affect the rate of transfection and impede selection of stably transfected cells. Moreover, it is almost impossible to propagate a large population of gene targeted primary cells to be used in homologous recombination techniques.

By contrast, the difficulties associated with retroviral systems should be eliminated by the use of RPSCs of the present invention, which are ES-like cells. Using known methods to introduced desired genes/mutations into ES cells, RPSCs may be genetically engineered, and the resulting engineered cells differentiated into desired cell types, e.g., heniatopoietic cells, neural cells, pancreatic cells, cartilage cells, etc. Genes which may be introduced into the RPSCs include, for example, epidermal growth factor, basic fibroblast growth factor, glial derived neurotrophic growth factor, insulin-like growth factor (I and II), neurotrophin3, neurotrophin-4/5, ciliary neurotrophic factor, AFT-1, cytokine genes (interleukins, interferons, colony stimulating factors, tumor necrosis factors (alpha and beta), etc.), genes encoding therapeutic enzymes, collagen, human serum albumin, etc.

In addition, it is also possible to use one of the negative selection systems now known in the art for eliminating therapeutic cells from a patient if necessary. For example, donor cells transfected with the thymidine kinase (TK) gene will lead to the production of embryonic cells containing the TK gene. Differentiation of these cells will lead to the isolation of therapeutic cells of interest which also express the TK gene. Such cells may be selectively eliminated at any time from a patient upon gancyclovir administration. Such a negative selection system is described in U.S. Pat. No. 5,698,446, and is herein incorporated by reference.

Examples of diseases, disorders, or conditions that may be treated or prevented include neurological, endocrine, structural, skeletal, vascular, urinary, digestive, integumentary, blood, immune, auto-immune, inflammatory, endocrine, kidney, bladder, cardiovascular, cancer, circulatory, digestive, hematopoeitic, and muscular diseases, disorders, and conditions. In addition, reprogrammed cells may be used for reconstructive applications, such as for repairing or replacing tissues or organs.

With respect to the therapeutic methods of the invention, it is not intended that the administration of RPSCs to a mammal be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration, including intramuscular, intravenous, intraarticular, intralesional, subcutaneous, or any other route sufficient to provide a dose adequate to prevent or treat a disease. The RPSCs may be administered to the mammal in a single dose or multiple doses. When multiple doses are administered, the doses may be separated from one another by, for example, one week, one month, one year, or ten years. One or more growth factors, hormones, interleukins, cytokines, or other cells may also be administered before, during, or after administration of the cells to further bias them towards a particular cell type.

The RPSCs of the present invention may be used as an in vitro model of differentiation, in particular for the study of genes which are involved in the regulation of early development. Differentiated cell tissues and organs using the RPSCs may be used in drug studies.

Furthermore, the RPSCs produced according to the invention maybe introduced into animals, e.g., SCID mice, cows, pigs, e.g., under the renal capsule or intramuscularly and used to produce a teratoma therein. This teratoma can be used to derive different tissue types. Also, the inner cell mass produced by X-species nuclear transfer may be introduced together with a biodegradable, biocompatible polymer matrix that provides for the formation of 3-dimensional tissues. After tissue formation, the polymer degrades, ideally just leaving the donor tissue, e.g., cardiac, pancreatic, neural, lung, liver. In some instances, it may be advantageous to include growth factors and proteins that promote angiogenesis. Alternatively, the formation of tissues can be effected totally in vitro, with appropriate culture media and conditions, growth factors, and biodegradable polymer matrices.

Applications of the Somatic Cell Reprogramming Methods and RPSCs in Animals

The reprogramming methods disclosed herein may be used to generate RPSCs for a variety of animal species. The RPSCs generated can be useful to produce desired animals. Animals include, for example, avians and mammals as well as any animal that is an endangered species. Exemplary birds include domesticated birds (e.g., quail, chickens, ducks, geese, turkeys, and guinea hens) as well as other birds such as birds of prey (e.g., hawks, falcons, ospreys, condors, etc.), endangered birds (e.g., parrots, California condor, etc.), ostriches etc. Exemplary mammals include murine, caprine,

ovine, bovine, porcine, canine, feline and primate. Of these, preferred members include domesticated animals, including, for examples, cattle, buffalo, pigs, horses, cows, rabbits, guinea pigs, sheep, and goats.

RPSCs generated by the reprogramming methods of the present invention allows one, for the first time, to genetically engineer animals other than mouse and human. RPSCs are ES-like cells, and are thus amenable to genetic manipulation. To date, no ES cells are available for animals other than mouse and human. As a result, for these animals, it is currently practically impossible to create genetically modified animals having targeted mutations. The ES-cell like RPSCs can be manipulated to introduce desired targeted genetic modifications. The resulting engineered RPSCs can then be used to generate a cloned animal with the desired genetic modifications in its germ line, using methods described for ES cells in mouse. See Capecchi and Thomas, U.S. Pat. Nos. 5,487,992, 5,627,059, 5,631,153, and 6,204,061. Genetic engineering in animals has potentially great applications in a variety of animals, especially farm animals.

The somatic cell reprogramming methods of the present invention provides at least two methods for delivering optimized farm animals. In the first, somatic cell reprogramming can be used to capture the best available phenotype for a farm animal stock. The current technologies used to deliver optimized farm animals are based on selective breeding, and expansion from preferred breeding stocks. Animals that have been selected on the basis of superior characteristics, including, for example, meat content, egg production (in the case of poultry), feed conversion ratio, are used to breed large numbers of animals that are in turn used in the human food supply. This traditional process has profound inherent inefficiencies. The phenotype observed in an individual animal is often only partially transmitted in the progeny of that animal. Therefore, traditional breeding schemes are inefficient in capturing the very best phenotype in all of the progeny animals. In contrast, the reprogramming methods of the present invention provides a controlled and efficient way to achieve the same goal, by generating RPSCs from somatic cells of an animal with the desired characteristics. The RPSCs generated may be used immediately to generate cloned animals derived from the RPSCs. Known methods for generating mice from ES cells can be used for this procedure. Alternatively, the RPSCs generated may be cryopreserved and thawed in response to a grower's needs.

In the second method, somatic cells from an animal with the desired characteristics are reprogrammed to produce RPSCs. The RPSCs are further genetically engineered to introduce desired genetic modification(s), before being placed into a recipient embryo to produce desired progeny.

The reprogramming methods can also be used to rescue endangered species. Somatic cell reprogramming provides an efficient method to generate RPSCs from somatic cells of an endangered animal. The resulting RPSCs can be used immediately to expand the numbers of the endangered animal. Alternatively, the RPSCs can be cryopreserved to generate a RPSC stock for the endangered species, as a safeguard measure against extinction of the endangered species.

Methods for Gene Identification

The present invention provides methods for identifying a gene that activates the expression of an endogenous pluripotency gene in somatic cells. The methods comprise: transfecting the somatic cells of the present invention with a cDNA library prepared from ES cells or oocytes, selecting for cells that express the first selectable marker, and assessing the expression of the first endogenous pluripotency gene in the transfected cells that express the first selectable marker. The expression of the first endogenous pluripotency gene indicates that the cDNA encodes a gene that activates the expression of an endogenous pluripotency gene in somatic cells.

The methods are applicable for identifying a gene that activates the expression of at least two endogenous pluripotency genes in somatic cells. The somatic cells used in the methods further comprise a second endogenous pluripotency gene linked to a second selectable marker. The methods are modified to select for transfected cells that express both selectable markers, among which the expression of the first and the second endogenous pluripotency genes are assessed. The expression of both the first and the second endogenous pluripotency genes indicates that the cDNA encodes a gene that activates the expression of at least two pluripotency genes in somatic cells.

The methods are further applicable for identifying a gene that activates the expression of at least three endogenous pluripotency genes in somatic cells. The somatic cells used in the methods further comprise a third endogenous pluripotency gene linked to a third selectable marker. The methods are modified to select for transfected cells that express all three selectable markers, among which the expression of all three endogenous pluripotency genes are assessed. The expression of all three endogenous pluripotency genes indicates that the cDNA encodes a gene that activates the expression of at least three pluripotency genes in somatic cells.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of mouse genetics, developmental biology, cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, Current Protocols in Cell Biology, ed. by Bonifacino, Dasso, Lippincott-Schwartz, Harford, and Yamada, John Wiley and Sons, Inc., New York, 1999; Manipulating the Mouse Embryos, A Laboratory Manual, 3rd Ed., by Hogan et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2003; Gene Targeting: A Practical Approach, IRL Press at Oxford University Press, Oxford, 1993; and Gene Targeting Protocols, Human Press, Totowa, N.J., 2000. All patents, patent applications and references cited herein are incorporated in their entirety by reference.

EXEMPLIFICATION

The invention now being generally described, it will be more readily understood by reference to the following example, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example

Oct4-Induced Fibroblasts are More Susceptible to Reprogramming than Unduced Fibroblasts as Demonstrated by Nuclear Transfer Experiment

A. Generation of Transgenic Mouse Carrying an Inducible Oct4 Transgene

An inducible Oct4 allele is constructed as the following: first, two integration vectors are constructed. The first integration vector, inducible Oct4 integration vector, contains an Oct4 gene driven by a tetracycline-inducible promoter (Tet-Op). The Tet-Op-Oct4 cassette is flanked by a splice-acceptor double poly-A signal (SA-dpA) at its 5' end and a SV40 polyA tail (SV40-pA) at its 3' end. The second integration vector, tetracycline activator integration vector, contains a

mutant form of tetracycline activator, M2-rtTA, which is more responsive to doxycycline (Dox) induction than the wild type activator. (Urlinger S. et al., 2000)

The two integration vectors are introduced into V6.5 ES cells: the inducible Oct4 integration vector and the tetracycline activator integration vector are introduced into the Collagen locus and the Rosa26 locus respectively via site-specific integration, as shown in FIG. **1**. The resulting ES cells are used to make Oct4-inducible mice by tetraploid complementation.

B. Expression of the Inducible Oct4 Transgene

Fibroblasts derived from tail biopsies of the Oct4-inducible mice were cultured. A fraction of the cultured fibroblasts were induced with doxycycline for 3 days (at 2 microgram/ml), and Oct4 expression was detected by Northern blot and Western blot analysis. As shown in FIG. **2**, the Oct4 expression level in fibroblasts treated with doxycycline is comparable to the Oct4 expression level in ES cells, and undetectable in fibroblasts not treated with doxycycline. The expression results demonstrate that the inducible Oct4 transgene is expressed as planned.

C. Nuclear Transfer Experiment

Nuclear transfer was performed on fibroblasts derived from tail biopsies of mice that carry the inducible Oct4 transgene. Dox induction was for 24 hours prior to nuclear transfer. Cloned embryos were then activated and cultured to the blastocyst stage to derive ES cells as described previously (Hochedlinger and Jaenisch, 2002). As shown in Table 1, on average, blastocyst formation and ES cell derivation (as measured as a fraction of eggs with pronucleus formation) is more efficient from Oct4 induced fibroblast than from uninduced fibroblasts. This result demonstrated that induced Oct4 expression in somatic cells such as fibroblasts make these cells more susceptible to reprogramming.

One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods, systems and kits are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention and are defined by the scope of the claims. It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

TABLE 1

In vitro development of clones derived from Oct4-induced fibroblasts

Expt.	Oct4	eggs w/PN	Blastocysts (% PN)		ES lines (% PN)	
#1	–	22	5 (23%)	19%	0 (0%)	3%
#2	–	35	5 (14%)		2 (6%)	
#3	+	37	10 (27%)	24%	2 (5%)	7%
#4	+	47	10 (21%)		4 (9%)	

PN . . . ProNucleus formation

Nuclear transfer was performed on fibroblasts derived from tail biopsies of mice that carry the inducible Oct4 transgene. Dox induction was for 24 hours prior to nuclear transfer. Cloned embryos were then activated and cultured to the blastocyst stage to derive ES cells as described previously (Hochedlinger and Jaenisch, Nature, 2002). These preliminary results show that on average blastocyst formation and ES cell derivation is more efficient from Oct4 induced than from uninduced fibroblasts.

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What is claimed is:

1. A method of making a primary somatic cell more susceptible to reprogramming to a less differentiated state, comprising: introducing an exogenous nucleic acid encoding an Oct 4 protein operably linked to at least one regulatory sequence into the somatic cell, wherein expression of the exogenously introduced nucleic acid results in making the somatic cell more susceptible to reprogramming to a less differentiated state.

2. The method of claim **1**, wherein the somatic cell does not comprise a selectable marker.

3. The method of claim **1**, wherein the somatic cell is a human cell or a mouse cell.

4. The method of claim **1**, wherein the somatic cell is an adult stem cell.

5. The method of claim **4**, wherein the adult stem cell is a hematopoietic stem cell, neural stem cell, or mesenchymal stem cell.

6. A method of making a primary somatic cell more susceptible to reprogramming to a less differentiated state, comprising: (a) contacting the somatic cell with a candidate agent of interest with respect to its potential to reprogram a somatic cell; and (b) introducing an exogenous nucleic acid encoding an Oct4 protein operably linked to at least one regulatory sequence into the somatic cell, wherein expression of the exogenously introduced pluripotency gene results in making the somatic cell more susceptible to reprogramming to a less differentiated state.

7. The method of claim **6**, wherein the somatic cell does not comprise a selectable marker.

8. The method of claim **6**, wherein the somatic cell is a human cell or a mouse cell.

9. The method of claim **6**, wherein the somatic cell is an adult stem cell.

10. The method of claim **9**, wherein the adult stem cell is a hematopoietic stem cell, neural stem cell, or mesenchymal stem cell.

11. The method of claim **6**, wherein the agent is a DNA methylation inhibitor, a histone deacetylase inhibitor or PD098059.

12. The method of claim **6**, wherein the agent is Sox-2.

13. A method of making a primary somatic cell more susceptible to reprogramming to a less differentiated state, comprising: introducing an Oct4 protein into the somatic cell, thereby making the somatic cell more susceptible to reprogramming to a less differentiated state.

14. The method of claim **13**, wherein the somatic cell does not comprise a selectable marker.

15. The method of claim **13**, wherein the somatic cell is a human cell or a mouse cell.

16. The method of claim **13**, wherein the somatic cell is an adult stem cell.

17. The method of claim **16**, wherein the adult stem cell is a hematopoietic stem cell, neural stem cell, or mesenchymal stem cell.

* * * * *

EXHIBIT E

US009169490B2

(12) **United States Patent**
Jaenisch et al.

(10) **Patent No.: US 9,169,490 B2**
(45) **Date of Patent: *Oct. 27, 2015**

(54) **METHODS FOR REPROGRAMMING SOMATIC CELLS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

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Related U.S. Application Data

(60) Continuation of application No. 12/703,061, filed on Feb. 9, 2010, now Pat. No. 8,940,536, which is a division of application No. 10/997,146, filed on Nov. 24, 2004, now Pat. No. 7,682,828.

(60) Provisional application No. 60/525,612, filed on Nov. 26, 2003, provisional application No. 60/530,042, filed on Dec. 15, 2003.

(51) **Int. Cl.**

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C12N 5/074	(2010.01)
A01K 67/027	(2006.01)
C07K 14/47	(2006.01)
C12N 15/877	(2010.01)

(52) **U.S. Cl.**

CPC ***C12N 15/85*** (2013.01); ***C12N 5/0696*** (2013.01); *A01K 67/0273* (2013.01); *A01K 67/0275* (2013.01); *A01K 2217/05* (2013.01); *A01K 2227/105* (2013.01); *C07K 14/4702* (2013.01); *C12N 15/8509* (2013.01); *C12N 15/8775* (2013.01); *C12N 2830/003* (2013.01); *C12N 2830/006* (2013.01)

(58) **Field of Classification Search**

CPC C12N 5/0696; C12N 15/85

See application file for complete search history.

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(74) *Attorney, Agent, or Firm* — Lisa M. Warren, Esq.; Morse, Barnes-Brown & Pendleton, P.C.

(57) **ABSTRACT**

The invention provides methods for reprogramming somatic cells to generate multipotent or pluripotent cells. Such methods are useful for a variety of purposes, including treating or preventing a medical condition in an individual. The invention further provides methods for identifying an agent that reprograms somatic cells to a less differentiated state.

11 Claims, 2 Drawing Sheets

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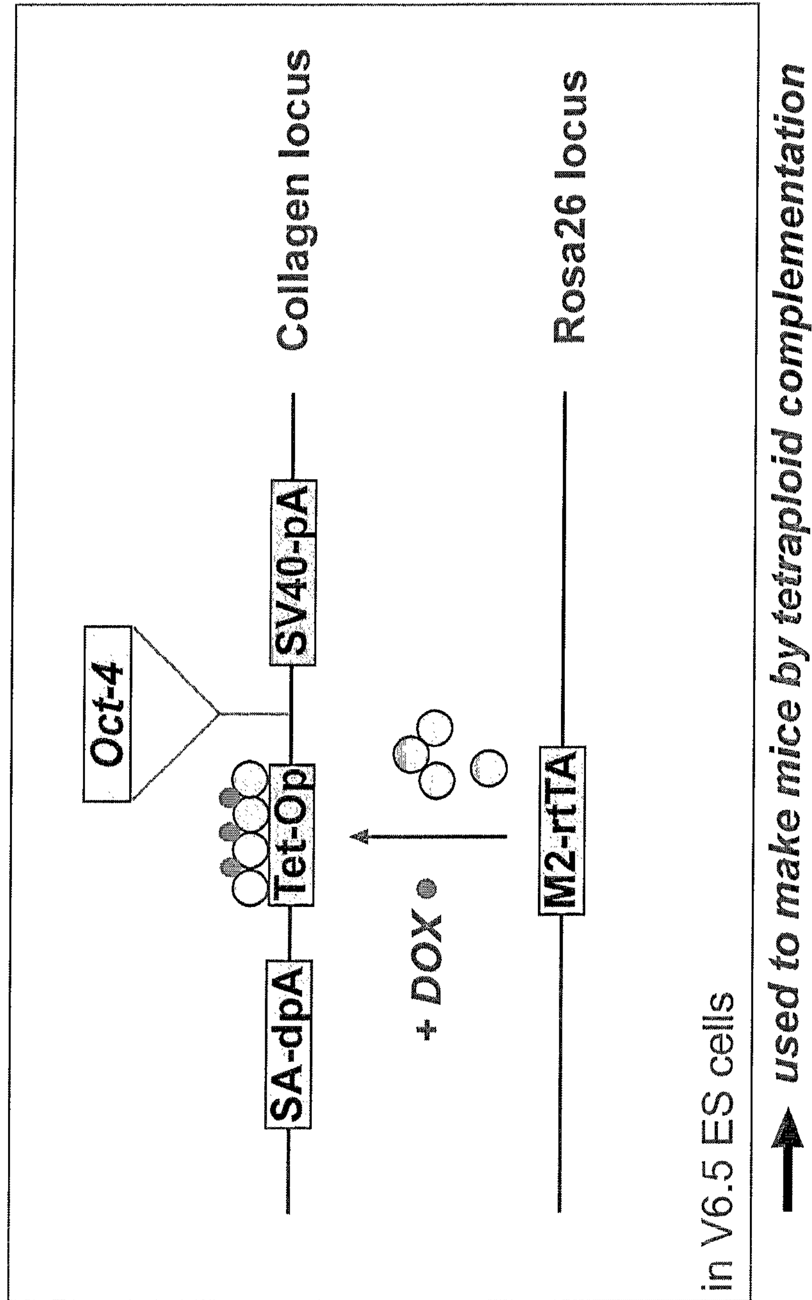

Figure 1. Inducible *Oct4* allele

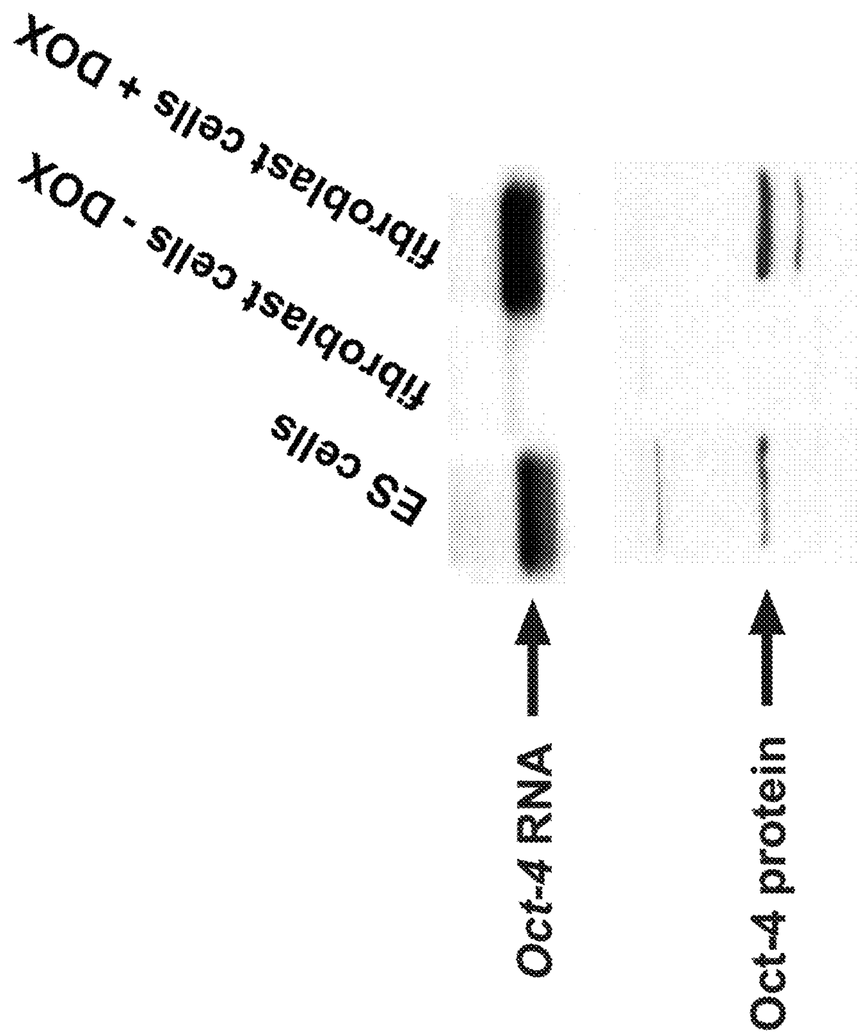
Figure 2. The system works...
fibroblast cells + DOX
fibroblast cells - DOX
ES cells
Oct-4 RNA
Oct-4 protein

METHODS FOR REPROGRAMMING SOMATIC CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 12/703,061, filed Feb. 9, 2010, which is a divisional of U.S. application Ser. No. 10/997,146, filed Nov. 24, 2004 (U.S. Pat. No. 7,682,828), which claims the benefit of U.S. Provisional Application No. 60/525,612, filed Nov. 26, 2003, and U.S. Provisional Application No. 60/530,042, filed Dec. 15, 2003, the specifications of which are incorporated herein by reference in their entirety.

GOVERNMENTAL FUNDING

This invention was made with government support under R37CA84198 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Pluripotent stem cells have the potential to differentiate into the full range of daughter cells having distinctly different morphological, cytological or functional phenotypes unique to a specific tissue. By contrast, descendants of pluripotent cells are restricted progressively in their differentiation potential, with some cells having only one fate. Pluripotent cells have extraordinary scientific and therapeutic potential, as they can be differentiated along the desired differentiation pathway in a precisely controlled manner and used in cell-based therapy.

Two categories of pluripotent stem cells are known to date: embryonic stem cells and embryonic germ cells. Embryonic stem cells are pluripotent stem cells that are derived directly from an embryo. Embryonic germ cells are pluripotent stem cells that are derived directly from the fetal tissue of aborted fetuses. For purposes of simplicity, embryonic stem cells and embryonic germ cells will be collectively referred to as "ES" cells herein.

ES cells are presently obtained via several methods. In a first method, an ES cell line is derived from the inner cell mass of a normal embryos in the blastocyst stage (See U.S. Pat. No. 6,200,806, Thompson, J. A. et al. Science, 282:1145-7, 1998 and Hogan et al., 2003). A second method for creating pluripotent ES cells utilizes the technique of somatic cell nuclear transfer (SCNT). In this technique, the nucleus is removed from a normal egg, thus removing the genetic material. Next, a donor diploid somatic cell is placed next to the enucleated egg and the two cells are fused, or the nucleus is introduced directly into the oocyte by micromanipulation. The fused cell has the potential to develop into a viable embryo, which may then be sacrificed to remove that portion of the embryo containing the stem cell producing inner cell mass.

In a third method, the nucleus of a human cell is transplanted into an entirely enucleated animal oocyte of a species different from the donor cell (referred to herein as animal stem cell nuclear transfer, or "ASCNT"). See U.S. Pat. application Ser. No. 20010012513 (2001). The resultant chimeric cells are used for the production of pluripotent ES cells, in particular human-like pluripotent ES cells. One disadvantage of this technique is that these chimeric cells may contain unknown non-human viruses and still contain the mitochondria of the animal species. Thus, there would be substantial risks of immune rejection if such cells were used in cell transplantation therapies.

In a fourth method, ES cells can be isolated from the primordial germ cells found in the genital ridges of post-implanted embryos.

As described above, all presently available methods depend on controversial sources—embryos (either created naturally or via cloning), fetal tissue and via the mixing of materials of multiple species. The controversy surrounding the sources for such cells, according to many leading scientists and public and private organizations including the NIH, has greatly compromised and slowed the study of such cells and their application.

There is thus a great demand for alternative methods of generating pluripotent cells.

SUMMARY OF THE INVENTION

The present invention provides engineered somatic cells, in which one or more endogenous pluripotency gene(s) is operably linked to a selectable marker in such a manner that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene to which the marker is linked. The invention also provides transgenic mice containing these engineered somatic cells.

The present invention also provides methods for reprogramming somatic cells to a less differentiated state. In the methods, engineered somatic cells of the invention are treated with an agent. Cells that express the selectable marker are then selected, and assessed for pluripotency characteristics. The treatment with an agent may be contacting the cells with an agent which alters chromatin structure, or may be transfecting the cells with at least one pluripotency gene, or both.

The present invention further provides methods for identifying an agent that reprograms somatic cells to a less differentiated state. In the methods, the engineered somatic cells described above are contacted with a candidate agent. Cells that express the selectable marker are then selected, and assessed for pluripotency characteristics. The presence of at least a subset of pluripotency characteristics indicates that the agent is capable of reprogramming somatic cells to a less-differentiated state. The agents identified by the present invention can then by used to reprogram somatic cells by contacting somatic cells with the agents.

The present invention also provides methods for identifying a gene that causes the expression of at least one endogenous pluripotency gene in somatic cells. In the methods, the engineered somatic cells are transfected with a cDNA library prepared from a pluripotent cell, such as an ES cell. The cells that express the appropriate selectable marker are then selected, and the expression of the appropriate endogenous pluripotency gene is examined. The expression of an endogenous pluripotency gene indicates that the cDNA encodes a protein whose expression in the cell results in, directly or indirectly, expression of the endogenous pluripotency gene.

The present invention further provides methods for treating a condition in an individual in need of such treatment. In certain embodiments, somatic cells are obtained from the individual and reprogrammed by the methods of the invention under conditions suitable for the cells to develop into cells of a desired cell type. The reprogrammed cells of a desired cell type are then harvested and introduced into the individual to treat the condition. In certain further embodiments, the somatic cells obtained from the individual contains a mutation in one or more genes. In these instances, the methods are modified so that the somatic cells obtained from the individual are first treated to restore the one or more normal gene(s) to the cells such that the resulting cells carry the normal endogenous gene, which are then introduced into the

individual. In certain other embodiments, methods of the invention can be used to treat individuals in need of a functional organ. In the methods, somatic cells are obtained from an individual in need of a functional organ, and reprogrammed by the methods of the invention to produce reprogrammed somatic cells. Such reprogrammed somatic cells are then cultured under conditions suitable for development of the reprogrammed somatic cells into a desired organ, which is then introduced into the individual. The methods are useful for treating any one of the following conditions: a neurological, endocrine, structural, skeletal, vascular, urinary, digestive, integumentary, blood, autoimmune, inflammatory, or muscular condition.

The present invention also provides methods for producing a cloned animal. In the methods, a somatic cell is isolated from an animal having desired characteristics, and reprogrammed using the methods of the invention to produce one or more reprogrammed pluripotent somatic cell ("RPSC"). The RPSCs are then inserted into a recipient embryo, and the resulting embryo is cultured to produce an embryo of suitable size for implantation into a recipient female, which is then transferred into a recipient female to produce a pregnant female. The pregnant female is maintained under conditions appropriate for carrying the embryo to term to produce chimeric animal progeny, which is then bred with a wild type animal to produce a cloned animal.

In certain embodiments, the RPSCs may alternatively be cryopreserved for future cloning uses. In certain other embodiments, genetic modification, such as a targeted mutation, may be introduced into the RPSCs prior to its insertion into a recipient embryo.

The present invention also provides methods for producing a cloned avian. In the methods, a somatic cell is isolated from an avian having desired characteristics, and reprogrammed using the methods of the invention to produce one or more reprogrammed pluripotent somatic cell ("RPSC"). The RPSCs are then inserted into eggs that are unable to develop into an embryo, and the resulting eggs are then incubated to produce avian offspring having the genotype of the RPSC, thereby producing a cloned avian.

It is contemplated that all embodiments described above are applicable to all different aspects of the invention. It is also contemplated that any of the above embodiments can be freely combined with one or more other such embodiments whenever appropriate.

Specific embodiments of the invention are described in more detail below. However, these are illustrative embodiments, and should not be construed as limiting in any respect.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts an inducible Oct4 allele.

FIG. **2** shows the expression of the inducible Oct4 transgene by Northern blot and Western blot analysis.

DETAILED DESCRIPTION OF THE INVENTION

Overview

Presently, human ES cells or ES-like cells can only be generated from controversial sources. It would be useful to reprogram somatic cells directly into pluripotent cells. Nuclei from somatic cells retain the totopotency potential to direct development of an animal, as demonstrated by nuclear transfer technology. It would be useful to reprogram somatic cells directly into ES cells without the use of oocytes and nuclear transfer technology.

Applicants have devised novel methods of reprogramming somatic cells to generate pluripotent cells or multipotent cells. Applicants have also devised novel methods to identify agents that reprogram somatic cells. The methods take advantage of the engineered somatic cells designed by Applicants, in which an endogenous gene typically associated with pluripotency ("pluripotency gene") is engineered to be operably linked to a selectable marker in a manner that the expression the endogenous pluripotency gene substantially matches the expression of the selectable marker. Because pluripotency genes are generally expressed only in pluripotent cells and not in somatic cells, the expression of an endogenous pluripotent gene(s) is an indication of successful reprogramming. Having a selectable marker operably linked to an endogenous pluripotency gene gives one a powerful mechanism to select for potentially reprogrammed somatic cells, which likely is a rare occurrence. The resulting cells may be further assessed for pluripotency characteristics to confirm whether a somatic cell has been successfully reprogrammed to pluripotency.

Generating pluripotent or multipotent cells by somatic cell reprogramming using the methods of the present invention has at least two advantages. First, the methods of the present invention allow one to generate autologous pluripotent cells, which are cells specific to a patient. The use of autologous cells in cell therapy offers a major advantage over the use of non-autologous cells, which are likely to be subject to immunological rejection. In contrast, autologous cells are unlikely to elicit significant immunological responses (See Munsie et al, 2000). Second, the methods of the present invention allow one to generate pluripotent without using embryos, oocytes and/or nuclear transfer technology.

A pluripotent cell is a cell that has the potential to divide in vitro for a long period of time (greater than one year) and has the unique ability to differentiate into cells derived from all three embryonic germ layers—endoderm, mesoderm and ectoderm.

A multipotent cell is a cell that is able to differentiate into some but not all of the cells derived from all three germ layers. Thus, a multipotent cell is a partially differentiated cell. Adult stem cells are multipotent cells. Known adult stem cells include, for example, hematopoietic stem cells and neural stem cells. A hematopoietic stem cell is multipotent because it has the ability to differentiate into all types of specific blood cells, but it is unlikely that they can differentiate into all cells of a given animal or human. Multipotent/adult stem cells have a great deal of promise in research and in the area of therapeutic applications. For example, multipotent/adult stem cells have already been used in humans in attempts to treat certain blood, neural and cancer diseases.

The term "pluripotency gene", as used herein, refers to a gene that is associated with pluripotency. The expression of a pluripotency gene is typically restricted to pluripotent stem cells, and is crucial for the functional identity of pluripotent stem cells. The transcription factor Oct-4 (also called Pou5fl, Oct-3, Oct3/4) is an example of a pluripotency gene. Oct-4 has been shown to be required for establishing and maintaining the undifferentiated phenotype of ES cells and plays a major role in determining early events in embryogenesis and cellular-differentiation (Nichols et al., 1998, Cell 95:379-391; Niwa et al., 2000, Nature Genet. 24:372-376). Oct-4 is down-regulated as stem cells differentiate into specialised cells. Other exemplary pluripotency genes include Nanog, and Stella (See Chambers et al., 2003, Cell 113: 643-655; Mitsui et al., Cell. 2003, 113(5):631-42; Bortvin et al. Development. 2003, 130(8):1673-80; Saitou et al., Nature. 2002, 418 (6895):293-300.

Engineered Somatic Cells and Transgenic Mice Comprising Such Cells

The present invention provides somatic cells comprising an endogenous pluripotency gene linked to DNA encoding a selectable marker in such a manner that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene. In one embodiment, the somatic cells of the present invention comprise a first endogenous pluripotency gene linked to DNA encoding a first selectable marker in such a manner that the expression of the first selectable marker substantially matches the expression of the first endogenous pluripotency gene. The somatic cells may also be engineered to comprise any number of endogenous pluripotency genes respectively linked to a distinct selectable marker. Thus, in another embodiment, the somatic cells of the present invention comprise two endogenous pluripotency genes, each of which is linked to DNA encoding a distinct selectable marker. In a further embodiment, the somatic cells of the present invention comprise three endogenous pluripotency genes, each of which is linked to DNA encoding a distinct selectable marker. The somatic cells described above will be collectively referred in this application as "engineered somatic cells." The engineered somatic cells may be further engineered to have one or more pluripotency gene expressed as a transgene under an inducible promoter.

The selectable marker is linked to an appropriate endogenous pluripotency gene such that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene. By "substantially match", it is meant that the expression of the selectable marker substantially reflects the expression pattern of the endogenous pluripotency gene. In other words, the selectable marker and the endogenous pluripotency gene are co-expressed. For purpose of the present invention, it is not necessary that the expression level of the endogenous gene and the selectable marker is the same or even similar. It is only necessary that the cells in which an endogenous pluripotency gene is activated will also express the selectable marker at a level sufficient to confer a selectable phenotype on the reprogrammed cells. For example, when the selectable marker is a marker that confers resistance to a lethal drug (a "drug resistance marker"), the cells are engineered in a way that allows cells in which an endogeneous pluripotency gene is activated to also express the drug resistance marker at a sufficient level to confer on reprogrammed cells resistance to lethal drugs. Thus, reprogrammed cells will survive and proliferate whereas non-reprogrammed cells will die.

The DNA encoding a selectable marker may be inserted downstream from the end of the open reading frame (ORF) encoding the desired endogenous pluripotency gene, anywhere between the last nucleotide of the ORF and the first nucleotide of the polyadenylation site. An internal ribosome entry site (IRES) may be placed in front of the DNA encoding the selectable marker. Alternatively, the DNA encoding a selectable marker may be inserted anywhere within the ORF of the desired endogenous pluripotency gene, downstream of the promoter, with a termination signal. An internal ribosome entry site (IRES) may be placed in front of the DNA encoding the selectable marker. The selectable marker may be inserted into only one allele, or both alleles, of the endogenous pluripotency gene.

The somatic cells in the invention may be primary cells or immortalized cells. Such cells may be primary cells (non-immortalized cells), such as those freshly isolated from an animal, or may be derived from a cell line (immortalized cells).

The somatic cells in the present invention are mammalian cells, such as, for example, human cells or mouse cells. They may be obtained by well-known methods, from different organs, e.g., skin, lung, pancreas, liver, stomach, intestine, heart, reproductive organs, bladder, kidney, urethra and other urinary organs, etc., generally from any organ or tissue containing live somatic cells. Mammalian somatic cells useful in the present invention include, by way of example, adult stem cells, sertoli cells, endothelial cells, granulosa epithelial, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc. generally any live somatic cells. The term "somatic cells", as used herein, also includes adult stem cells. An adult stem cell is a cell that is capable of giving rise to all cell types of a particular tissue. Exemplary adult stem cells include hematopoietic stem cells, neural stem cells, and mesenchymal stem cells.

In one embodiment, the engineered somatic cells are obtained from a transgenic mouse comprising such engineered somatic cells. Such transgenic mouse can be produced using standard techniques known in the art. For example, Bronson et al. describe a technique for inserting a single copy of a transgene into a chosen chromosomal site. See Bronson et al., 1996. Briefly, a vector containing the desired integration construct (for example, a construct containing a selectable marker linked to a pluripotency gene) is introduced into ES cells by standard techniques known in the art. The resulting ES cells are screened for the desired integration event, in which the knock-in vector is integrated into the desired endogenous pluripotency gene locus such that the selectable marker is integrated into the genomic locus of the pluripotency gene and is under the control of the pluripotency gene promoter. The desired ES cell is then used to produce transgenic mouse in which all cell types contain the correct integration event. Desired types of cells may be selectively obtained from the transgenic mouse and maintained in vitro. In one embodiment, two or more transgenic mice may be created, each carrying a distinct integration construct. These mice may then be bred to generate mice that carry multiple desired integration construct. For example, one type of transgenic mouse may be created to carry an endogenous pluripotency gene linked to a selectable marker, while a second type of transgenic mouse may be created to carry a pluripotency gene expressed as a transgene under an inducible promoter. These two types of mice may then be bred to generate transgenic mice that have both a selectable marker linked to an endogenous pluripotency gene and an additional pluripotency gene expressed as a transgene under an inducible promoter. These two pluripotency genes may or may not be the same. Many variables are contemplated: the identity of the endogenous pluripotency gene linked to marker, the identity of the pluripotency gene expressed as a transgene, and the number of the endogenous pluripotency gene linked to a selectable marker, and the number of pluripotency gene expressed as a transgene. The present invention encompasses all possible combinations of these variables.

Alternatively, engineered somatic cells of the present invention may be produced by direct introduction of the desired construct into somatic cells. DNA construct may be introduced into cells by any standard technique known in the art, such as viral transfection (eg. using an adenoviral system) or liposome-mediated transfection. Any means known in the art to generate somatic cells with targeted integration can be used to produce somatic cells of the invention. In mammalian

cells, homologous recombination occurs at much lower frequency compared to non-homologous recombination. To facilitate the selection of homologous recombination events over the non-homologous recombination events, at least two enrichment methods have been developed: the positive-negative selection (PNS) method and the "promoterless" selection method (Sedivy and Dutriaux, 1999). Briefly, PNS, the first method, is in genetic terms a negative selection: it selects against recombination at the incorrect (non-homologous) loci by relying on the use of a negatively selectable gene that is placed on the flanks of a targeting vector. On the other hand, the second method, the "promoterless" selection, is a positive selection in genetic terms: it selects for recombination at the correct (homologous) locus by relying on the use of a positively selectable gene whose expression is made conditional on recombination at the homologous target site. The disclosure of Sedivy and Dutriaux is incorporated herein.

A selectable marker, as used herein, is a marker that, when expressed, confers upon recipient cells a selectable phenotype, such as antibiotic resistance, resistance to a cytotoxic agent, nutritional prototrophy or expression of a surface protein. The presence of a selectable marker linked to an endogenous pluripotency gene makes it possible to identify and select reprogrammed cells in which the endogenous pluripotency gene is expressed. A variety of selectable marker genes can be used, such as neomycin resistance gene (neo), puromycin resistance gene (puro), guanine phosphoribosyl transferase (gpt), dihydrofolate reductase (DHFR), adenosine deaminase (ada), puromycin-N-acetyltransferase (PAC), hygromycin resistance gene (hyg), multidrug resistance gene (mdr), and hisD gene.

The present invention further provides transgenic mice comprising the somatic cells of the invention.

Methods for Reprogramming Somatic Cells

The present invention further provides methods for reprogramming somatic cells to a less differentiated state. The resulting cells are termed "reprogrammed somatic cells" ("RSC") herein. A RSC may be a reprogrammed pluripotent somatic cell ("RPSC"), a reprogrammed multipotent somatic cell ("RMSC"), or a reprogrammed somatic cell of varying differentiation status.

In general, the methods comprise treating the engineered somatic cells with an agent. The treatment with an agent may be contacting the cells with an agent which alters chromatin structure, or may be transfecting the cells with one or more pluripotency gene, or both. The above two treatments may be concurrent, or may be sequential, with no particular preference for order. In a further embodiment, reprogrammed somatic cells are identified by selecting for cells that express the appropriate selectable marker. In still a further embodiment, reprogrammed somatic cells are further assessed for pluripotency characteristics. The presence of pluripotency characteristics indicates that the somatic cells have been reprogrammed to a pluripotent state.

Differentiation status of cells is a continuous spectrum, with terminally differentiated state at one end of this spectrum and de-differentiated state (pluripotent state) at the other end. Reprogramming, as used herein, refers to a process that alters or reverses the differentiation status of a somatic cell, which can be either partially or terminally differentiated. Reprogramming includes complete reversion, as well as partial reversion, of the differentiation status of a somatic cell. In other words, the term "reprogramming", as used herein, encompasses any movement of the differentiation status of a cell along the spectrum toward a less-differentiated state. For example, reprogramming includes reversing a multipotent cell back to a pluripotent cell, reversing a terminally differentiated cell back to either a multipotent cell or a pluripotent cell. In one embodiment, reprogramming of a somatic cell turns the somatic cell all the way back to a pluripotent state. In another embodiment, reprogramming of a somatic cell turns the somatic cell back to a multipotent state. The term "less-differentiated state", as used herein, is thus a relative term and includes a completely de-differentiated state and a partially differentiated state.

The term "pluripotency characteristics", as used herein, refers to many characteristics associated with pluripotency, including, for example, the ability to differentiate into all types of cells and an expression pattern distinct for a pluripotent cell, including expression of pluripotency genes, expression of other ES cell markers, and on a global level, a distinct expression profile known as "stem cell molecular signature" or "stemness."

Thus, to assess reprogrammed somatic cells for pluripotency characteristics, one may analyze such cells for different growth characteristics and ES cell-like morphology. Cells may be injected subcutaneously into immunocompromised SCID mice to induce teratomas (a standard assay for ES cells). ES-like cells can be differentiated into embryoid bodies (another ES specific feature). Moreover, ES-like cells can be differentiated in vitro by adding certain growth factors known to drive differentiation into specific cell types. Self-renewing capacity, marked by induction of telomerase activity, is another pluripotency characteristics that can be monitored. One may carry out functional assays of the reprogrammed somatic cells by introducing them into blastocysts and determine whether the cells are capable of giving rise to all cell types. See Hogan et al., 2003. If the reprogrammed cells are capable of forming a few cell types of the body, they are multipotent; if the reprogrammed cells are capable of forming all cell types of the body including germ cells, they are pluripotent.

One may also examine the expression of an individual pluripotency gene in the reprogrammed somatic cells to assess their pluripotency characteristics. Additionally, one may assess the expression of other ES cell markers. Stage-specific embryonic 1 5 antigens-1, -3, and -4 (SSEA-1, SSEA-3, SSEA-4) are glycoproteins specifically expressed in early embryonic development and are markers for ES cells (Solter and Knowles, 1978, Proc. Natl. Acad. Sci. USA 75:5565-5569; Kannagi et al., 1983, EMBO J. 2:2355-2361). Elevated expression of the enzyme Alkaline Phosphatase (AP) is another marker associated with undifferentiated embryonic stem cells (Wobus et al., 1984, Exp. Cell 152:212-219; Pease et al., 1990, Dev. Biol. 141:322-352). Other stem/progenitor cells markers include the intermediate neurofilament nestin (Lendahl et al., 1990, Cell 60:585-595; Dah-Istrand et al., 1992, J. Cell Sci. 103:589-597), the membrane glycoprotein prominin/AC133 (Weigmann et al., 1997, Proc. Natl. Acad. USA 94:12425-12430; Corbeil et al., 1998, Blood 91:2625-22626), the transcription factor Tcf-4 (Korinek et al, 1998, Nat. Genet. 19: 379-383; Lee et al., 1999, J. Biol. Chem. 274.1 566-1 572), and the transcription factor Cdx1 (Duprey et al., 1 988, Genes Dev. 2:1647-1654; Subramania'n et al., 1998, Differentiation 64:11-1 8).

One may additionally conduct expression profiling of the reprogrammed somatic cells to assess their pluripotency characteristics. Pluripotent cells, such as embryonic stem cells, and multipotent cells, such as adult stem cells, are known to have a distinct pattern of global gene expression profile. This distinct pattern is termed "stem cell molecular signature", or "stemness". See, for example, Ramalho-Santos et al., Science 298: 597-600 (2002); Ivanova et al., Science 298: 601-604.

Somatic cells may be reprogrammed to gain either a complete set of the pluripotency characteristics and are thus pluripotent. Alternatively, somatic cells may be reprogrammed to gain only a subset of the pluripotency characteristics. In another alternative, somatic cells may be reprogrammed to be multipotent.

In a further embodiment, in conjunction with contacting the somatic cells of the invention with an agent which alters chromatin structure, at least one gene that affects pluripotent state of a cell may be further introduced into the same cells. This may be carried out sequentially. For example, the somatic cells of the invention may be first contacted with an agent which alters chromatin structure. Then at least one pluripotency gene can be introduced into the same cells, or vice versa. Alternatively, the two steps may be carried out simultaneously.

Genes that affect pluripotent state of a cell includes pluripotency genes, genes involved in chromatin remodeling, and genes that are important for maintaining pluripotency, such as LIF, BMP, and PD098059 (See Cell, 115: 281-292 (2003); Philos Trans R Soc Lond B Biol Sci. 2003 Aug. 29; 358 (1436):1397-402).

The exogenously introduced pluripotency gene may be carried out in several ways. In one embodiment, the exogenously introduced pluripotency gene may be expressed from a chromosomal locus different from the endogenous chromosomal locus of the pluripotency gene. Such chromosomal locus may be a locus with open chromatin structure, and contain gene(s) dispensible for a somatic cell. In other words, the desirable chromosomal locus contains gene(s) whose disruption will not cause cells to die. Exemplary chromosomal loci include, for example, the mouse ROSA 26 locus and type II collagen (Col2a1) locus (See Zambrowicz et al., 1997) The exogenously introduced pluripotency gene may be expressed from an inducible promoter such that their expression can be regulated as desired.

In an alternative embodiment, the exogenously introduced pluripotency gene may be transiently transfected into cells, either individually or as part of a cDNA expression library, prepared from pluripotent cells. Such pluripotent cells may be embryonic stem cells, oocytes, blastomeres, inner cell mass cells, embryonic germ cells, embryoid body (embryonic) cells, morula-derived cells, teratoma (teratocarcinoma) cells, and multipotent partially differentiated embryonic stem cells taken from later in the embryonic development process.

The cDNA library is prepared by conventional techniques. Briefly, mRNA is isolated from an organism of interest. An RNA-directed DNA polymerase is employed for first strand synthesis using the mRNA as template. Second strand synthesis is carried out using a DNA-directed DNA polymerase which results in the cDNA product. Following conventional processing to facilitate cloning of the cDNA, the cDNA is inserted into an expression vector such that the cDNA is operably linked to at least one regulatory sequence. The choice of expression vectors for use in connection with the cDNA library is not limited to a particular vector. Any expression vector suitable for use in mouse cells is appropriate. In one embodiment, the promoter which drives expression from the cDNA expression construct is an inducible promoter. The term regulatory sequence includes promoters, enhancers and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology*, Academic Press, San Diego, Calif. (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express cDNAs. Such useful expression control sequences, include, for example, the early and late promoters of SV40, tet promoter, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

The exogenously introduced pluripotency gene may be expressed from an inducible promoter. The term "inducible promoter", as used herein, refers to a promoter that, in the absence of an inducer (such as a chemical and/or biological agent), does not direct expression, or directs low levels of expression of an operably linked gene (including cDNA), and, in response to an inducer, its ability to direct expression is enhanced. Exemplary inducible promoters include, for example, promoters that respond to heavy metals (CRC Boca Raton, Fla. (1991), 167-220; Brinster et al. Nature (1982), 296, 39-42), to thermal shocks, to hormones (Lee et al. P.N.A.S. USA (1988), 85, 1204-1208; (1981), 294, 228-232; Klock et al. Nature (1987), 329, 734-736; Israel and Kaufman, Nucleic Acids Res. (1989), 17, 2589-2604), promoters that respond to chemical agents, such as glucose, lactose, galactose or antibiotic.

A tetracycline-inducible promoter is an example of an inducible promoter that responds to an antibiotics. See Gossen et al., 2003. The tetracycline-inducible promoter comprises a minimal promoter linked operably to one or more tetracycline operator(s). The presence of tetracycline or one of its analogues leads to the binding of a transcription activator to the tetracycline operator sequences, which activates the minimal promoter and hence the transcription of the associated cDNA. Tetracycline analogue includes any compound that displays structural homologies with tetracycline and is capable of activating a tetracycline-inducible promoter. Exemplary tetracycline analogues includes, for example, doxycycline, chlorotetracycline and anhydrotetracycline.

Thus, in one embodiment, the present invention provides mice and somatic cells carrying at least one pluripotency gene expressed as a transgene under an inducible promoter. It is possible that somatic cells with such inducible pluripotency transgene(s) are more prone to be reprogrammed.

Any of the engineered somatic cells of the present invention may be used in the methods. In one embodiment, somatic cells used in the methods comprise only one endogenous pluripotency gene linked to a first selectable marker, and the selection step is carried out to select for the expression of the first selectable marker. In an alternative embodiment, the somatic cells used in the methods comprise any number of endogenous pluripotency genes, each of which is linked to a distinct selectable marker respectively, and the selection step is carried out to select for at least a subset of the selectable markers. For example, the selection step may be carried out to select for all the selectable markers linked to the various endogenous pluripotency genes.

In an alternative embodiment, somatic cells used in the method comprise a selectable marker linked to an endogenous pluripotency gene and an additional pluripotency gene expressed as a transgene under an inducible promoter. For these cells, the method of reprogramming may comprises induce the expression of the pluripotency transgene and select for the expression of the selectable marker. The method may further comprise contacting the somatic cells with an agent that alter chromatic structure.

Without wishing to be bound by theory, the agents used in the method may cause chromatin to take on a more open structure, which is more permissive for gene expression. DNA methylation and histone acetylation are two known events that alter chromatin toward a more closed structure. For example, loss of methylation by genetic deletion of DNA methylation enzyme Dnmt1 in fibroblasts results in reactivation of endogenous Oct4 gene. See J. Biol. Chem. 277: 34521-30, 2002; and Bergman and Mostoslaysky, Biol. Chem. 1990. Thus, DNA methylation inhibitors and histone deacetyation inhibitors are two classes of agents that may be used in the methods of the invention. Exemplary agents include 5-aza-cytidine, TSA and valproic acid.

In another embodiment, methods of the invention may further include repeating the steps of treating the cells with an agent. The agent used in the repeating treatment may be the same as, or different from, the one used during the first treatment.

Methods for Screening for an Agent that Reprograms Somatic Cells

The present invention also provides methods for identifying an agent that reprograms somatic cells to a less-differentiated state, as well as the agents thus identified. In one embodiment, the methods comprise contacting the engineered somatic cells of the invention with a candidate agent, selecting for cells that express the appropriate selectable marker. The presence of cells that express the appropriate selectable marker indicates that the agent reprograms somatic cells. Such an agent is referred as a "reprogramming agent" for purpose of this application.

In a further embodiment, the methods comprise contacting the engineered somatic cells of the invention with a candidate agent, selecting for cells that express the appropriate selectable marker, and assessing the cells so selected for pluripotency characteristics. The presence of a complete set of pluripotency characteristics indicates that the agent reprograms somatic cells to become pluripotent.

Candidate agents used in the invention encompass numerous chemical classes, though typically they are organic molecules, including small organic compounds. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, nucleic acids and derivatives, structural analogs or combinations thereof.

Candidate agents may be naturally arising, recombinant or designed in the laboratory. The candidate agents may be isolated from microorganisms, animals, or plants, or may be produced recombinantly, or synthesized by chemical methods known in the art. In some embodiments, candidate agents are isolated from libraries of synthetic or natural compounds using the methods of the present invention. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, including acylation, alkylation, esterification, amidification, to produce structural analogs.

There are numerous commercially available compound libraries, including, for example, the Chembridge DIVERSet. Libraries are also available from academic investigators, such as the Diversity set from the NCI developmental therapeutics program.

The screening methods mentioned above are based on assays performed on cells. These cell-based assays may be performed in a high throughput screening (HTS) format, which has been described in the art. For example, Stockwell et al. described a high-throughput screening of small molecules in miniaturized mammalian cell-based assays involving post-translational modifications (Stockwell et al., 1999). Likewise, Qian et al. described a leukemia cell-based assay for high-throughput screening for anti-cancer agents (Qian et al., 2001). Both references are incorporated herein in their entirety.

A reprogramming agent may belong to any one of many different categories. For example, a reprogramming agent may be a chromatin remodeling agent. A chromatin remodeling agent may be a protein involved in chromatin remodeling or an agent known to alter chromatin toward a more open structure, such as a DNA methylation inhibitor or a histone deacetyation inhibitor. Exemplary compounds include 5-aza-cytidine, TSA and valproic acid. For another example, such an agent may be a pluripotency protein, including, for example, Nanog, Oct-4 and Stella. Such an agent may also be a gene essential for pluripotency, including, for example, Sox2, FoxD3, and LIF, and Stat3. See Smith et al. 1988, William et al., 1988, Ihle, 1996, Avilion et al., 2003, and Hanna et al., 2002)

Methods for Reprogramming Somatic Cells with a Reprogramming Agent

The reprogramming agent identified by the methods of the present invention is useful for reprogramming somatic cells into pluripotent or multipotent cells. Accordingly, the present invention provides methods for reprogramming somatic cells to a less differentiated state, comprising contacting somatic cells with a reprogramming agent. The somatic cells used may be native somatic cells, or engineered somatic cells. It is not necessary for these cells to carry a selectable marker integrated into the endogenous locus of a pluripotency gene.

Reprogrammed Somatic Cells and these Uses

The present invention also provides reprogrammed somatic cells (RSCs), including reprogrammed pluripotent somatic cells (RPSCs), produced by the methods of the invention. These methods, useful for the generation of cells of a desired cell type, have wide range of applications. For one example, these methods have applications in livestock management, involving the precise genetic manipulation of animals for economic or health purposes. For another example, these methods have medical application in treating or preventing a condition.

Accordingly, the invention provides methods for the treatment or prevention of a condition in a mammal. In one embodiment, the methods start with obtaining somatic cells from the individual, reprogramming the somatic cells so obtained by methods of the present invention to obtain RPSCs. The RPSCs are then cultured under conditions suitable for development of the RPSCs into cells of a desired cell type. The developed cells of the desired cell type are harvested and introduced into the individual to treat the condi-

tion. In an alternative embodiment, the methods start with obtaining somatic cells from the individual, reprogramming the somatic cells so obtained by methods of the present invention. The RPSCs are then cultured under conditions suitable for development of the RPSCs into a desired organ, which is harvested and introduced into the individual to treat the condition.

The RPSCs of the present invention are ES-like cells, and thus may be induced to differentiate to obtain the desired cell types according to known methods to differentiate ES cells. For example, the RPSCs may be induced to differentiate into hematopoietic stem cells, muscle cells, cardiac muscle cells, liver cells, cartilage cells, epithelial cells, urinary tract cells, etc., by culturing such cells in differentiation medium and under conditions which provide for cell differentiation. Medium and methods which result in the differentiation of embryonic stem cells are known in the art as are suitable culturing conditions.

For example, Palacios et al., Proc. Natl. Acad. Sci., USA, 92: 7530-37 (1995) teaches the production of hematopoietic stem cells from an embryonic cell line by subjecting stem cells to an induction procedure comprising initially culturing aggregates of such cells in a suspension culture medium lacking retinoic acid followed by culturing in the same medium containing retinoic acid, followed by transferal of cell aggregates to a substrate which provides for cell attachment.

Moreover, Pedersen, J. Reprod. Fertil. Dev., 6: 543-52 (1994) is a review article which references numerous articles disclosing methods for in vitro differentiation of embryonic stem cells to produce various differentiated cell types including hematopoietic cells, muscle, cardiac muscle, nerve cells, among others.

Further, Bain et al., Dev. Biol., 168:342-357 (1995) teaches in vitro differentiation of embryonic stem cells to produce neural cells which possess neuronal properties. These references are exemplary of reported methods for obtaining differentiated cells from embryonic or stem-like cells. These references and in particular the disclosures therein relating to methods for differentiating embryonic stem cells are incorporated by reference in their entirety herein.

Thus, using known methods and culture medium, one skilled in the art may culture the subject embryonic or stem-like cells to obtain desired differentiated cell types, e.g., neural cells, muscle cells, hematopoietic cells, etc. In addition, the use of inducible Bcl-2 or Bcl-xl might be useful for enhancing in vitro development of specific cell lineages. In vivo, BcI-2 prevents many, but not all, forms of apoptotic cell death that occur during lymphoid and neural development. A thorough discussion of how Bcl-2 expression might be used to inhibit apoptosis of relevant cell lineages following transfection of donor cells is disclosed in U.S. Pat. No. 5,646,008, which is herein incorporated by reference.

The subject RPSCs may be used to obtain any desired differentiated cell type. Therapeutic usages of such differentiated human cells are unparalleled. For example, human hematopoietic stem cells may be used in medical treatments requiring bone marrow transplantation. Such procedures are used to treat many diseases, e.g., late stage cancers such as ovarian cancer and leukemia, as well as diseases that compromise the immune system, such as AIDS. Hematopoietic stem cells can be obtained, e.g., by fusing adult somatic cells of a cancer or AIDS patient, e.g., epithelial cells or lymphocytes with an enucleated oocyte, e.g., bovine oocyte, obtaining embryonic or stem-like cells as described above, and culturing such cells under conditions which favor differentiation, until hematopoietic stem cells are obtained. Such hematopoietic cells may be used in the treatment of diseases including cancer and AIDS.

The methods of the present invention can also be used to treat, prevent, or stabilize a neurological disease such as Alzheimer's disease, Parkinson's disease, Huntington's disease, or ALS, lysosomal storage diseases, multiple sclerosis, or a spinal cord injury. For example, somatic cells may be obtained from the individual in need of treatment, and reprogrammed to gain pluripotency, and cultured to derive neurectoderm cells that may be used to replace or assist the normal function of diseased or damaged tissue.

For the treatment or prevention of endocrine conditions, RPSCs that produce a hormone, such as a growth factor, thyroid hormone, thyroid-stimulating hormone, parathyroid hormone, steroid, serotonin, epinephrine, or norepinephrine may be administered to a mammal. Additionally, reprogrammed epithelial cells may be administered to repair damage to the lining of a body cavity or organ, such as a lung, gut, exocrine gland, or urogenital tract. It is also contemplated that RPSCs may be administered to a mammal to treat damage or deficiency of cells in an organ such as the bladder, brain, esophagus, fallopian tube, heart, intestines, gallbladder, kidney, liver, lung, ovaries, pancreas, prostate, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, ureter, urethra, or uterus.

The great advantage of the present invention is that it provides an essentially limitless supply of isogenic or synegenic human cells suitable for transplantation. Therefore, it will obviate the significant problem associated with current transplantation methods, i.e., rejection of the transplanted tissue which may occur because of host versus graft or graft versus host rejection. Conventionally, rejection is prevented or reduced by the administration of anti-rejection drugs such as cyclosporin. However, such drugs have significant adverse side-effects, e.g., immunosuppression, carcinogenic properties, as well as being very expensive. The present invention should eliminate, or at least greatly reduce, the need for anti-rejection drugs, such as cyclosporine, imulan, FK-506, glucocorticoids, and rapamycin, and derivatives thereof.

RPSCs may also be combined with a matrix to form a tissue or organ in vitro or in vivo that may be used to repair or replace a tissue or organ in a recipient mammal. For example, RPSCs may be cultured in vitro in the presence of a matrix to produce a tissue or organ of the urogenital system, such as the bladder, clitoris, corpus cavermosum, kidney, testis, ureter, uretal valve, or urethra, which may then be transplanted into a mammal (Atala, Curr. Opin. Urol. 9(6):517-526, 1999). In another transplant application, synthetic blood vessels are formed in vitro by culturing reprogrammed cells in the presence of an appropriate matrix, and then the vessels are transplanted into a mammal for the treatment or prevention of a cardiovascular or circulatory condition. For the generation of donor cartilage or bone tissue, RPSCs such as chondrocytes or osteocytes are cultured in vitro in the presence of a matrix under conditions that allow the formation of cartilage or bone, and then the matrix containing the donor tissue is administered to a mammal. Alternatively, a mixture of the cells and a matrix may be administered to a mammal for the formation of the desired tissue in vivo. Preferably, the cells are attached to the surface of the matrix or encapsulated by the matrix. Examples of matrices that may be used for the formation of donor tissues or organs include collagen matrices, carbon fibers, polyvinyl alcohol sponges, acrylateamide sponges, fibrin-thrombin gels, hyaluronic acid-based polymers, and synthetic polymer matrices containing polyanhydride, poly-

orthoester, polyglycolic acid, or a combination thereof (see, for example, U.S. Pat. Nos. 4,846,835; 4,642,120; 5,786,217; and 5,041,138).

The RPSCs produced according to the invention may be used to produce genetically engineered or transgenic differentiated cells. Essentially, this will be effected by introducing a desired gene or genes, or removing all or part of an endogenous gene or genes of RPSCs produced according to the invention, and allowing such cells to differentiate into the desired cell type. A preferred method for achieving such modification is by homologous recombination because such technique can be used to insert, delete or modify a gene or genes at a specific site or sites in the stem-like cell genome.

This methodology can be used to replace defective genes, e.g., defective immune system genes, cystic fibrosis genes, or to introduce genes which result in the expression of therapeutically beneficial proteins such as growth factors, lymphokines, cytokines, enzymes, etc. For example, the gene encoding brain derived growth factor maybe introduced into human embryonic or stem-like cells, the cells differentiated into neural cells and the cells transplanted into a Parkinson's patient to retard the loss of neural cells during such disease. Examples of mutations that may be rescued using these methods include mutations in the cystic fibrosis gene; mutations associated with Dunningan's disease such as the R482W, R482Q, and R584H mutations in the lamin A gene; and mutations associated with the autosomal-dominant form of Emery Deyfuss muscular dystrophy such as the R249Q, R453W, and Q6STOP mutations in the lamin A gene. In the Q6STOP mutation, the codon for Gln6 is mutated to a stop codon.

Previously, cell types transfected with BDNF varied from primary cells to immortalized cell lines, either neural or non-neural (myoblast and fibroblast) derived cells. For example, astrocytes have been transfected with BDNF gene using retroviral vectors, and the cells grafted into a rat model of Parkinson's disease (Yoshimoto et al., Brain Research, 691:25-36, (1995)). This ex vivo therapy reduced Parkinson's-like symptoms in the rats up to 45% 32 days after transfer. Also, the tyrosine hydroxylase gene has been placed into astrocytes with similar results (Lundberg et al., Develop. Neurol., 139: 39-53 (1996) and references cited therein).

However, such ex vivo systems have problems. In particular, retroviral vectors currently used are down-regulated in vivo and the transgene is only transiently expressed (review by Mulligan, Science, 260: 926-932 (1993)). Also, such studies used primary cells, astrocytes, which have finite life span and replicate slowly. Such properties adversely affect the rate of transfection and impede selection of stably transfected cells. Moreover, it is almost impossible to propagate a large population of gene targeted primary cells to be used in homologous recombination techniques.

By contrast, the difficulties associated with retroviral systems should be eliminated by the use of RPSCs of the present invention, which are ES-like cells. Using known methods to introduced desired genes/mutations into ES cells, RPSCs may be genetically engineered, and the resulting engineered cells differentiated into desired cell types, e.g., heniatopoietic cells, neural cells, pancreatic cells, cartilage cells, etc. Genes which may be introduced into the RPSCs include, for example, epidermal growth factor, basic fibroblast growth factor, glial derived neurotrophic growth factor, insulin-like growth factor (I and II), neurotrophin3, neurotrophin-4/5, ciliary neurotrophic factor, AFT-1, cytokine genes (interleukins, interferons, colony stimulating factors, tumor necrosis factors (alpha and beta), etc.), genes encoding therapeutic enzymes, collagen, human serum albumin, etc.

In addition, it is also possible to use one of the negative selection systems now known in the art for eliminating therapeutic cells from a patient if necessary. For example, donor cells transfected with the thymidine kinase (TK) gene will lead to the production of embryonic cells containing the TK gene. Differentiation of these cells will lead to the isolation of therapeutic cells of interest which also express the TK gene. Such cells may be selectively eliminated at any time from a patient upon gancyclovir administration. Such a negative selection system is described in U.S. Pat. No. 5,698,446, and is herein incorporated by reference.

Examples of diseases, disorders, or conditions that may be treated or prevented include neurological, endocrine, structural, skeletal, vascular, urinary, digestive, integumentary, blood, immune, auto-immune, inflammatory, endocrine, kidney, bladder, cardiovascular, cancer, circulatory, digestive, hematopoeitic, and muscular diseases, disorders, and conditions. In addition, reprogrammed cells may be used for reconstructive applications, such as for repairing or replacing tissues or organs.

With respect to the therapeutic methods of the invention, it is not intended that the administration of RPSCs to a mammal be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration, including intramuscular, intravenous, intraarticular, intralesional, subcutaneous, or any other route sufficient to provide a dose adequate to prevent or treat a disease. The RPSCs may be administered to the mammal in a single dose or multiple doses. When multiple doses are administered, the doses may be separated from one another by, for example, one week, one month, one year, or ten years. One or more growth factors, hormones, interleukins, cytokines, or other cells may also be administered before, during, or after administration of the cells to further bias them towards a particular cell type.

The RPSCs of the present invention may be used as an in vitro model of differentiation, in particular for the study of genes which are involved in the regulation of early development. Differentiated cell tissues and organs using the RPSCs may be used in drug studies.

Furthermore, the RPSCs produced according to the invention maybe introduced into animals, e.g., SCID mice, cows, pigs, e.g., under the renal capsule or intramuscularly and used to produce a teratoma therein. This teratoma can be used to derive different tissue types. Also, the inner cell mass produced by X-species nuclear transfer may be introduced together with a biodegradable, biocompatible polymer matrix that provides for the formation of 3-dimensional tissues. After tissue formation, the polymer degrades, ideally just leaving the donor tissue, e.g., cardiac, pancreatic, neural, lung, liver. In some instances, it may be advantageous to include growth factors and proteins that promote angiogenesis. Alternatively, the formation of tissues can be effected totally in vitro, with appropriate culture media and conditions, growth factors, and biodegradable polymer matrices.

Applications of the Somatic Cell Reprogramming Methods and RPSCs in Animals

The reprogramming methods disclosed herein may be used to generate RPSCs for a variety of animal species. The RPSCs generated can be useful to produce desired animals. Animals include, for example, avians and mammals as well as any animal that is an endangered species. Exemplary birds include domesticated birds (e.g., quail, chickens, ducks, geese, turkeys, and guinea hens) as well as other birds such as birds of prey (e.g., hawks, falcons, ospreys, condors, etc.), endangered birds (e.g., parrots, California condor, etc.), ostriches etc. Exemplary mammals include murine, caprine,

ovine, bovine, porcine, canine, feline and primate. Of these, preferred members include domesticated animals, including, for examples, cattle, buffalo, pigs, horses, cows, rabbits, guinea pigs, sheep, and goats.

RPSCs generated by the reprogramming methods of the present invention allows one, for the first time, to genetically engineer animals other than mouse and human. RPSCs are ES-like cells, and are thus amenable to genetic manipulation. To date, no ES cells are available for animals other than mouse and human. As a result, for these animals, it is currently practically impossible to create genetically modified animals having targeted mutations. The ES-cell like RPSCs can be manipulated to introduce desired targeted genetic modifications. The resulting engineered RPSCs can then be used to generate a cloned animal with the desired genetic modifications in its germ line, using methods described for ES cells in mouse. See Capecchi and Thomas, U.S. Pat. Nos. 5,487,992, 5,627,059, 5,631,153, and 6,204,061. Genetic engineering in animals has potentially great applications in a variety of animals, especially farm animals.

The somatic cell reprogramming methods of the present invention provides at least two methods for delivering optimized farm animals. In the first, somatic cell reprogramming can be used to capture the best available phenotype for a farm animal stock. The current technologies used to deliver optimized farm animals are based on selective breeding, and expansion from preferred breeding stocks. Animals that have been selected on the basis of superior characteristics, including, for example, meat content, egg production (in the case of poultry), feed conversion ratio, are used to breed large numbers of animals that are in turn used in the human food supply. This traditional process has profound inherent inefficiencies. The phenotype observed in an individual animal is often only partially transmitted in the progeny of that animal. Therefore, traditional breeding schemes are inefficient in capturing the very best phenotype in all of the progeny animals. In contrast, the reprogramming methods of the present invention provides a controlled and efficient way to achieve the same goal, by generating RPSCs from somatic cells of an animal with the desired characteristics. The RPSCs generated may be used immediately to generate cloned animals derived from the RPSCs. Known methods for generating mice from ES cells can be used for this procedure. Alternatively, the RPSCs generated may be cryopreserved and thawed in response to a grower's needs.

In the second method, somatic cells from an animal with the desired characteristics are reprogrammed to produce RPSCs. The RPSCs are further genetically engineered to introduce desired genetic modification(s), before being placed into a recipient embryo to produce desired progeny.

The reprogramming methods can also be used to rescue endangered species. Somatic cell reprogramming provides an efficient method to generate RPSCs from somatic cells of an endangered animal. The resulting RPSCs can be used immediately to expand the numbers of the endangered animal. Alternatively, the RPSCs can be cryopreserved to generate a RPSC stock for the endangered species, as a safeguard measure against extinction of the endangered species.

Methods for Gene Identification

The present invention provides methods for identifying a gene that activates the expression of an endogenous pluripotency gene in somatic cells. The methods comprise: transfecting the somatic cells of the present invention with a cDNA library prepared from ES cells or oocytes, selecting for cells that express the first selectable marker, and assessing the expression of the first endogenous pluripotency gene in the transfected cells that express the first selectable marker. The expression of the first endogenous pluripotency gene indicates that the cDNA encodes a gene that activates the expression of an endogenous pluripotency gene in somatic cells.

The methods are applicable for identifying a gene that activates the expression of at least two endogenous pluripotency genes in somatic cells. The somatic cells used in the methods further comprise a second endogenous pluripotency gene linked to a second selectable marker. The methods are modified to select for transfected cells that express both selectable markers, among which the expression of the first and the second endogenous pluripotency genes are assessed. The expression of both the first and the second endogenous pluripotency genes indicates that the cDNA encodes a gene that activates the expression of at least two pluripotency genes in somatic cells.

The methods are further applicable for identifying a gene that activates the expression of at least three endogenous pluripotency genes in somatic cells. The somatic cells used in the methods further comprise a third endogenous pluripotency gene linked to a third selectable marker. The methods are modified to select for transfected cells that express all three selectable markers, among which the expression of all three endogenous pluripotency genes are assessed. The expression of all three endogenous pluripotency genes indicates that the cDNA encodes a gene that activates the expression of at least three pluripotency genes in somatic cells.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of mouse genetics, developmental biology, cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, Current Protocols in Cell Biology, ed. by Bonifacino, Dasso, Lippincott-Schwartz, Harford, and Yamada, John Wiley and Sons, Inc., New York, 1999; Manipulating the Mouse Embryos, A Laboratory Manual, 3rd Ed., by Hogan et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2003; Gene Targeting: A Practical Approach, IRL Press at Oxford University Press, Oxford, 1993; and Gene Targeting Protocols, Human Press, Totowa, N.J., 2000. All patents, patent applications and references cited herein are incorporated in their entirety by reference.

EXEMPLIFICATION

The invention now being generally described, it will be more readily understood by reference to the following example, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example

Oct4-Induced Fibroblasts are More Susceptible to Reprogramming than Unduced Fibroblasts as Demonstrated by Nuclear Transfer Experiment

A. Generation of Transgenic Mouse Carrying an Inducible Oct4 Transgene

An inducible Oct4 allele is constructed as the following: first, two integration vectors are constructed. The first integration vector, inducible Oct4 integration vector, contains an Oct4 gene driven by a tetracycline-inducible promoter (Tet-Op). The Tet-Op-Oct4 cassette is flanked by a splice-acceptor double poly-A signal (SA-dpA) at its 5' end and a SV40 polyA tail (SV40-pA) at its 3' end. The second integration vector, tetracycline activator integration vector, contains a

mutant form of tetracycline activator, M2-rtTA, which is more responsive to doxycycline (Dox) induction than the wild type activator. (Urlinger S. et al., 2000)

The two integration vectors are introduced into V6.5 ES cells: the inducible Oct4 integration vector and the tetracycline activator integration vector are introduced into the Collagen locus and the Rosa26 locus respectively via site-specific integration, as shown in FIG. **1**. The resulting ES cells are used to make Oct4-inducible mice by tetraploid complementation.

B. Expression of the Inducible Oct4 Transgene

Fibroblasts derived from tail biopsies of the Oct4-inducible mice were cultured. A fraction of the cultured fibroblasts were induced with doxycycline for 3 days (at 2 microgram/ml), and Oct4 expression was detected by Northern blot and Western blot analysis. As shown in FIG. **2**, the Oct4 expression level in fibroblasts treated with doxycycline is comparable to the Oct4 expression level in ES cells, and undetectable in fibroblasts not treated with doxycycline. The expression results demonstrate that the inducible Oct4 transgene is expressed as planned.

C. Nuclear Transfer Experiment

Nuclear transfer was performed on fibroblasts derived from tail biopsies of mice that carry the inducible Oct4 transgene. Dox induction was for 24 hours prior to nuclear transfer. Cloned embryos were then activated and cultured to the blastocyst stage to derive ES cells as described previously (Hochedlinger and Jaenisch, 2002). As shown in Table 1, on average, blastocyst formation and ES cell derivation (as measured as a fraction of eggs with pronucleus formation) is more efficient from Oct4 induced fibroblast than from uninduced fibroblasts. This result demonstrated that induced Oct4 expression in somatic cells such as fibroblasts make these cells more susceptible to reprogramming.

One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods, systems and kits are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention and are defined by the scope of the claims. It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

TABLE 1

In vitro development of clones derived from Oct4-induced fibroblasts

Expt.	Oct4	eggs w/PN	Blastocysts (% PN)		ES lines (% PN)	
#1	−	22	5 (23%)	19%	0 (0%)	3%
#2	−	35	5 (14%)		2 (6%)	
#3	+	37	10 (27%)	24%	2 (5%)	7%
#4	+	47	10 (21%)		4 (9%)	

PN . . . ProNucleus formation

Nuclear transfer was performed on fibroblasts derived from tail biopsies of mice that carry the inducible Oct4 transgene. Dox induction was for 24 hours prior to nuclear transfer. Cloned embryos were then activated and cultured to the blastocyst stage to derive ES cells as described previously (Hochedlinger and Jaenisch, Nature, 2002). These preliminary results show that on average blastocyst formation and ES cell derivation is more efficient from Oct4 induced than from uninduced fibroblasts.

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What is claimed is:

1. A somatic cell comprising an exogenous nucleic acid encoding Oct4 and an amount of Oct4 expression comparable to the amount of Oct4 expression in an embryonic stem cell.

2. A somatic cell comprising an exogenous nucleic acid encoding Oct4 and an amount of Oct4 protein sufficient to make the cell more susceptible to reprogramming.

3. The somatic cell of any one of claims **1** to **2**, wherein the somatic cell is a mammalian cell.

4. The somatic cell of any one of claims **1** to **2**, wherein the somatic cell is a human cell or a mouse cell.

5. The somatic cell of any one of claims **1** to **2**, wherein the cell is an adult stem cell.

6. The somatic cell of claim **5**, wherein the adult stem cell is selected from the group consisting of: a hematopoietic stem cell, a neural stem cell, and a mesenchymal stem cell.

7. A composition comprising an isolated cDNA encoding an Oct4 protein operably linked to at least one regulatory sequence and DNA methylation inhibitor, a histone deacetylase inhibitor, or PD09859.

8. A composition comprising an isolated cDNA encoding an Oct4 protein operably linked to at least one regulatory sequence, an isolated cDNA encoding a Sox2 protein operably linked to at least one regulatory sequence, and an isolated adult stem cell.

9. A composition comprising an isolated cDNA encoding an Oct4 protein operably linked to at least one regulatory sequence, an isolated cDNA encoding a Nanog protein operably linked to at least one regulatory sequence, and an isolated adult stem cell.

10. The composition of any one of claims **8** and **9**, wherein the adult stem cell is a human adult stem cell or a mouse adult stem cell.

11. The composition of any one of claims **8** and **9**, wherein the composition further comprises a DNA methylation inhibitor, a histone deacetylase inhibitor, or PD09859.

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EXHIBIT F

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(12) **United States Patent**
Jaenisch et al.

(10) **Patent No.: US 10,457,917 B2**
(45) **Date of Patent: *Oct. 29, 2019**

(54) **METHODS FOR REPROGRAMMING SOMATIC CELLS**

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(60) Continuation of application No. 15/588,062, filed on May 5, 2017, now Pat. No. 10,017,744, which is a continuation of application No. 14/923,321, filed on Oct. 26, 2015, now Pat. No. 9,670,464, which is a continuation of application No. 13/646,430, filed on Oct. 5, 2012, now Pat. No. 9,169,490, which is a continuation of application No. 12/703,061, filed on Feb. 9, 2010, now Pat. No. 8,940,536, which is a division of application No. 10/997,146, filed on Nov. 24, 2004, now Pat. No. 7,682,828.

(60) Provisional application No. 60/525,612, filed on Nov. 26, 2003, provisional application No. 60/530,042, filed on Dec. 15, 2003.

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(52) **U.S. Cl.**

CPC ***C12N 5/0696*** (2013.01); ***C12N 15/85*** (2013.01); *A01K 67/0273* (2013.01); *A01K 67/0275* (2013.01); *A01K 2217/05* (2013.01); *A01K 2227/105* (2013.01); *C07K 14/4702* (2013.01); *C12N 15/8509* (2013.01); *C12N 15/8775* (2013.01); *C12N 2501/602* (2013.01); *C12N 2501/603* (2013.01); *C12N 2501/605* (2013.01); *C12N 2506/1307* (2013.01); *C12N 2510/00* (2013.01); *C12N 2830/003* (2013.01); *C12N 2830/006* (2013.01)

(58) **Field of Classification Search**

CPC C12N 5/0696; C12N 15/85

See application file for complete search history.

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(57) **ABSTRACT**

The invention provides methods for reprogramming somatic cells to generate multipotent or pluripotent cells. Such methods are useful for a variety of purposes, including treating or preventing a medical condition in an individual. The invention further provides methods for identifying an agent that reprograms somatic cells to a less differentiated state.

18 Claims, 2 Drawing Sheets

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Final Office Action issued in U.S. Appl. No. 15/354,604, dated Oct. 9, 2018.

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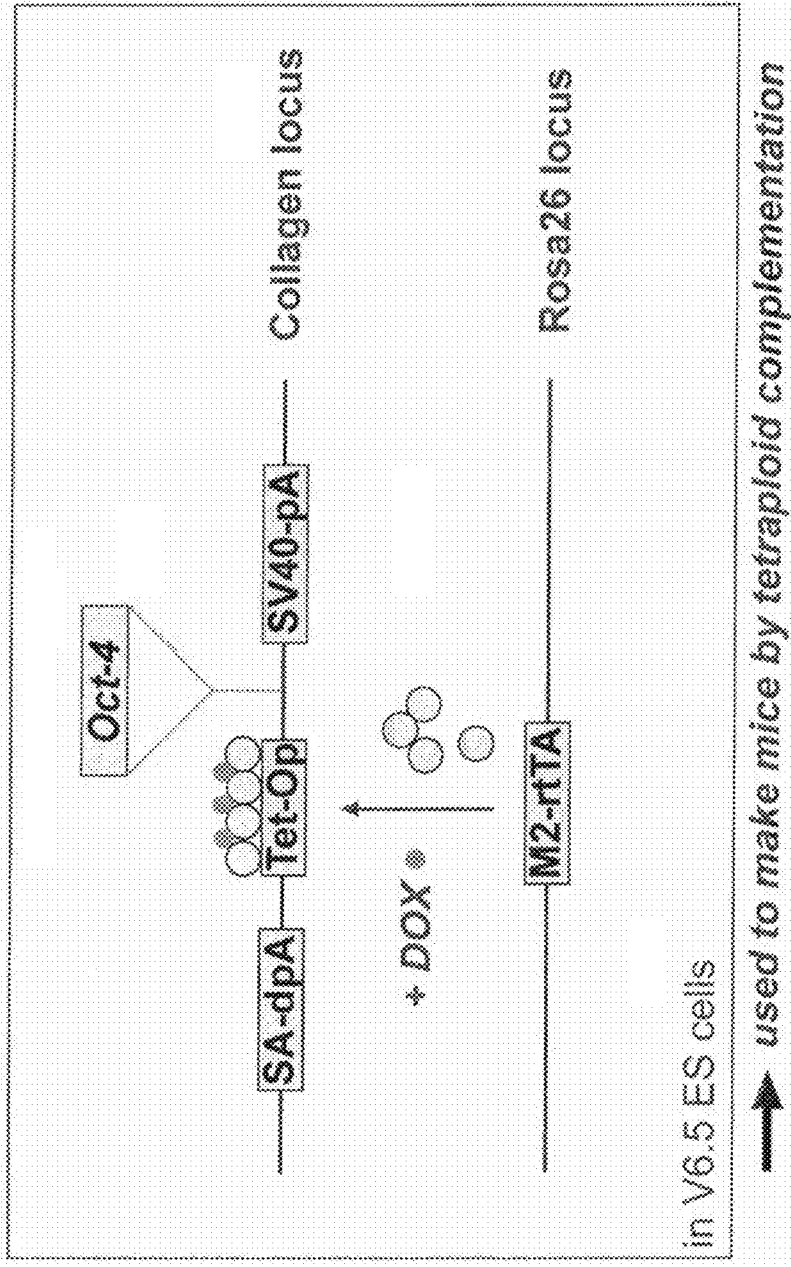
Figure 1. Inducible *Oct4* allele



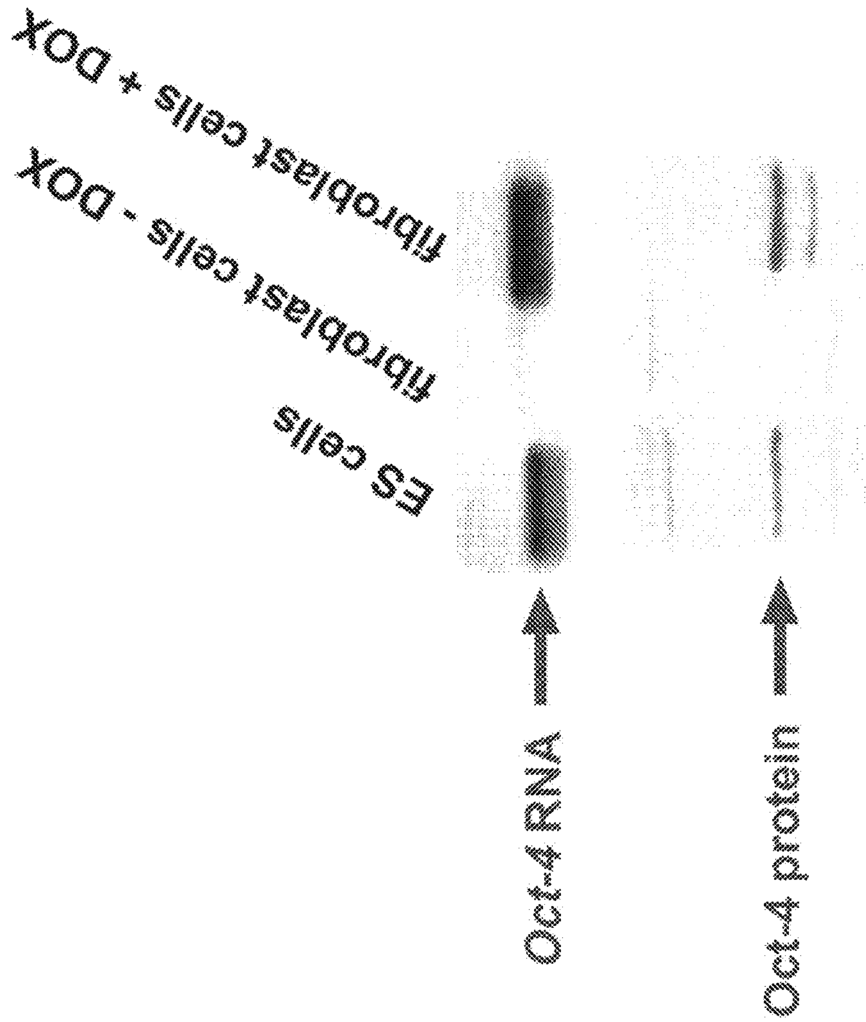


Figure 2. The system works...
fibroblast cells + DOX
fibroblast cells - DOX
ES cells
Oct-4 RNA
Oct-4 protein

METHODS FOR REPROGRAMMING SOMATIC CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 15/588,062, filed May 5, 2017, which is a continuation of U.S. application Ser. No. 14/923,321, filed Oct. 26, 2015 (U.S. Pat. No. 9,670,464), which is a continuation of U.S. application Ser. No. 13/646,430, filed Oct. 5, 2012 (U.S. Pat. No. 9,169,490), which is a continuation of U.S. application Ser. No. 12/703,061, filed Feb. 9, 2010 (U.S. Pat. No. 8,940,536), which is a divisional of U.S. application Ser. No. 10/997,146, filed Nov. 24, 2004 (U.S. Pat. No. 7,682,828), which claims the benefit of U.S. Provisional Application No. 60/525,612, filed Nov. 26, 2003, and U.S. Provisional Application No. 60/530,042, filed Dec. 15, 2003, the specifications of which are incorporated herein by reference in their entirety.

GOVERNMENTAL FUNDING

This invention was made with government support under R37 CA84198 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Pluripotent stem cells have the potential to differentiate into the full range of daughter cells having distinctly different morphological, cytological or functional phenotypes unique to a specific tissue. By contrast, descendants of pluripotent cells are restricted progressively in their differentiation potential, with some cells having only one fate. Pluripotent cells have extraordinary scientific and therapeutic potential, as they can be differentiated along the desired differentiation pathway in a precisely controlled manner and used in cell-based therapy.

Two categories of pluripotent stem cells are known to date: embryonic stem cells and embryonic germ cells. Embryonic stem cells are pluripotent stem cells that are derived directly from an embryo. Embryonic germ cells are pluripotent stem cells that are derived directly from the fetal tissue of aborted fetuses. For purposes of simplicity, embryonic stem cells and embryonic germ cells will be collectively referred to as “ES” cells herein.

ES cells are presently obtained via several methods. In a first method, an ES cell line is derived from the inner cell mass of a normal embryo in the blastocyst stage (See U.S. Pat. No. 6,200,806, Thompson, J. A. et al. Science, 282: 1145-7, 1998 and Hogan et al., 2003). A second method for creating pluripotent ES cells utilizes the technique of somatic cell nuclear transfer (SCNT). In this technique, the nucleus is removed from a normal egg, thus removing the genetic material. Next, a donor diploid somatic cell is placed next to the enucleated egg and the two cells are fused, or the nucleus is introduced directly into the oocyte by micromanipulation. The fused cell has the potential to develop into a viable embryo, which may then be sacrificed to remove that portion of the embryo containing the stem cell producing inner cell mass.

In a third method, the nucleus of a human cell is transplanted into an entirely enucleated animal oocyte of a species different from the donor cell (referred to herein as animal stem cell nuclear transfer, or “ASCNT”). See U.S. Pat. Application Ser. No. 20010012513 (2001). The resultant chimeric cells are used for the production of pluripotent ES cells, in particular human-like pluripotent ES cells. One disadvantage of this technique is that these chimeric cells may contain unknown non-human viruses and still contain the mitochondria of the animal species. Thus, there would be substantial risks of immune rejection if such cells were used in cell transplantation therapies.

In a fourth method, ES cells can be isolated from the primordial germ cells found in the genital ridges of post-implanted embryos.

As described above, all presently available methods depend on controversial sources—embryos (either created naturally or via cloning), fetal tissue and via the mixing of materials of multiple species. The controversy surrounding the sources for such cells, according to many leading scientists and public and private organizations including the NIH, has greatly compromised and slowed the study of such cells and their application.

There is thus a great demand for alternative methods of generating pluripotent cells.

SUMMARY OF THE INVENTION

The present invention provides engineered somatic cells, in which one or more endogenous pluripotency gene(s) is operably linked to a selectable marker in such a manner that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene to which the marker is linked. The invention also provides transgenic mice containing these engineered somatic cells.

The present invention also provides methods for reprogramming somatic cells to a less differentiated state. In the methods, engineered somatic cells of the invention are treated with an agent. Cells that express the selectable marker are then selected, and assessed for pluripotency characteristics. The treatment with an agent may be contacting the cells with an agent which alters chromatin structure, or may be transfecting the cells with at least one pluripotency gene, or both.

The present invention further provides methods for identifying an agent that reprograms somatic cells to a less differentiated state. In the methods, the engineered somatic cells described above are contacted with a candidate agent. Cells that express the selectable marker are then selected, and assessed for pluripotency characteristics. The presence of at least a subset of pluripotency characteristics indicates that the agent is capable of reprogramming somatic cells to a less-differentiated state. The agents identified by the present invention can then by used to reprogram somatic cells by contacting somatic cells with the agents.

The present invention also provides methods for identifying a gene that causes the expression of at least one endogenous pluripotency gene in somatic cells. In the methods, the engineered somatic cells are transfected with a cDNA library prepared from a pluripotent cell, such as an ES cell. The cells that express the appropriate selectable marker are then selected, and the expression of the appropriate endogenous pluripotency gene is examined. The expression of an endogenous pluripotency gene indicates that the cDNA encodes a protein whose expression in the cell results in, directly or indirectly, expression of the endogenous pluripotency gene.

The present invention further provides methods for treating a condition in an individual in need of such treatment. In certain embodiments, somatic cells are obtained from the individual and reprogrammed by the methods of the invention under conditions suitable for the cells to develop into

cells of a desired cell type. The reprogrammed cells of a desired cell type are then harvested and introduced into the individual to treat the condition. In certain further embodiments, the somatic cells obtained from the individual contains a mutation in one or more genes. In these instances, the methods are modified so that the somatic cells obtained from the individual are first treated to restore the one or more normal gene(s) to the cells such that the resulting cells carry the normal endogenous gene, which are then introduced into the individual. In certain other embodiments, methods of the invention can be used to treat individuals in need of a functional organ. In the methods, somatic cells are obtained from an individual in need of a functional organ, and reprogrammed by the methods of the invention to produce reprogrammed somatic cells. Such reprogrammed somatic cells are then cultured under conditions suitable for development of the reprogrammed somatic cells into a desired organ, which is then introduced into the individual. The methods are useful for treating any one of the following conditions: a neurological, endocrine, structural, skeletal, vascular, urinary, digestive, integumentary, blood, autoimmune, inflammatory, or muscular condition.

The present invention also provides methods for producing a cloned animal. In the methods, a somatic cell is isolated from an animal having desired characteristics, and reprogrammed using the methods of the invention to produce one or more reprogrammed pluripotent somatic cell ("RPSC"). The RPSCs are then inserted into a recipient embryo, and the resulting embryo is cultured to produce an embryo of suitable size for implantation into a recipient female, which is then transferred into a recipient female to produce a pregnant female. The pregnant female is maintained under conditions appropriate for carrying the embryo to term to produce chimeric animal progeny, which is then bred with a wild type animal to produce a cloned animal.

In certain embodiments, the RPSCs may alternatively be cryopreserved for future cloning uses. In certain other embodiments, genetic modification, such as a targeted mutation, may be introduced into the RPSCs prior to its insertion into a recipient embryo.

The present invention also provides methods for producing a cloned avian. In the methods, a somatic cell is isolated from an avian having desired characteristics, and reprogrammed using the methods of the invention to produce one or more reprogrammed pluripotent somatic cell ("RPSC"). The RPSCs are then inserted into eggs that are unable to develop into an embryo, and the resulting eggs are then incubated to produce avian offspring having the genotype of the RPSC, thereby producing a cloned avian.

It is contemplated that all embodiments described above are applicable to all different aspects of the invention. It is also contemplated that any of the above embodiments can be freely combined with one or more other such embodiments whenever appropriate.

Specific embodiments of the invention are described in more detail below. However, these are illustrative embodiments, and should not be construed as limiting in any respect.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts an inducible Oct4 allele.

FIG. **2** shows the expression of the inducible Oct4 transgene by Northern blot and Western blot analysis.

DETAILED DESCRIPTION OF THE INVENTION

Overview

Presently, human ES cells or ES-like cells can only be generated from controversial sources. It would be useful to reprogram somatic cells directly into pluripotent cells. Nuclei from somatic cells retain the totopotency potential to direct development of an animal, as demonstrated by nuclear transfer technology. It would be useful to reprogram somatic cells directly into ES cells without the use of oocytes and nuclear transfer technology.

Applicants have devised novel methods of reprogramming somatic cells to generate pluripotent cells or multipotent cells. Applicants have also devised novel methods to identify agents that reprogram somatic cells. The methods take advantage of the engineered somatic cells designed by Applicants, in which an endogenous gene typically associated with pluripotency ("pluripotency gene") is engineered to be operably linked to a selectable marker in a manner that the expression the endogenous pluripotency gene substantially matches the expression of the selectable marker. Because pluripotency genes are generally expressed only in pluripotent cells and not in somatic cells, the expression of an endogenous pluripotent gene(s) is an indication of successful reprogramming. Having a selectable marker operably linked to an endogenous pluripotency gene gives one a powerful mechanism to select for potentially reprogrammed somatic cells, which likely is a rare occurrence. The resulting cells may be further assessed for pluripotency characteristics to confirm whether a somatic cell has been successfully reprogrammed to pluripotency.

Generating pluripotent or multipotent cells by somatic cell reprogramming using the methods of the present invention has at least two advantages. First, the methods of the present invention allow one to generate autologous pluripotent cells, which are cells specific to a patient. The use of autologous cells in cell therapy offers a major advantage over the use of non-autologous cells, which are likely to be subject to immunological rejection. In contrast, autologous cells are unlikely to elicit significant immunological responses (See Munsie et al, 2000). Second, the methods of the present invention allow one to generate pluipotent without using embryos, oocytes and/or nuclear transfer technology.

A pluripotent cell is a cell that has the potential to divide in vitro for a long period of time (greater than one year) and has the unique ability to differentiate into cells derived from all three embryonic germ layers—endoderm, mesoderm and ectoderm.

A multipotent cell is a cell that is able to differentiate into some but not all of the cells derived from all three germ layers. Thus, a multipotent cell is a partially differentiated cell. Adult stem cells are multipotent cells. Known adult stem cells include, for example, hematopoietic stem cells and neural stem cells. A hematopoietic stem cell is multipotent because it has the ability to differentiate into all types of specific blood cells, but it is unlikely that they can differentiate into all cells of a given animal or human. Multipotent/adult stem cells have a great deal of promise in research and in the area of therapeutic applications. For example, multipotent/adult stem cells have already been used in humans in attempts to treat certain blood, neural and cancer diseases.

The term "pluripotency gene", as used herein, refers to a gene that is associated with pluripotency. The expression of a pluripotency gene is typically restricted to pluripotent stem

cells, and is crucial for the functional identity of pluripotent stem cells. The transcription factor Oct-4 (also called Pou5fl, Oct-3, Oct3/4) is an example of a pluripotency gene. Oct-4 has been shown to be required for establishing and maintaining the undifferentiated phenotype of ES cells and plays a major role in determining early events in embryogenesis and cellular-.differentiation (Nichols et al., 1998, Cell 95:379-391; Niwa et al., 2000, Nature Genet. 24:372-376). Oct-4 is down-regulated as stem cells differentiate into specialised cells. Other exemplary pluripotency genes include Nanog, and Stella (See Chambers et al., 2003, Cell 113: 643-655; Mitsui et al., Cell. 2003, 113(5):631-42; Bortvin et al. Development. 2003, 130(8):1673-80; Saitou et al., Nature. 2002, 418 (6895):293-300.

Engineered Somatic Cells and Transgenic Mice Comprising Such Cells

The present invention provides somatic cells comprising an endogenous pluripotency gene linked to DNA encoding a selectable marker in such a manner that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene. In one embodiment, the somatic cells of the present invention comprise a first endogenous pluripotency gene linked to DNA encoding a first selectable marker in such a manner that the expression of the first selectable marker substantially matches the expression of the first endogenous pluripotency gene. The somatic cells may also be engineered to comprise any number of endogenous pluripotency genes respectively linked to a distinct selectable marker. Thus, in another embodiment, the somatic cells of the present invention comprise two endogenous pluripotency genes, each of which is linked to DNA encoding a distinct selectable marker. In a further embodiment, the somatic cells of the present invention comprise three endogenous pluripotency genes, each of which is linked to DNA encoding a distinct selectable marker. The somatic cells described above will be collectively referred in this application as “engineered somatic cells.” The engineered somatic cells may be further engineered to have one or more pluripotency gene expressed as a transgene under an inducible promoter.

The selectable marker is linked to an appropriate endogenous pluripotency gene such that the expression of the selectable marker substantially matches the expression of the endogenous pluripotency gene. By “substantially match”, it is meant that the expression of the selectable marker substantially reflects the expression pattern of the endogenous pluripotency gene. In other words, the selectable marker and the endogenous pluripotency gene are co-expressed. For purpose of the present invention, it is not necessary that the expression level of the endogenous gene and the selectable marker is the same or even similar. It is only necessary that the cells in which an endogenous pluripotency gene is activated will also express the selectable marker at a level sufficient to confer a selectable phenotype on the reprogrammed cells. For example, when the selectable marker is a marker that confers resistance to a lethal drug (a “drug resistance marker”), the cells are engineered in a way that allows cells in which an endogeneous pluripotency gene is activated to also express the drug resistance marker at a sufficient level to confer on reprogrammed cells resistance to lethal drugs. Thus, reprogrammed cells will survive and proliferate whereas non-reprogrammed cells will die.

The DNA encoding a selectable marker may be inserted downstream from the end of the open reading frame (ORF) encoding the desired endogenous pluripotency gene, anywhere between the last nucleotide of the ORF and the first nucleotide of the polyadenylation site. An internal ribosome entry site (IRES) may be placed in front of the DNA encoding the selectable marker. Alternatively, the DNA encoding a selectable marker may be inserted anywhere within the ORF of the desired endogenous pluripotency gene, downstream of the promoter, with a termination signal. An internal ribosome entry site (IRES) may be placed in front of the DNA encoding the selectable marker. The selectable marker may be inserted into only one allele, or both alleles, of the endogenous pluripotency gene.

The somatic cells in the invention may be primary cells or immortalized cells. Such cells may be primary cells (non-immortalized cells), such as those freshly isolated from an animal, or may be derived from a cell line (immortalized cells).

The somatic cells in the present invention are mammalian cells, such as, for example, human cells or mouse cells. They may be obtained by well-known methods, from different organs, e.g., skin, lung, pancreas, liver, stomach, intestine, heart, reproductive organs, bladder, kidney, urethra and other urinary organs, etc., generally from any organ or tissue containing live somatic cells. Mammalian somatic cells useful in the present invention include, by way of example, adult stem cells, sertoli cells, endothelial cells, granulosa epithelial, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc. generally any live somatic cells. The term “somatic cells”, as used herein, also includes adult stem cells. An adult stem cell is a cell that is capable of giving rise to all cell types of a particular tissue. Exemplary adult stem cells include hematopoietic stem cells, neural stem cells, and mesenchymal stem cells.

In one embodiment, the engineered somatic cells are obtained from a transgenic mouse comprising such engineered somatic cells. Such transgenic mouse can be produced using standard techniques known in the art. For example, Bronson et al. describe a technique for inserting a single copy of a transgene into a chosen chromosomal site. See Bronson et al., 1996. Briefly, a vector containing the desired integration construct (for example, a construct containing a selectable marker linked to a pluripotency gene) is introduced into ES cells by standard techniques known in the art. The resulting ES cells are screened for the desired integration event, in which the knock-in vector is integrated into the desired endogenous pluripotency gene locus such that the selectable marker is integrated into the genomic locus of the pluripotency gene and is under the control of the pluripotency gene promoter. The desired ES cell is then used to produce transgenic mouse in which all cell types contain the correct integration event. Desired types of cells may be selectively obtained from the transgenic mouse and maintained in vitro. In one embodiment, two or more transgenic mice may be created, each carrying a distinct integration construct. These mice may then be bred to generate mice that carry multiple desired integration construct. For example, one type of transgenic mouse may be created to carry an endogenous pluripotency gene linked to a selectable marker, while a second type of transgenic mouse may be created to carry a pluripotency gene expressed as a transgene under an inducible promoter. These two types of mice may then be bred to generate transgenic mice that have both a selectable marker linked to an endogenous pluripotency gene and an additional pluripotency gene expressed as a transgene under an inducible promoter. These two pluripotency genes may or

may not be the same. Many variables are contemplated: the identity of the endogenous pluripotency gene linked to marker, the identity of the pluripotency gene expressed as a transgene, and the number of the endogenous pluripotency gene linked to a selectable marker, and the number of pluripotency gene expressed as a transgene. The present invention encompasses all possible combinations of these variables.

Alternatively, engineered somatic cells of the present invention may be produced by direct introduction of the desired construct into somatic cells. DNA construct may be introduced into cells by any standard technique known in the art, such as viral transfection (eg. using an adenoviral system) or liposome-mediated transfection. Any means known in the art to generate somatic cells with targeted integration can be used to produce somatic cells of the invention. In mammalian cells, homologous recombination occurs at much lower frequency compared to non-homologous recombination. To facilitate the selection of homologous recombination events over the non-homologous recombination events, at least two enrichment methods have been developed: the positive-negative selection (PNS) method and the "promoterless" selection method (Sedivy and Dutriaux, 1999). Briefly, PNS, the first method, is in genetic terms a negative selection: it selects against recombination at the incorrect (non-homologous) loci by relying on the use of a negatively selectable gene that is placed on the flanks of a targeting vector. On the other hand, the second method, the "promoterless" selection, is a positive selection in genetic terms: it selects for recombination at the correct (homologous) locus by relying on the use of a positively selectable gene whose expression is made conditional on recombination at the homologous target site. The disclosure of Sedivy and Dutriaux is incorporated herein.

A selectable marker, as used herein, is a marker that, when expressed, confers upon recipient cells a selectable phenotype, such as antibiotic resistance, resistance to a cytotoxic agent, nutritional prototrophy or expression of a surface protein. The presence of a selectable marker linked to an endogenous pluripotency gene makes it possible to identify and select reprogrammed cells in which the endogenous pluripotency gene is expressed. A variety of selectable marker genes can be used, such as neomycin resistance gene (neo), puromycin resistance gene (puro), guanine phosphoribosyl transferase (gpt), dihydrofolate reductase (DHFR), adenosine deaminase (ada), puromycin-N-acetyltransferase (PAC), hygromycin resistance gene (hyg), multidrug resistance gene (mdr), and hisD gene.

The present invention further provides transgenic mice comprising the somatic cells of the invention.

Methods for Reprogramming Somatic Cells

The present invention further provides methods for reprogramming somatic cells to a less differentiated state. The resulting cells are termed "reprogrammed somatic cells" ("RSC") herein. A RSC may be a reprogrammed pluripotent somatic cell ("RPSC"), a reprogrammed multipotent somatic cell ("RMSC"), or a reprogrammed somatic cell of varying differentiation status.

In general, the methods comprise treating the engineered somatic cells with an agent. The treatment with an agent may be contacting the cells with an agent which alters chromatin structure, or may be transfecting the cells with one or more pluripotency gene, or both. The above two treatments may be concurrent, or may be sequential, with no particular preference for order. In a further embodiment, reprogrammed somatic cells are identified by selecting for cells that express the appropriate selectable marker. In still a further embodiment, reprogrammed somatic cells are further assessed for pluripotency characteristics. The presence of pluripotency characteristics indicates that the somatic cells have been reprogrammed to a pluripotent state.

Differentiation status of cells is a continuous spectrum, with terminally differentiated state at one end of this spectrum and de-differentiated state (pluripotent state) at the other end. Reprogramming, as used herein, refers to a process that alters or reverses the differentiation status of a somatic cell, which can be either partially or terminally differentiated. Reprogramming includes complete reversion, as well as partial reversion, of the differentiation status of a somatic cell. In other words, the term "reprogramming", as used herein, encompasses any movement of the differentiation status of a cell along the spectrum toward a less-differentiated state. For example, reprogramming includes reversing a multipotent cell back to a pluripotent cell, reversing a terminally differentiated cell back to either a multipotent cell or a pluripotent cell. In one embodiment, reprogramming of a somatic cell turns the somatic cell all the way back to a pluripotent state. In another embodiment, reprogramming of a somatic cell turns the somatic cell back to a multipotent state. The term "less-differentiated state", as used herein, is thus a relative term and includes a completely de-differentiated state and a partially differentiated state.

The term "pluripotency characteristics", as used herein, refers to many characteristics associated with pluripotency, including, for example, the ability to differentiate into all types of cells and an expression pattern distinct for a pluripotent cell, including expression of pluripotency genes, expression of other ES cell markers, and on a global level, a distinct expression profile known as "stem cell molecular signature" or "stemness."

Thus, to assess reprogrammed somatic cells for pluripotency characteristics, one may analyze such cells for different growth characteristics and ES cell-like morphology. Cells may be injected subcutaneously into immunocompromised SCID mice to induce teratomas (a standard assay for ES cells). ES-like cells can be differentiated into embryoid bodies (another ES specific feature). Moreover, ES-like cells can be differentiated in vitro by adding certain growth factors known to drive differentiation into specific cell types. Self-renewing capacity, marked by induction of telomerase activity, is another plutipotency characteristics that can be monitored. One may carry out functional assays of the reprogrammed somatic cells by introducing them into blastocysts and determine whether the cells are capable of giving rise to all cell types. See Hogan et al., 2003. If the reprogrammed cells are capable of forming a few cell types of the body, they are multipotent; if the reprogrammed cells are capable of forming all cell types of the body including germ cells, they are pluripotent.

One may also examine the expression of an individual pluripotency gene in the reprogrammed somatic cells to assess their pluripotency characteristics. Additionally, one may assess the expression of other ES cell markers. Stage-specific embryonic 1 5 antigens-1, -3, and -4 (SSEA-1, SSEA-3, SSEA-4) are glycoproteins specifically expressed in early embryonic development and are markers for ES cells (Solter and Knowles, 1978, Proc. Natl. Acad. Sci. USA 75:5565-5569; Kannagi et al., 1983, EMBO J 2:2355-2361). Elevated expression of the enzyme Alkaline Phosphatase (AP) is another marker associated with undifferentiated embryonic stem cells (Wobus et al., 1984, Exp. Cell 152: 212-219; Pease et al., 1990, Dev. Biol. 141:322-352). Other stem/progenitor cells markers include the intermediate neurofilament nestin (Lendahl et al., 1990, Cell 60:585-595;

Dah-Istrand et al., 1992, J. Cell Sci. 103:589-597), the membrane glycoprotein prominin/AC133 (Weigmann et al., 1997, Proc. Natl. Acad. USA 94:12425-12430; Corbeil et al., 1998, Blood 91:2625-22626), the transcription factor Tcf-4 (Korinek et al, 1998, Nat. Genet. 19: 379-383; Lee et al., 1999, J. Biol. Chem. 274.1 566-1 572), and the transcription factor Cdx1 (Duprey et al., 1 988, Genes Dev. 2:1647-1654; Subramania'n et al., 1998, Differentiation 64:11-1 8).

One may additionally conduct expression profiling of the reprogrammed somatic cells to assess their pluripotency characteristics. Pluripotent cells, such as embryonic stem cells, and multipotent cells, such as adult stem cells, are known to have a distinct pattern of global gene expression profile. This distinct pattern is termed "stem cell molecular signature", or "stemness". See, for example, Ramalho-Santos et al., Science 298: 597-600 (2002); Ivanova et al., Science 298: 601-604.

Somatic cells may be reprogrammed to gain either a complete set of the pluripotency characteristics and are thus pluripotent. Alternatively, somatic cells may be reprogrammed to gain only a subset of the pluripotency characteristics. In another alternative, somatic cells may be reprogrammed to be multipotent.

In a further embodiment, in conjunction with contacting the somatic cells of the invention with an agent which alters chromatin structure, at least one gene that affects pluripotent state of a cell may be further introduced into the same cells. This may be carried out sequentially. For example, the somatic cells of the invention may be first contacted with an agent which alters chromatin structure. Then at least one pluripotency gene can be introduced into the same cells, or vice versa. Alternatively, the two steps may be carried out simultaneously.

Genes that affect pluripotent state of a cell includes pluripotency genes, genes involved in chromatin remodeling, and genes that are important for maintaining pluripotency, such as LIF, BMP, and PD098059 (See Cell, 115: 281-292 (2003); Philos Trans R Soc Lond B Biol Sci. 2003 Aug. 29; 358(1436):1397-402).

The exogenously introduced pluripotency gene may be carried out in several ways. In one embodiment, the exogenously introduced pluripotency gene may be expressed from a chromosomal locus different from the endogenous chromosomal locus of the pluripotency gene. Such chromosomal locus may be a locus with open chromatin structure, and contain gene(s) dispensible for a somatic cell. In other words, the desirable chromosomal locus contains gene(s) whose disruption will not cause cells to die. Exemplary chromosomal loci include, for example, the mouse ROSA 26 locus and type II collagen (Col2a1) locus (See Zambrowicz et al., 1997) The exogenously introduced pluripotency gene may be expressed from an inducible promoter such that their expression can be regulated as desired.

In an alternative embodiment, the exogenously introduced pluripotency gene may be transiently transfected into cells, either individually or as part of a cDNA expression library, prepared from pluripotent cells. Such pluripotent cells may be embryonic stem cells, oocytes, blastomeres, inner cell mass cells, embryonic germ cells, embryoid body (embryonic) cells, morula-derived cells, teratoma (teratocarcinoma) cells, and multipotent partially differentiated embryonic stem cells taken from later in the embryonic development process.

The cDNA library is prepared by conventional techniques. Briefly, mRNA is isolated from an organism of interest. An RNA-directed DNA polymerase is employed for first strand synthesis using the mRNA as template. Second strand synthesis is carried out using a DNA-directed DNA polymerase which results in the cDNA product. Following conventional processing to facilitate cloning of the cDNA, the cDNA is inserted into an expression vector such that the cDNA is operably linked to at least one regulatory sequence. The choice of expression vectors for use in connection with the cDNA library is not limited to a particular vector. Any expression vector suitable for use in mouse cells is appropriate. In one embodiment, the promoter which drives expression from the cDNA expression construct is an inducible promoter. The term regulatory sequence includes promoters, enhancers and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology*, Academic Press, San Diego, Calif. (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express cDNAs. Such useful expression control sequences, include, for example, the early and late promoters of SV40, tet promoter, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

The exogenously introduced pluripotency gene may be expressed from an inducible promoter. The term "inducible promoter", as used herein, refers to a promoter that, in the absence of an inducer (such as a chemical and/or biological agent), does not direct expression, or directs low levels of expression of an operably linked gene (including cDNA), and, in response to an inducer, its ability to direct expression is enhanced. Exemplary inducible promoters include, for example, promoters that respond to heavy metals (CRC Boca Raton, Fla. (1991), 167-220; Brinster et al. Nature (1982), 296, 39-42), to thermal shocks, to hormones (Lee et al. P.N.A.S. USA (1988), 85, 1204-1208; (1981), 294, 228-232; Klock et al. Nature (1987), 329, 734-736; Israel and Kaufman, Nucleic Acids Res. (1989), 17, 2589-2604), promoters that respond to chemical agents, such as glucose, lactose, galactose or antibiotic.

A tetracycline-inducible promoter is an example of an inducible promoter that responds to an antibiotics. See Gossen et al., 2003. The tetracycline-inducible promoter comprises a minimal promoter linked operably to one or more tetracycline operator(s). The presence of tetracycline or one of its analogues leads to the binding of a transcription activator to the tetracycline operator sequences, which activates the minimal promoter and hence the transcription of the associated cDNA. Tetracycline analogue includes any compound that displays structural homologies with tetracycline and is capable of activating a tetracycline-inducible

promoter. Exemplary tetracycline analogues includes, for example, doxycycline, chlorotetracycline and anhydrotetracycline.

Thus, in one embodiment, the present invention provides mice and somatic cells carrying at least one pluripotency gene expressed as a transgene under an inducible promoter. It is possible that somatic cells with such inducible pluripotency transgene(s) are more prone to be reprogrammed.

Any of the engineered somatic cells of the present invention may be used in the methods. In one embodiment, somatic cells used in the methods comprise only one endogenous pluripotency gene linked to a first selectable marker, and the selection step is carried out to select for the expression of the first selectable marker. In an alternative embodiment, the somatic cells used in the methods comprise any number of endogenous pluripotency genes, each of which is linked to a distinct selectable marker respectively, and the selection step is carried out to select for at least a subset of the selectable markers. For example, the selection step may be carried out to select for all the selectable markers linked to the various endogenous pluripotency genes.

In an alternative embodiment, somatic cells used in the method comprise a selectable marker linked to an endogenous pluripotency gene and an additional pluripotency gene expressed as a transgene under an inducible promoter. For these cells, the method of reprogramming may comprises induce the expression of the pluripotency transgene and select for the expression of the selectable marker. The method may further comprise contacting the somatic cells with an agent that alter chromatic structure.

Without wishing to be bound by theory, the agents used in the method may cause chromatin to take on a more open structure, which is more permissive for gene expression. DNA methylation and histone acetylation are two known events that alter chromatin toward a more closed structure. For example, loss of methylation by genetic deletion of DNA methylation enzyme Dnmt1 in fibroblasts results in reactivation of endogenous Oct4 gene. See J. Biol. Chem. 277: 34521-30, 2002; and Bergman and Mostoslavsky, Biol. Chem. 1990. Thus, DNA methylation inhibitors and histone deacelyation inhibitors are two classes of agents that may be used in the methods of the invention. Exemplary agents include 5-aza-cytidine, TSA and valproic acid.

In another embodiment, methods of the invention may further include repeating the steps of treating the cells with an agent. The agent used in the repeating treatment may be the same as, or different from, the one used during the first treatment.

Methods for Screening for an Agent that Reprograms Somatic Cells

The present invention also provides methods for identifying an agent that reprograms somatic cells to a less-differentiated state, as well as the agents thus identified. In one embodiment, the methods comprise contacting the engineered somatic cells of the invention with a candidate agent, selecting for cells that express the appropriate selectable marker. The presence of cells that express the appropriate selectable marker indicates that the agent reprograms somatic cells. Such an agent is referred as a “reprogramming agent” for purpose of this application.

In a further embodiment, the methods comprise contacting the engineered somatic cells of the invention with a candidate agent, selecting for cells that express the appropriate selectable marker, and assessing the cells so selected for pluripotency characteristics. The presence of a complete set of pluripotency characteristics indicates that the agent reprograms somatic cells to become pluripotent.

Candidate agents used in the invention encompass numerous chemical classes, though typically they are organic molecules, including small organic compounds. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, nucleic acids and derivatives, structural analogs or combinations thereof.

Candidate agents may be naturally arising, recombinant or designed in the laboratory. The candidate agents may be isolated from microorganisms, animals, or plants, or may be produced recombinantly, or synthesized by chemical methods known in the art. In some embodiments, candidate agents are isolated from libraries of synthetic or natural compounds using the methods of the present invention. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, including acylation, alkylation, esterification, amidification, to produce structural analogs.

There are numerous commercially available compound libraries, including, for example, the Chembridge DIVERSet. Libraries are also available from academic investigators, such as the Diversity set from the NCI developmental therapeutics program.

The screening methods mentioned above are based on assays performed on cells. These cell-based assays may be performed in a high throughput screening (HTS) format, which has been described in the art. For example, Stockwell et al. described a high-throughput screening of small molecules in miniaturized mammalian cell-based assays involving post-translational modifications (Stockwell et al., 1999). Likewise, Qian et al. described a leukemia cell-based assay for high-throughtput screening for anti-cancer agents (Qian et al., 2001). Both references are incorporated herein in their entirety.

A reprogramming agent may belong to any one of many different categories. For example, a reprogramming agent may be a chromatin remodeling agent. A chromatin remodeling agent may be a protein involved in chromatin remodeling or an agent known to alter chromatin toward a more open structure, such as a DNA methylation inhibitor or a histone deacelyation inhibitor. Exemplary compounds include 5-aza-cytidine, TSA and valproic acid. For another example, such an agent may be a pluripotency protein, including, for example, Nanog, Oct-4 and Stella. Such an agent may also be a gene essential for pluripotency, including, for example, Sox2, FoxD3, and LIF, and Stat3. See Smith et al. 1988, William et al., 1988, Ihle, 1996, Avilion et al., 2003, and Hanna et al., 2002)

Methods for Reprogramming Somatic Cells with a Reprogramming Agent

The reprogramming agent identified by the methods of the present invention is useful for reprogramming somatic cells into pluripotent or multipotent cells. Accordingly, the present invention provides methods for reprogramming somatic cells to a less differentiated state, comprising contacting somatic cells with a reprogramming agent. The somatic cells used may be native somatic cells, or engineered somatic

cells. It is not necessary for these cells to carry a selectable marker integrated into the endogenous locus of a pluripotency gene.

Reprogrammed Somatic Cells and these Uses

The present invention also provides reprogrammed somatic cells (RSCs), including reprogrammed pluripotent somatic cells (RPSCs), produced by the methods of the invention. These methods, useful for the generation of cells of a desired cell type, have wide range of applications. For one example, these methods have applications in livestock management, involving the precise genetic manipulation of animals for economic or health purposes. For another example, these methods have medical application in treating or preventing a condition.

Accordingly, the invention provides methods for the treatment or prevention of a condition in a mammal. In one embodiment, the methods start with obtaining somatic cells from the individual, reprogramming the somatic cells so obtained by methods of the present invention to obtain RPSCs. The RPSCs are then cultured under conditions suitable for development of the RPSCs into cells of a desired cell type. The developed cells of the desired cell type are harvested and introduced into the individual to treat the condition. In an alternative embodiment, the methods start with obtaining somatic cells from the individual, reprogramming the somatic cells so obtained by methods of the present invention. The RPSCs are then cultured under conditions suitable for development of the RPSCs into a desired organ, which is harvested and introduced into the individual to treat the condition.

The RPSCs of the present invention are ES-like cells, and thus may be induced to differentiate to obtain the desired cell types according to known methods to differentiate ES cells. For example, the RPSCs may be induced to differentiate into hematopoietic stem cells, muscle cells, cardiac muscle cells, liver cells, cartilage cells, epithelial cells, urinary tract cells, etc., by culturing such cells in differentiation medium and under conditions which provide for cell differentiation. Medium and methods which result in the differentiation of embryonic stem cells are known in the art as are suitable culturing conditions.

For example, Palacios et al., Proc. Natl. Acad. Sci., USA, 92: 7530-37 (1995) teaches the production of hematopoietic stem cells from an embryonic cell line by subjecting stem cells to an induction procedure comprising initially culturing aggregates of such cells in a suspension culture medium lacking retinoic acid followed by culturing in the same medium containing retinoic acid, followed by transferral of cell aggregates to a substrate which provides for cell attachment.

Moreover, Pedersen, J. Reprod. Fertil. Dev., 6: 543-52 (1994) is a review article which references numerous articles disclosing methods for in vitro differentiation of embryonic stem cells to produce various differentiated cell types including hematopoietic cells, muscle, cardiac muscle, nerve cells, among others.

Further, Bain et al., Dev. Biol., 168:342-357 (1995) teaches in vitro differentiation of embryonic stem cells to produce neural cells which possess neuronal properties. These references are exemplary of reported methods for obtaining differentiated cells from embryonic or stem-like cells. These references and in particular the disclosures therein relating to methods for differentiating embryonic stem cells are incorporated by reference in their entirety herein.

Thus, using known methods and culture medium, one skilled in the art may culture the subject embryonic or stem-like cells to obtain desired differentiated cell types, e.g., neural cells, muscle cells, hematopoietic cells, etc. In addition, the use of inducible Bcl-2 or Bcl-xl might be useful for enhancing in vitro development of specific cell lineages. In vivo, Bcl-2 prevents many, but not all, forms of apoptotic cell death that occur during lymphoid and neural development. A thorough discussion of how Bcl-2 expression might be used to inhibit apoptosis of relevant cell lineages following transfection of donor cells is disclosed in U.S. Pat. No. 5,646,008, which is herein incorporated by reference.

The subject RPSCs may be used to obtain any desired differentiated cell type. Therapeutic usages of such differentiated human cells are unparalleled. For example, human hematopoietic stem cells may be used in medical treatments requiring bone marrow transplantation. Such procedures are used to treat many diseases, e.g., late stage cancers such as ovarian cancer and leukemia, as well as diseases that compromise the immune system, such as AIDS. Hematopoietic stem cells can be obtained, e.g., by fusing adult somatic cells of a cancer or AIDS patient, e.g., epithelial cells or lymphocytes with an enucleated oocyte, e.g., bovine oocyte, obtaining embryonic or stem-like cells as described above, and culturing such cells under conditions which favor differentiation, until hematopoietic stem cells are obtained. Such hematopoietic cells may be used in the treatment of diseases including cancer and AIDS.

The methods of the present invention can also be used to treat, prevent, or stabilize a neurological disease such as Alzheimer's disease, Parkinson's disease, Huntington's disease, or ALS, lysosomal storage diseases, multiple sclerosis, or a spinal cord injury. For example, somatic cells may be obtained from the individual in need of treatment, and reprogrammed to gain pluripotency, and cultured to derive neurectoderm cells that may be used to replace or assist the normal function of diseased or damaged tissue.

For the treatment or prevention of endocrine conditions, RPSCs that produce a hormone, such as a growth factor, thyroid hormone, thyroid-stimulating hormone, parathyroid hormone, steroid, serotonin, epinephrine, or norepinephrine may be administered to a mammal. Additionally, reprogrammed epithelial cells may be administered to repair damage to the lining of a body cavity or organ, such as a lung, gut, exocrine gland, or urogenital tract. It is also contemplated that RPSCs may be administered to a mammal to treat damage or deficiency of cells in an organ such as the bladder, brain, esophagus, fallopian tube, heart, intestines, gallbladder, kidney, liver, lung, ovaries, pancreas, prostate, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, ureter, urethra, or uterus.

The great advantage of the present invention is that it provides an essentially limitless supply of isogenic or synegenic human cells suitable for transplantation. Therefore, it will obviate the significant problem associated with current transplantation methods, i.e., rejection of the transplanted tissue which may occur because of host versus graft or graft versus host rejection. Conventionally, rejection is prevented or reduced by the administration of anti-rejection drugs such as cyclosporin. However, such drugs have significant adverse side-effects, e.g., immunosuppression, carcinogenic properties, as well as being very expensive. The present invention should eliminate, or at least greatly reduce, the need for anti-rejection drugs, such as cyclosporine, imulan, FK-506, glucocorticoids, and rapamycin, and derivatives thereof.

RPSCs may also be combined with a matrix to form a tissue or organ in vitro or in vivo that may be used to repair or replace a tissue or organ in a recipient mammal. For

example, RPSCs may be cultured in vitro in the presence of a matrix to produce a tissue or organ of the urogenital system, such as the bladder, clitoris, corpus cavermosum, kidney, testis, ureter, uretal valve, or urethra, which may then be transplanted into a mammal (Atala, Curr. Opin. Urol. 9(6):517-526, 1999). In another transplant application, synthetic blood vessels are formed in vitro by culturing reprogrammed cells in the presence of an appropriate matrix, and then the vessels are transplanted into a mammal for the treatment or prevention of a cardiovascular or circulatory condition. For the generation of donor cartilage or bone tissue, RPSCs such as chondrocytes or osteocytes are cultured in vitro in the presence of a matrix under conditions that allow the formation of cartilage or bone, and then the matrix containing the donor tissue is administered to a mammal. Alternatively, a mixture of the cells and a matrix may be administered to a mammal for the formation of the desired tissue in vivo. Preferably, the cells are attached to the surface of the matrix or encapsulated by the matrix. Examples of matrices that may be used for the formation of donor tissues or organs include collagen matrices, carbon fibers, polyvinyl alcohol sponges, acrylateamide sponges, fibrin-thrombin gels, hyaluronic acid-based polymers, and synthetic polymer matrices containing polyanhydride, polyorthoester, polyglycolic acid, or a combination thereof (see, for example, U.S. Pat. Nos. 4,846,835; 4,642,120; 5,786,217; and 5,041,138).

The RPSCs produced according to the invention may be used to produce genetically engineered or transgenic differentiated cells. Essentially, this will be effected by introducing a desired gene or genes, or removing all or part of an endogenous gene or genes of RPSCs produced according to the invention, and allowing such cells to differentiate into the desired cell type. A preferred method for achieving such modification is by homologous recombination because such technique can be used to insert, delete or modify a gene or genes at a specific site or sites in the stem-like cell genome.

This methodology can be used to replace defective genes, e.g., defective immune system genes, cystic fibrosis genes, or to introduce genes which result in the expression of therapeutically beneficial proteins such as growth factors, lymphokines, cytokines, enzymes, etc. For example, the gene encoding brain derived growth factor may be introduced into human embryonic or stem-like cells, the cells differentiated into neural cells and the cells transplanted into a Parkinson's patient to retard the loss of neural cells during such disease. Examples of mutations that may be rescued using these methods include mutations in the cystic fibrosis gene; mutations associated with Dunningan's disease such as the R482W, R482Q, and R584H mutations in the lamin A gene; and mutations associated with the autosomal-dominant form of Emery Deyfuss muscular dystrophy such as the R249Q, R453W, and Q6STOP mutations in the lamin A gene. In the Q6STOP mutation, the codon for Gln6 is mutated to a stop codon.

Previously, cell types transfected with BDNF varied from primary cells to immortalized cell lines, either neural or non-neural (myoblast and fibroblast) derived cells. For example, astrocytes have been transfected with BDNF gene using retroviral vectors, and the cells grafted into a rat model of Parkinson's disease (Yoshimoto et al., Brain Research, 691:25-36, (1995)). This ex vivo therapy reduced Parkinson's-like symptoms in the rats up to 45% 32 days after transfer. Also, the tyrosine hydroxylase gene has been placed into astrocytes with similar results (Lundberg et al., Develop. Neurol., 139:39-53 (1996) and references cited therein).

However, such ex vivo systems have problems. In particular, retroviral vectors currently used are down-regulated in vivo and the transgene is only transiently expressed (review by Mulligan, Science, 260: 926-932 (1993)). Also, such studies used primary cells, astrocytes, which have finite life span and replicate slowly. Such properties adversely affect the rate of transfection and impede selection of stably transfected cells. Moreover, it is almost impossible to propagate a large population of gene targeted primary cells to be used in homologous recombination techniques.

By contrast, the difficulties associated with retroviral systems should be eliminated by the use of RPSCs of the present invention, which are ES-like cells. Using known methods to introduced desired genes/mutations into ES cells, RPSCs may be genetically engineered, and the resulting engineered cells differentiated into desired cell types, e.g., heniatopoietic cells, neural cells, pancreatic cells, cartilage cells, etc. Genes which may be introduced into the RPSCs include, for example, epidermal growth factor, basic fibroblast growth factor, glial derived neurotrophic growth factor, insulin-like growth factor (I and II), neurotrophin3, neurotrophin-4/5, ciliary neurotrophic factor, AFT-1, cytokine genes (interleukins, interferons, colony stimulating factors, tumor necrosis factors (alpha and beta), etc.), genes encoding therapeutic enzymes, collagen, human serum albumin, etc.

In addition, it is also possible to use one of the negative selection systems now known in the art for eliminating therapeutic cells from a patient if necessary. For example, donor cells transfected with the thymidine kinase (TK) gene will lead to the production of embryonic cells containing the TK gene. Differentiation of these cells will lead to the isolation of therapeutic cells of interest which also express the TK gene. Such cells may be selectively eliminated at any time from a patient upon gancyclovir administration. Such a negative selection system is described in U.S. Pat. No. 5,698,446, and is herein incorporated by reference.

Examples of diseases, disorders, or conditions that may be treated or prevented include neurological, endocrine, structural, skeletal, vascular, urinary, digestive, integumentary, blood, immune, auto-immune, inflammatory, endocrine, kidney, bladder, cardiovascular, cancer, circulatory, digestive, hematopoeitic, and muscular diseases, disorders, and conditions. In addition, reprogrammed cells may be used for reconstructive applications, such as for repairing or replacing tissues or organs.

With respect to the therapeutic methods of the invention, it is not intended that the administration of RPSCs to a mammal be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration, including intramuscular, intravenous, intraarticular, intralesional, subcutaneous, or any other route sufficient to provide a dose adequate to prevent or treat a disease. The RPSCs may be administered to the mammal in a single dose or multiple doses. When multiple doses are administered, the doses may be separated from one another by, for example, one week, one month, one year, or ten years. One or more growth factors, hormones, interleukins, cytokines, or other cells may also be administered before, during, or after administration of the cells to further bias them towards a particular cell type.

The RPSCs of the present invention may be used as an in vitro model of differentiation, in particular for the study of genes which are involved in the regulation of early development. Differentiated cell tissues and organs using the RPSCs may be used in drug studies.

Furthermore, the RPSCs produced according to the invention may be introduced into animals, e.g., SCID mice, cows, pigs, e.g., under the renal capsule or intramuscularly and used to produce a teratoma therein. This teratoma can be used to derive different tissue types. Also, the inner cell mass produced by X-species nuclear transfer may be introduced together with a biodegradable, biocompatible polymer matrix that provides for the formation of 3-dimensional tissues. After tissue formation, the polymer degrades, ideally just leaving the donor tissue, e.g., cardiac, pancreatic, neural, lung, liver. In some instances, it may be advantageous to include growth factors and proteins that promote angiogenesis. Alternatively, the formation of tissues can be effected totally in vitro, with appropriate culture media and conditions, growth factors, and biodegradable polymer matrices.

Applications of the Somatic Cell Reprogramming Methods and RPSCs in Animals

The reprogramming methods disclosed herein may be used to generate RPSCs for a variety of animal species. The RPSCs generated can be useful to produce desired animals. Animals include, for example, avians and mammals as well as any animal that is an endangered species. Exemplary birds include domesticated birds (e.g., quail, chickens, ducks, geese, turkeys, and guinea hens) as well as other birds such as birds of prey (e.g., hawks, falcons, ospreys, condors, etc.), endangered birds (e.g., parrots, California condor, etc.), ostriches etc. Exemplary mammals include murine, caprine, ovine, bovine, porcine, canine, feline and primate. Of these, preferred members include domesticated animals, including, for examples, cattle, buffalo, pigs, horses, cows, rabbits, guinea pigs, sheep, and goats.

RPSCs generated by the reprogramming methods of the present invention allows one, for the first time, to genetically engineer animals other than mouse and human. RPSCs are ES-like cells, and are thus amenable to genetic manipulation. To date, no ES cells are available for animals other than mouse and human. As a result, for these animals, it is currently practically impossible to create genetically modified animals having targeted mutations. The ES-cell like RPSCs can be manipulated to introduce desired targeted genetic modifications. The resulting engineered RPSCs can then be used to generate a cloned animal with the desired genetic modifications in its germ line, using methods described for ES cells in mouse. See Capecchi and Thomas, U.S. Pat. Nos. 5,487,992, 5,627,059, 5,631,153, and 6,204,061. Genetic engineering in animals has potentially great applications in a variety of animals, especially farm animals.

The somatic cell reprogramming methods of the present invention provides at least two methods for delivering optimized farm animals. In the first, somatic cell reprogramming can be used to capture the best available phenotype for a farm animal stock. The current technologies used to deliver optimized farm animals are based on selective breeding, and expansion from preferred breeding stocks. Animals that have been selected on the basis of superior characteristics, including, for example, meat content, egg production (in the case of poultry), feed conversion ratio, are used to breed large numbers of animals that are in turn used in the human food supply. This traditional process has profound inherent inefficiencies. The phenotype observed in an individual animal is often only partially transmitted in the progeny of that animal. Therefore, traditional breeding schemes are inefficient in capturing the very best phenotype in all of the progeny animals. In contrast, the reprogramming methods of the present invention provides a controlled and efficient way to achieve the same goal, by generating RPSCs from somatic cells of an animal with the desired characteristics. The RPSCs generated may be used immediately to generate cloned animals derived from the RPSCs. Known methods for generating mice from ES cells can be used for this procedure. Alternatively, the RPSCs generated may be cryopreserved and thawed in response to a grower's needs.

In the second method, somatic cells from an animal with the desired characteristics are reprogrammed to produce RPSCs. The RPSCs are further genetically engineered to introduce desired genetic modification(s), before being placed into a recipient embryo to produce desired progeny.

The reprogramming methods can also be used to rescue endangered species. Somatic cell reprogramming provides an efficient method to generate RPSCs from somatic cells of an endangered animal. The resulting RPSCs can be used immediately to expand the numbers of the endangered animal. Alternatively, the RPSCs can be cryopreserved to generate a RPSC stock for the endangered species, as a safeguard measure against extinction of the endangered species.

Methods for Gene Identification

The present invention provides methods for identifying a gene that activates the expression of an endogenous pluripotency gene in somatic cells. The methods comprise: transfecting the somatic cells of the present invention with a cDNA library prepared from ES cells or oocytes, selecting for cells that express the first selectable marker, and assessing the expression of the first endogenous pluripotency gene in the transfected cells that express the first selectable marker. The expression of the first endogenous pluripotency gene indicates that the cDNA encodes a gene that activates the expression of an endogenous pluripotency gene in somatic cells.

The methods are applicable for identifying a gene that activates the expression of at least two endogenous pluripotency genes in somatic cells. The somatic cells used in the methods further comprise a second endogenous pluripotency gene linked to a second selectable marker. The methods are modified to select for transfected cells that express both selectable markers, among which the expression of the first and the second endogenous pluripotency genes are assessed. The expression of both the first and the second endogenous pluripotency genes indicates that the cDNA encodes a gene that activates the expression of at least two pluripotency genes in somatic cells.

The methods are further applicable for identifying a gene that activates the expression of at least three endogenous pluripotency genes in somatic cells. The somatic cells used in the methods further comprise a third endogenous pluripotency gene linked to a third selectable marker. The methods are modified to select for transfected cells that express all three selectable markers, among which the expression of all three endogenous pluripotency genes are assessed. The expression of all three endogenous pluripotency genes indicates that the cDNA encodes a gene that activates the expression of at least three pluripotency genes in somatic cells.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of mouse genetics, developmental biology, cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, Current Protocols in Cell Biology, ed. by Bonifacino, Dasso, Lippincott-Schwartz, Harford, and Yamada, John Wiley and Sons, Inc., New York, 1999; Manipulating the Mouse Embryos, A Laboratory Manual, 3rd Ed., by Hogan et al., Cold Spring Harbor Laboratory

Press, Cold Spring Harbor, N.Y., 2003; Gene Targeting: A Practical Approach, IRL Press at Oxford University Press, Oxford, 1993; and Gene Targeting Protocols, Human Press, Totowa, N.J., 2000. All patents, patent applications and references cited herein are incorporated in their entirety by reference.

EXEMPLIFICATION

The invention now being generally described, it will be more readily understood by reference to the following example, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example

Oct4-Induced Fibroblasts are More Susceptible to Reprogramming than Unduced Fibroblasts as Demonstrated by Nuclear Transfer Experiment

A. Generation of Transgenic Mouse Carrying an Inducible Oct4 Transgene

An inducible Oct4 allele is constructed as the following: first, two integration vectors are constructed. The first integration vector, inducible Oct4 integration vector, contains an Oct4 gene driven by a tetracycline-inducible promoter (Tet-Op). The Tet-Op-Oct4 cassette is flanked by a splice-acceptor double poly-A signal (SA-dpA) at its 5' end and a SV40 polyA tail (SV40-pA) at its 3' end. The second integration vector, tetracycline activator integration vector, contains a mutant form of tetracycline activator, M2-rtTA, which is more responsive to doxycycline (Dox) induction than the wild type activator. (Urlinger S. et al., 2000)

The two integration vectors are introduced into V6.5 ES cells: the inducible Oct4 integration vector and the tetracycline activator integration vector are introduced into the Collagen locus and the Rosa26 locus respectively via site-specific integration, as shown in FIG. **1**. The resulting ES cells are used to make Oct4-inducible mice by tetraploid complementation.

B. Expression of the Inducible Oct4 Transgene

Fibroblasts derived from tail biopsies of the Oct4-inducible mice were cultured. A fraction of the cultured fibroblasts were induced with doxycycline for 3 days (at 2 microgram/ml), and Oct4 expression was detected by Northern blot and Western blot analysis. As shown in FIG. **2**, the Oct4 expression level in fibroblasts treated with doxycycline is comparable to the Oct4 expression level in ES cells, and undetectable in fibroblasts not treated with doxycycline. The expression results demonstrate that the inducible Oct4 transgene is expressed as planned.

C. Nuclear Transfer Experiment

Nuclear transfer was performed on fibroblasts derived from tail biopsies of mice that carry the inducible Oct4 transgene. Dox induction was for 24 hours prior to nuclear transfer. Cloned embryos were then activated and cultured to the blastocyst stage to derive ES cells as described previously (Hochedlinger and Jaenisch, 2002). As shown in Table 1, on average, blastocyst formation and ES cell derivation (as measured as a fraction of eggs with pronucleus formation) is more efficient from Oct4 induced fibroblast than from uninduced fibroblasts. This result demonstrated that induced Oct4 expression in somatic cells such as fibroblasts make these cells more susceptible to reprogramming.

One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods, systems and kits are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention and are defined by the scope of the claims. It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

TABLE 1

In vitro development of clones derived from Oct4-induced fibroblasts

Expt.	Oct4	eggs w/ PN	Blastocysts (% PN)		ES lines (% PN)	
#1	−	22	5 (23%)	19%	0 (0%)	3%
#2	−	35	5 (14%)		2 (6%)	
#3	+	37	10 (27%)	24%	2 (5%)	7%
#4	+	47	10 (21%)		4 (9%)	

PN . . . ProNucleus formation

Nuclear transfer was performed on fibroblasts derived from tail biopsies of mice that carry the inducible Oct4 transgene. Dox induction was for 24 hours prior to nuclear transfer. Cloned embryos were then activated and cultured to the blastocyst stage to derive ES cells as described previously (Hochedlinger and Jaenisch, Nature, 2002). These preliminary results show that on average blastocyst formation and ES cell derivation is more efficient from Oct4 induced than from uninduced fibroblasts.

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What is claimed is:

1. A method of making a somatic cell more susceptible to reprogramming to a less differentiated state, comprising: introducing an exogenous nucleic acid encoding an Oct 4 protein operably linked to at least one regulatory sequence into the somatic cell, thereby increasing expression of Oct4 protein in the somatic cell, wherein increased expression of Oct4 protein makes the cell more susceptible to reprogramming; and wherein the exogenous nucleic acid is transiently transfected into the somatic cell.

2. The method of claim **1**, wherein the somatic cell does not comprise a selectable marker.

3. The method of claim **1**, wherein the somatic cell is a human cell or a mouse cell.

4. The method of claim **1**, wherein the somatic cell is an adult stem cell.

5. The method of claim **4**, wherein the adult stem cell is a hematopoietic stem cell, neural stem cell, or mesenchymal stem cell.

6. The method of claim **1**, wherein the less differentiated state comprises pluripotent state.

7. The method of claim **1**, further comprises: contacting the somatic cell with a candidate agent of interest with respect to its potential to reprogram a somatic cell.

8. The method of claim **7**, wherein the agent is Sox-2 or Nanog.

9. The method of claim **1**, wherein the somatic cell does not comprise a selectable marker integrated into an endogenous locus of a pluripotency gene.

10. A method of making a somatic cell more susceptible to reprogramming to a cell having a less differentiated state, comprising: obtaining a somatic cell that comprises an exogenously introduced polynucleic acid encoding Oct4 protein, and an exogenously introduced polynucleic acid encoding Sox2 or Nanog protein; wherein the exogenously introduced polynucleic acids result in making the somatic cell more susceptible to reprogramming to a less differentiated state; and wherein the exogenous polynucleic acids are transiently transfected into the cell.

11. The method of claim **10**, the step of obtaining further comprises: introducing an exogenous polynucleic acid encoding Oct4 protein, and an exogenously introduced polynucleic acid encoding Sox2 or Nanog protein into the somatic cell.

12. The method of claim **10** or **11**, further comprising: contacting the somatic cell with a candidate agent of interest with respect to its potential to reprogram a somatic cell.

13. The method of claim **10**, wherein the exogenous polynucleic acids increase Oct4 expression in the somatic cell.

14. The method of claim **10**, wherein the somatic cell is a mammalian cell.

15. The method of claim **10**, wherein the somatic cell is a human cell or a mouse cell.

16. The method of claim **10**, wherein the somatic cell is an adult stem cell.

17. The method of claim **16**, wherein the adult stem cell is selected from the group consisting of: a hematopoietic stem cell, a neural stem cell, and a mesenchymal stem cell.

18. The method of claim **10**, wherein the less differentiated state comprises pluripotent state.

* * * * *

EXHIBIT G

Source: *Fate Therapeutics, Inc.*

December 13, 2021 16:31 ET

Fate Therapeutics Showcases Positive Interim Phase 1 Data from FT596 Off-the-shelf, iPSC-derived CAR NK Cell Program for Relapsed / Refractory B-cell Lymphoma at 2021 ASH Annual Meeting

5 of 6 Patients Achieve Objective Response, including 4 Patients with Complete Response, with Single Dose of FT596 at 900 Million Cells in Combination with Rituximab

13 of 19 Patients Achieve Objective Response with Single Dose of FT596 at 90 Million and 300 Million Cell Dose; 10 of 11 Patients Treated with a Second FT596 Cycle Continue in Ongoing Response, with 3 Patients in Ongoing Complete Response at ≥6 Months Follow-up; Additional 2 Patients Reach 6 Months in Complete Response

FT596 Treatment Regimens were Well-tolerated; No Dose-limiting Toxicities, and No Adverse Events of Any Grade of ICANS or GVHD, were Observed; Three Low-grade Adverse Events of CRS Resolved without Intensive Care Treatment

Company to Host Virtual Investor Event Tomorrow at 8:00 AM Eastern Time

SAN DIEGO, Dec. 13, 2021 (GLOBE NEWSWIRE) -- Fate Therapeutics, Inc. (NASDAQ: FATE), a clinical-stage biopharmaceutical company dedicated to the development of programmed cellular immunotherapies for cancer, today showcased positive interim Phase 1 data from the Company's FT596 program for patients with relapsed / refractory B-cell lymphoma (BCL) at the 63rd American Society of Hematology (ASH) Annual Meeting and Exposition. FT596 is the Company's off-the-shelf, multi-antigen targeted, iPSC-derived natural killer (NK) cell product candidate derived from a clonal master induced pluripotent stem cell (iPSC) line engineered with three anti-tumor functional modalities: a proprietary chimeric antigen receptor (CAR) optimized for NK cell biology that targets B-cell antigen CD19; a novel high-affinity, non-cleavable CD16 (hnCD16) Fc receptor that has been modified to prevent its down-regulation and to enhance its binding to tumor-targeting antibodies; and an IL-15 receptor fusion (IL-15RF) that augments NK cell activity.

"The interim dose-escalation clinical data from our FT596 program in relapsed / refractory B-cell lymphoma demonstrate that off-the-shelf, iPSC-derived CAR NK cells can bring substantial therapeutic benefit to heavily pre-treated patients in urgent need of therapy, with high response rates and meaningful duration of responses," said Scott Wolchko, President and Chief Executive Officer of Fate Therapeutics. "We are particularly pleased with the therapeutic profile that has emerged with FT596 in combination with rituximab, where over half of the patients treated with a single dose of FT596 at higher dose levels achieved a complete response with a favorable safety profile that is clearly differentiated from CAR T-cell therapy. We look forward to assessing a two-dose treatment schedule for FT596 to further define its potential best-in-class therapeutic profile and ability to reach more patients, including those earlier in care."

The ongoing Phase 1 study in relapsed / refractory BCL is assessing a single dose of FT596 as monotherapy (Monotherapy Arm) and in combination with a single dose of rituximab (375 mg/m^2) (Combination Arm) following three days of conditioning chemotherapy (500 mg/m^2 of cyclophosphamide and 30 mg/m^2 of fludarabine). Certain patients are eligible for re-treatment with a second, single-dose cycle.

The ASH presentation (*Session 704—Cellular Immunotherapies: Expanding Targets and Cellular Sources for Immunotherapies, Abstract 823*) includes clinical data from 25 evaluable patients for safety (n=12 in Monotherapy Arm; n=13 in Combination Arm) in the first, second, and third single-dose cohorts of 30 million, 90 million, and 300 million cells, respectively, of which 24 patients were also evaluable for efficacy (n=12 in Monotherapy Arm; n=12 in Combination Arm), as of the data cutoff date of October 11, 2021. These 25 patients had received a median of

four prior lines of therapy and a median of two prior lines containing CD20-targeted therapy. Of the 25 patients, 15 patients (60%) had aggressive B-cell lymphoma, 15 patients (60%) were refractory to most recent prior therapy, and 8 patients (32%) were previously treated with autologous CD19-targeted CAR T-cell therapy. Subsequent to the data cutoff date for the ASH presentation, an additional patient in the third single-dose cohort of the Combination Arm was evaluable for initial anti-tumor response, and seven patients in the fourth single-dose cohort of 900 million cells (n=1 in Monotherapy Arm; n=6 in Combination Arm) were evaluable for safety and initial anti-tumor response.

Single-dose, Single-cycle Response Data

In the second, third, and fourth dose cohorts of the Monotherapy and Combination Arms comprising a total of 26 patients, 18 patients (69%) achieved an objective response, including 12 patients (46%) that achieved a complete response, on Day 29 following a single dose of FT596 (see Table 1). Nine of these 26 patients were previously treated with autologous CD19-targeted CAR T-cell therapy and, of these nine patients, six achieved an objective response (67%) on Day 29 following a single dose of FT596. Notably, in the third and fourth dose cohorts of the Combination Arm comprising a total of 12 patients, nine patients (75%) achieved an objective response, including seven patients (58%) that achieved a complete response, on Day 29 following a single dose of FT596.

Durability of Response Data

The ASH presentation includes durability of response data from 13 responding patients in the second and third single-dose cohorts of 90 million cells and 300 million cells (n=9 in Monotherapy Arm; n=10 in Combination Arm). As of the data cutoff date of October 11, 2021, 10 patients continued in ongoing response, including three patients in ongoing complete response at least six months from initiation of treatment; two patients reached six months in complete response and subsequently had disease progression; and one patient had disease progression prior to six months. Of these 13 responding patients:

- *Monotherapy Arm (n=7 responding patients).* Five patients, all of whom were treated with a second FT596 single-dose cycle with the consent of the U.S. Food and Drug Administration (FDA), continued in ongoing response at a median follow-up of 4.1 months, including one patient in ongoing complete response at 8.1 months; one patient, who was treated with only one FT596 single-dose cycle, reached six months in complete response and subsequently had disease progression at 6.5 months; and one patient, who was treated with only one FT596 single-dose cycle, had disease progression at 1.7 months.
- *Combination Arm (n=6 responding patients).* Five patients, all of whom were treated with a second FT596 single-dose cycle with the consent of the FDA, continued in ongoing response at a median follow-up of 4.6 months, including two patients in ongoing complete response at 6.0 and 10.8 months; and one patient, who was treated with a second FT596 single-dose cycle with the consent of the FDA, reached six months in complete response and subsequently had disease progression at 6.7 months.

Table 1. FT596 Interim Phase 1 Data – Day 29 Response Assessment [1]

1 Dose x 1 Cycle	**Monotherapy (n=13)**		**Combination (n=19)**	
Single-dose Level Cohorts (Cells)	**OR**	**CR**	**OR**	**CR**
30M	1/3 (33%)	0	0/3 (0%)	0
90M	3/4 (75%)	2	2/4 (50%)	2
300M [2]	4/5 (80%)	1	4/6 (67%)	3
900M [2]	0/1 (0%)	0	5/6 (83%)	4
aCD19 History (≥90M Cells)	n=10		n=16	
Naïve	7/9 (78%)	3	5/8 (63%)	4
Prior	0/1 (0%)	0	6/8 (75%)	5
Disease Histology (≥90M Cells)	n=10		n=16	
Aggressive	1/3 (33%)	0	6/11 (55%)	4
Mantle cell	0/1 (0%)	0	2/2 (100%)	2
Indolent	6/6 (100%)	3	3/3 (100%)	3

aCD19 = autologous CD19-targeted CAR T-cell therapy; **Aggressive** = diffuse large B-cell lymphoma, Grade 3b follicular lymphoma, Richter's transformation, and high-grade B-cell lymphoma; **CR** = complete response; **Indolent** = splenic diffuse red pulp small B-cell lymphoma, non-Grade 3b follicular lymphoma, Waldenstrom's macroglobulinemia, and small lymphocytic lymphoma; **M** = million; **OR** = objective response

[1] As of data cutoff date of October 11, 2021, unless otherwise noted. Objective response and complete response are based on Cycle 1 Day 29 protocol-defined response assessment per Lugano 2014 criteria.

Data subject to source document verification.

[2] Cycle 1 Day 29 protocol-defined response assessment completed subsequent to data cutoff date for one patient in the third single-dose cohort of 300 million cells in the Combination Arm and seven patients in the fourth single-dose cohort of 900 million cells (n=1 in Monotherapy Arm; n=6 in Combination Arm).

Safety Data

The FT596 treatment regimens were well tolerated, including in those patients treated with a second, single-dose cycle. No dose-limiting toxicities, and no treatment-emergent adverse events (TEAEs) of any grade of immune effector cell-associated neurotoxicity syndrome (ICANS) or graft-versus-host disease (GvHD) were observed. Three low-grade adverse events (two Grade 1, one Grade 2) of cytokine release syndrome (CRS) were reported, which were of limited duration and resolved without intensive care treatment (see Table 2).

The Company has initiated enrollment of a two-dose treatment schedule in the Combination Arm, with FT596 administered on Day 1 and Day 15 at 900 million cells per dose. Patients with clinical benefit following administration of the first two-dose cycle are eligible for re-treatment with a second two-dose cycle. Additionally, patients with clinical response are eligible for re-treatment following disease progression.

Table 2. FT596 Interim Phase 1 Data – TEAEs of Interest

n (%)	**Monotherapy (n=13)**		**Combination (n=19)**	
	All Grade	**Grade 3+**	**All Grade**	**Grade 3+**
CRS	1 (8%)	---	2 (11%)	---
ICANS	---	---	---	---
GvHD	---	---	---	---
Infections	6 (46%)	3 (23%)	6 (32%)	3 (16%)
FT596-related SAEs	---	---	1 (5%) [a]	---

CRS = Cytokine Release Syndrome; **GvHD** = Graft vs. Host Disease; **ICANS** = Immune Cell-Associated Neurotoxicity Syndrome; **TEAE** = Treatment-Emergent Adverse Event; **SAE** = Severe Adverse Events

[a] Grade 2 CRS

Investor Event Webcast

The Company will host a live audio webcast on Tuesday, December 14, 2021 at 8:00 a.m. ET to highlight interim Phase 1 clinical data from the Company's FT516 and FT596 programs for the treatment of relapsed / refractory B-cell lymphoma. The live webcast can be accessed under "Events & Presentations" in the Investors section of the Company's website at www.fatetherapeutics.com. The archived webcast will be available on the Company's website beginning approximately two hours after the event.

About Fate Therapeutics' iPSC Product Platform

The Company's proprietary induced pluripotent stem cell (iPSC) product platform enables mass production of off-the-shelf, engineered, homogeneous cell products that are designed to be administered with multiple doses to deliver more effective pharmacologic activity, including in combination with other cancer treatments. Human iPSCs possess the unique dual properties of unlimited self-renewal and differentiation potential into all cell types of the body. The Company's first-of-kind approach involves engineering human iPSCs in a one-time genetic modification event and selecting a single engineered iPSC for maintenance as a clonal master iPSC line. Analogous to master cell lines used to manufacture biopharmaceutical drug products such as monoclonal antibodies, clonal master iPSC lines are a renewable source for manufacturing cell therapy products which are well-defined and uniform in composition, can be mass produced at significant scale in a cost-effective manner, and can be delivered off-the-shelf for patient treatment. As a result, the Company's platform is uniquely designed to overcome numerous limitations associated with the production of cell therapies using patient- or donor-sourced cells, which is logistically complex and expensive and is subject to batch-to-batch and cell-to-cell variability that can affect clinical safety and efficacy. Fate Therapeutics' iPSC product platform is supported by an intellectual property portfolio of over 350 issued patents and 150 pending patent applications.

About FT596

FT596 is an investigational, universal, off-the-shelf natural killer (NK) cell cancer immunotherapy derived from a clonal master induced pluripotent stem cell (iPSC) line engineered with three anti-tumor functional modalities: a proprietary chimeric antigen receptor (CAR) optimized for NK cell biology that targets B-cell antigen CD19; a novel high-affinity 158V, non-cleavable CD16 (hnCD16) Fc receptor, which has been modified to prevent its down-regulation and to enhance its binding to tumor-targeting antibodies; and an IL-15 receptor fusion (IL-15RF) that augments NK cell activity. In preclinical studies of FT596, the Company has demonstrated that dual activation of the CAR19 and hnCD16 targeting receptors enhances cytotoxic activity, indicating that multi-antigen

engagement may elicit a deeper and more durable response. Additionally, in a humanized mouse model of lymphoma, FT596 in combination with the anti-CD20 monoclonal antibody rituximab showed enhanced killing of tumor cells *in vivo* as compared to rituximab alone. FT596 is being investigated in a multi-center Phase 1 clinical trial for the treatment of relapsed / refractory B-cell lymphoma as a monotherapy and in combination with rituximab, and for the treatment of relapsed / refractory chronic lymphocytic leukemia (CLL) as a monotherapy and in combination with obinutuzumab (NCT04245722).

About Fate Therapeutics, Inc.

Fate Therapeutics is a clinical-stage biopharmaceutical company dedicated to the development of first-in-class cellular immunotherapies for patients with cancer. The Company has established a leadership position in the clinical development and manufacture of universal, off-the-shelf cell products using its proprietary induced pluripotent stem cell (iPSC) product platform. The Company's immuno-oncology pipeline includes off-the-shelf, iPSC-derived natural killer (NK) cell and T-cell product candidates, which are designed to synergize with well-established cancer therapies, including immune checkpoint inhibitors and monoclonal antibodies, and to target tumor-associated antigens using chimeric antigen receptors (CARs). Fate Therapeutics is headquartered in San Diego, CA. For more information, please visit www.fatetherapeutics.com.

Forward-Looking Statements

This release contains "forward-looking statements" within the meaning of the Private Securities Litigation Reform Act of 1995 including statements regarding the safety and therapeutic potential of the Company's iPSC-derived NK cell product candidates, including FT596, its ongoing and planned clinical studies, and the expected clinical development plans for FT596. These and any other forward-looking statements in this release are based on management's current expectations of future events and are subject to a number of risks and uncertainties that could cause actual results to differ materially and adversely from those set forth in or implied by such forward-looking statements. These risks and uncertainties include, but are not limited to, the risk that results observed in studies of its product candidates, including preclinical studies and clinical trials of any of its product candidates, will not be observed in ongoing or future studies involving these product candidates, the risk that the Company may cease or delay clinical development of any of its product candidates for a variety of reasons (including requirements that may be imposed by regulatory authorities on the initiation or conduct of clinical trials, the amount and type of data to be generated, or otherwise to support regulatory approval, difficulties or delays in subject enrollment and continuation in current and planned clinical trials, difficulties in manufacturing or supplying the Company's product candidates for clinical testing, and any adverse events or other negative results that may be observed during preclinical or clinical development), and the risk that its product candidates may not produce therapeutic benefits or may cause other unanticipated adverse effects. For a discussion of other risks and uncertainties, and other important factors, any of which could cause the Company's actual results to differ from those contained in the forward-looking statements, see the risks and uncertainties detailed in the Company's periodic filings with the Securities and Exchange Commission, including but not limited to the Company's most recently filed periodic report, and from time to time in the Company's press releases and other investor communications. Fate Therapeutics is providing the information in this release as of this date and does not undertake any obligation to update any forward-looking statements contained in this release as a result of new information, future events or otherwise.

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EXHIBIT H

Intelligent Cells: Shoreline Builds Immunotherapy Platform, Pipeline with $140M Financing

Posted on November 8, 2021 | In the News

GENEDGE

CEO Kleanthis Xanthopoulos touts the San Diego biotech's engineering prowess in developing iPSC-derived NK cells and macrophages against cancer

By Alex Philippidis
November 8, 2021

Shoreline Biosciences has developed induced pluripotent stem cell (iPSC)-derived natural killer (NK) cells and macrophages that are optimized by applying gene editing to target specific genes with properties sought by the company. San Diego-based Shoreline says its "intelligently engineered" NK cells can target and kill tumors more effectively and efficiently.

Shoreline Biosciences has nearly doubled its total capital by completing a $140-million financing that will help it further build its platform and pipeline, focused on developing natural killer (NK) and macrophage cellular immunotherapies derived from induced pluripotent stem cells (iPSCs).

The financing also gives Shoreline capital toward creating potent and persistent NK cell-specific Chimeric Antigen Receptors (CARs) as well as switchable CAR-NK cell engagers and macrophage-specific CARs to treat blood cancers, solid tumors, and other disorders.

Shoreline has developed iPSC-derived NK cells and macrophages that are optimized by applying gene editing to target specific genes with properties sought by the company. Shoreline says its "intelligently engineered" NK cells can target and kill tumors more effectively and efficiently.

"It is natural to think that we can take advantage of the biology of natural killer cells, and if we can make them more persistent, arm them with specific target missiles that we call chimeric antigen receptors and direct them towards the site of the tumor cell, it will have an effect," Kleanthis G. Xanthopoulos, PhD, Shoreline's chairman and CEO, told *GEN Edge*.

Shoreline reasons it can produce NK cells faster, less expensively, and with potential for re-dosing compared with CAR T cell therapies. Despite their effectiveness fighting blood tumors, CAR-T therapies have shown significant side effects, notably cytokine release syndrome and graft versus host disease. And while T cells require activation before they can be sicced on cancer cells, being part of the adaptive immune system, NK cells do not since they are within the innate immune system.

"We found out that you can engineer the cells to be a better athlete, metabolically better fitted. They can persist longer. They can withstand the tumor micro-environment, which is a hostile environment typically for any cell therapy approaches," he added. "Armed with these additional properties, we're hoping that we can have a huge impact on the tumor cells."

In NK cells, for example, Shoreline has removed the cytokine-inducible SH2 (CIS) protein encoded by the CISH protein coding gene and known to block the interleukin (IL)-2 and IL15 signaling that is critically important for activating cell proliferation.

"If we can intelligently remove that CIS protein, we see an NK cell that responds better to cytokines and at much lower concentrations, is metabolically more active, is a metabolically better fitted cell. It kills better because it produces many more different cytokines and it also withstands the tumor micro-environment better," Xanthopoulos explained. "That's the whole idea of intelligent-designed allogeneic cells."

Looking beyond cancer

Shoreline has chosen to focus on cancer given the huge unmet medical need and the innate ability of NK cells to fight tumors. Long-term, however, the company envisions fighting cancer as well as chronic and infectious diseases through cell therapies based on macrophages.

"We know that there are chronic diseases that eventually can be addressed with macrophage cell therapies that have the innate ability to repair damaged tissue, and you can think of a number of diseases down the road that potentially macrophages can have the ability to do that," Xanthopoulos said.

"What stands between our goals and moving non-oncology clinical programs forward is the need to understand better the so-called immunodepletion and conditioning of these patients, which we're doing in the oncology setting, but you can't necessarily consider doing that for chronic disease," he added. "We can address that in in the future but the immediate focus is on oncology."

Could Shoreline someday create cell therapies that combine NKs and macrophages?

"The short answer is yes, they indeed can complement each other," Xanthopoulos replied. "Down the road we can potentially consider combining those two, but obviously we need to study them in the clinic individually and understand how they behave before we take that step."

Shoreline's core cell therapy platform is already designed to produce a homogenous population of optimized, fully functional immune cells. By cloning a select master iPSC-derived NK cell with its new optimized genetic signature, the company said, it can amplify that superior NK cell to create an unlimited number of homogenous quantities of the living cell therapy. The cells are then cryopreserved and stored, ready to treat any number of patients within hours.

"The clonal expansion capabilities of pluripotent stem cells give us the ability to do all the engineering we want at the pluripotent stem cell level, then select the clones or the clone that has the phenotype and the genotype that we are interested in," Xanthopoulos said.

"From that clone, we can engineer trillions of cells. The process is relatively straightforward. It's a clonal expansion of the clone that we have identified, and it gives us the ability to then create from a single run currently up to 500 doses, and it the yield will certainly increase as we optimize and we move into what we call smart manufacturing, which we plan to bring online in a couple of years from now."

To satisfy its initial manufacturing need to get its candidates into the clinic for Phase I studies, Shoreline has partnered with UC San Diego's Advanced Cell Therapy Laboratory (ACTL). The company says its partnership with ACTL allows it to bring in-house GMP grade iPSCs to bank and rapidly initiate preclinical development and IND-enabling studies. [Shoreline Biosciences]To satisfy its initial manufacturing need to get its candidates into the clinic for Phase I studies, Shoreline has partnered with UC San Diego's (UCSD) Advanced Cell Therapy Laboratory (ACTL). The company says its partnership with ACTL allows it to bring in-house GMP grade iPSCs to bank and rapidly initiate preclinical development and IND-enabling studies.

$300M in capitalization

Shoreline said the financing, announced Tuesday, brings its total capitalization to more than $300 million. Roughly half of that capital consists of upfront cash that Shoreline received when it signed a pair of cell therapy collaborations in June with big-name partners Kite, a Gilead Company, and BeiGene—collaborations that could generate more than 10 times Shoreline's current capitalization.

Kite is also partnering with oNKo-innate to develop NK cells. Numerous other companies have expanded into NK cell drug development; Artiva Biotherapeutics, for example, is building a pipeline of NK cell products that include antibody-dependent cellular cytotoxicity (ADCC) enhancers and targeted NK cells engineered to express proprietary CARs. Artiva, Catamaran Bio, Dragonfly Therapeutics, Fate Therapeutics, Kiadis, and Nkarta are among other companies focused on NK cell development.

"These transformative corporate partnerships with Kite and BeiGene have tripled the size of our aspirations and clinical trials," and thus the number of treatment candidates in Shoreline's pipeline, Xanthopoulos said. "As such, we are expanding our capabilities in manufacturing." Shoreline is already building a "smart" manufacturing facility in San Diego designed to enable full automation. Xanthopoulos said the facility will enable the company to keep costs low. "We will announce in the near future some additional collaborations to secure additional manufacturing capabilities," Xanthopoulos added.

Kite and Shoreline are partnering to develop novel allogeneic candidates for several blood cancers. The companies' collaboration is initially focusing on CAR NK targets, with Kite having an option to expand the collaboration to include an iPSC CAR macrophage program for an undisclosed target.

"We already have constructs that we received from Kite. They're now engineered in the backbone of our NK cells that have the CISH knockout. And we're putting them together, and advancing with the aim to be in the clinic with the KITE collaborations programs in the next couple of years," Xanthopoulos said.

Kite—which participated in Shoreline's $43-million Series A round completed last April—selected Shoreline as its strategic partner for a strategic expansion into allogeneic iPSC therapies based around NK cells, in due to the expertise of the laboratory of Dan S. Kaufman, MD, PhD, a UCSD investigator and Shoreline co-founder, who serves as the company's Chief Scientific Officer.

Last year, Kaufman and Xanthopoulos joined 30-year biotech veteran Steven Holtzman, the former CEO of Decibel Therapeutics, and William Sandborn, MD, in co-founding Shoreline to commercialize research and tech developed in the labs of Kaufman and Sandborn, who is Director of the Inflammatory Bowel Disease Center at UCSD and Shoreline's Chief Medical Officer. Shoreline is also developing and commercializing additional technologies from Scripps Research's Calibr division.

NK Cell Partnership

With BeiGene, Shoreline agreed to develop and commercialize NK-based cell therapeutics by combining its research and clinical development capabilities with Shoreline's iPSC NK cell technology. BeiGene will retain worldwide development and commercialization rights for up to four targets—two solid tumors two blood tumors—with Shoreline holding an option to retain exclusive U.S. and Canadian rights for two of those four, as well as royalties.

BeiGene paid $45 million upfront to launch their collaboration, which could generate more than $1.3 billion for Shoreline in additional R&D funding, milestone payments, plus royalties. Shoreline is overseeing clinical manufacturing, while clinical development will be led by BeiGene globally.

"What we show in partnering with BeiGene is their amazing global clinical development and protein engineering capabilities as we plan to potentially combine our cells with antibodies, or so-called engagers that further enhance the activity of our NK cells," Xanthopoulos said. "Those are core strategic capabilities—not just from the financial and validation perspective, but importantly from the clinical focus perspective—therefore amplifying Shoreline's capabilities."

BeiGene received an option to acquire an equity stake in a subsequent Shoreline financing—an option BeiGene exercised when it joined Kite as an investor in the $140-million financing. The round was led by Aly Bridge Group—whose founder, CEO and CIO Frank Yu joined Shoreline's board in connection with the financing.

Yu stated that Ally Bridge Group, which focuses its investments on what it deems best-in-class cell therapy companies from oncology to autoimmune diseases, "expects Shoreline to be a new category leader."

Other new investors in the $140-million round included Eventide Asset Management, Irving Investors, Kingdon, NS Investment, Piper Heartland Healthcare Capital, and Superstring. They were joined by previous investors including Boxer Capital, BVF Partners, Commodore Capital, Cormorant Asset Management, Janus Henderson Investors, Stork Capital, and Wedbush Healthcare Partners.

The latest financing will help Shoreline fund an ongoing expansion: The company plans to double its workforce, now close to 50 full-time equivalents, and anticipates moving in the second quarter of 2022 into a new 60,000-square-foot headquarters in San Diego.

Shoreline has mostly completed its executive suite with the appointment of Scott Forrest, PhD as Chief Business Officer; he was previously CFO of Autobahn Therapeutics. Further expansion will focus on R&D, clinical operations, and Chemistry, Manufacturing and Controls (CMC).

Recommended Posts

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Harnessing the body's natural killers to target cancer

California Life Sciences awarded Kite Pharma, a Gilead company, and Shoreline Biosciences the 2021 Strategic Partnership of the Year Award

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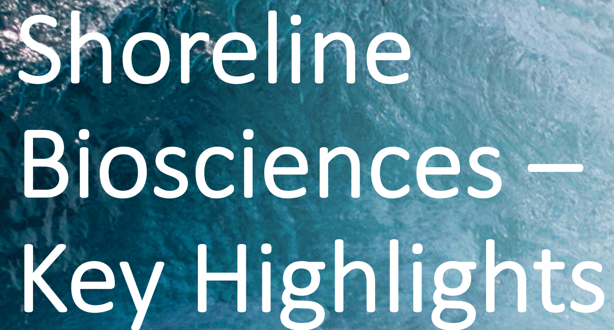
EXHIBIT I

Intelligently Engineered iPSC Derived
Allogeneic Cell–based Therapies

August 2020



Shoreline Biosciences – Key Highlights

- Deep and unique iPSC know how
- Differentiated iPSC Allogeneic CISH $^{-/-}$ NK-Cell Technology
    - Targeted
    - Standardized
    - Off-the-shelf
- Superior Preclinical Data in AML
- Differentiated IP
- iPSC derived CAR Macrophages with novel CAR signaling constructs
- GMP Manufacturing Capabilities Secured
- Two Programs in Clinic within 2.5 Years
- Experienced leadership team, well known Investigators & rapid access to clinical trial sites

Shoreline is built by a very experienced team

Kleanthis G. Xanthopoulos, Ph.D.
Founder and Executive Chairman

- Founding CEO, Anadys (2004 IPO), Regulus (2012 IPO), IRRAS AB (2017 IPO)
- VP Aurora Biosciences
- Chairman, Stork Capital Life Sciences

Dan Kaufman, M.D., Ph.D.
Scientific Founder

- Professor of Regenerative Medicine, UCSD
- Runs Advanced Cell Therapy Lab – GMP cell therapy facility
- Renowned leader in iPSC engineering

Steven Holtzman
Founder and Director

- Founding CEO, Decibel, Infinity
- EVP, Biogen
- CBO, Millennium
- Co-founder DNX Corp

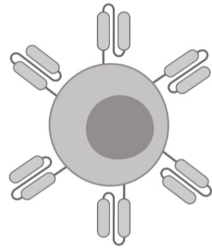
William Sandborn, M.D.
Scientific Founder

- Professor of Medicine, Chief, Gastroenterology, UCSD
- 2019 Sherman Prize awardee
- Renowned leader inflammatory diseases and clinical development
- Santarus, Receptos

Multiple cell therapy modalities are bringing promising treatments to patients

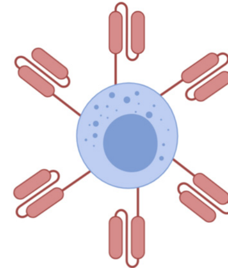
Shoreline Biosciences

Engineered CAR-T cells



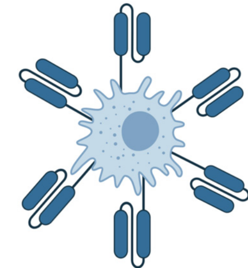
- CAR-T therapies; first major advance in cell therapy since BMT
- Majority autologous resulting in manufacturing & COGS issues
- Toxicities include CRS, GvHD, neuro

iPSC-derived CAR-NK cells



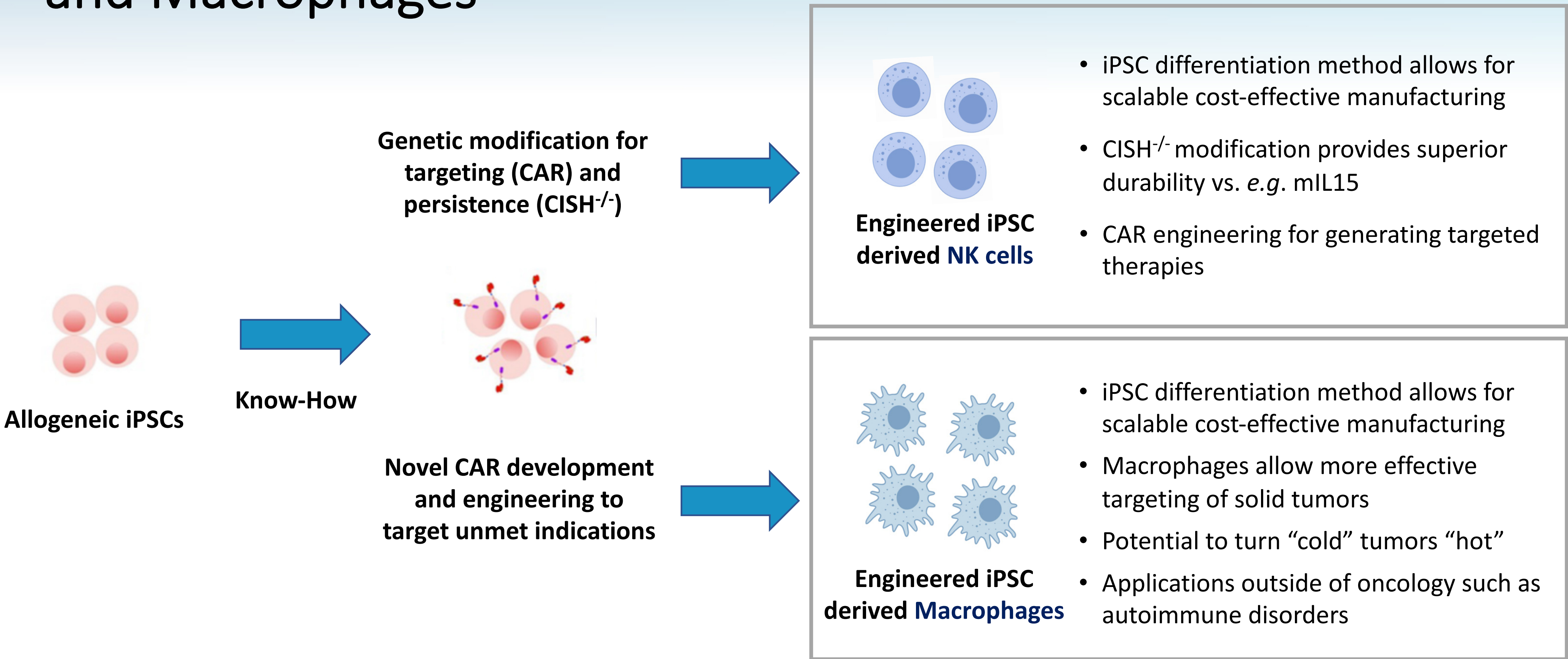
- First generation approaches showing promising efficacy
- Potential for repeat dose due to low toxicities
- Fewer players, room to differentiate based on iPSC

iPSC-derived CAR-Macrophages



- Macrophages bring unique functionality including targeting solid tumors and non-oncology indications
- Very few players engineering these cell types; even fewer with scalable manufacturing such as Shoreline

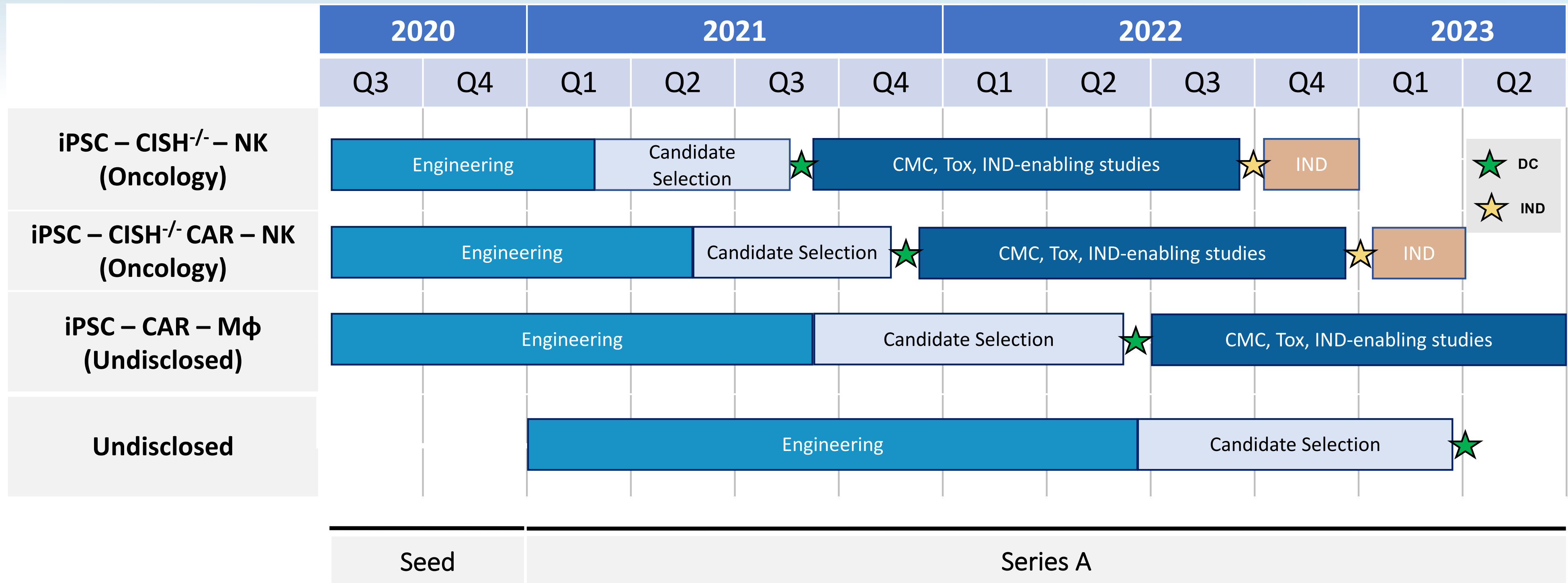
Shoreline is developing iPSC derived CAR-NK cells and Macrophages



- iPSC differentiation method allows for scalable cost-effective manufacturing
- CISH$^{-/-}$ modification provides superior durability vs. *e.g.* mIL15
- CAR engineering for generating targeted therapies

- iPSC differentiation method allows for scalable cost-effective manufacturing
- Macrophages allow more effective targeting of solid tumors
- Potential to turn “cold” tumors “hot”
- Applications outside of oncology such as autoimmune disorders

Pipeline



- IND for two lead programs across NK and Macrophage modalities. Possibility for RMAT Designation
- Series A funds meaningful clinical inflection points

Shoreline's iPSC-NK Cell Platform

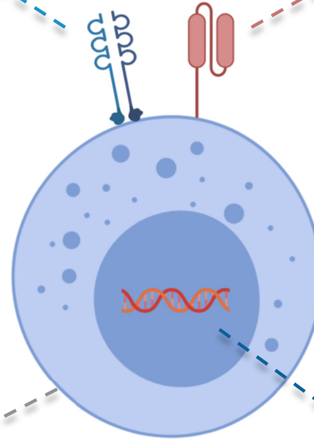
Major Pillars of Development

Signaling Domains for Improved Activation

Development of NK cell-specific signaling domains for improved cell function

NK cell Specific CAR Development

NK-cell specific CARs optimized for tumor targeting and cell activation



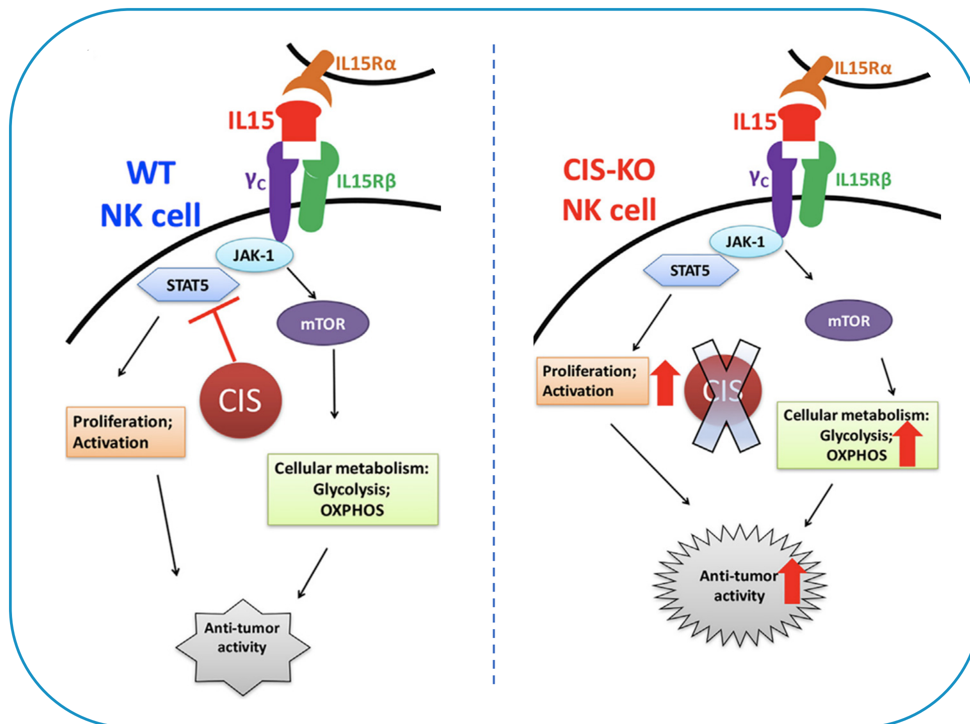
Off-the-Shelf Production

Proprietary Know-How to develop iPSC-derived NK cells for universal application

CISH$^{-/-}$ Engineered NK Cells

Intellectual Property to enhance NK cell persistence via improved ImmunoMetabolism

Deletion of IL-15 negative regulator improves Persistence and ImmunoMetabolism, culminating in enhanced Anti-tumor Activity



- **Cytokine-inducible SH2-containing protein** (CIS; encoded by the gene CISH) is an intracellular protein that acts as a key signaling checkpoint and is negative regulator of interleukin-15 (IL-15)
- IL-15 is an important stimulatory cytokine for differentiation, proliferation, activation, and survival of NK cells

Unique iPSC derived NK cell therapy platform

Cell Stem Cell

CellPress

Clinical and Translational Report

Metabolic Reprograming via Deletion of *CISH* in Human iPSC-Derived NK Cells Promotes *In Vivo* Persistence and Enhances Anti-tumor Activity

Huang Zhu,[1] Robert H. Blum,[1] Davide Bernareggi,[1] Eivind Heggernes Ask,[3] Zhengming Wu,[2] Hanna Julie Hoel,[3] Zhipeng Meng,[2] Chengsheng Wu,[2] Kun-Liang Guan,[2] Karl-Johan Malmberg,[3] and Dan S. Kaufman[1,4,*]
[1]Department of Medicine, Division of Regenerative Medicine, University of California, San Diego, La Jolla, CA, USA
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[3]Department of Cancer Immunology, Institute for Cancer Research, Oslo University Hospital, Oslo, Norway
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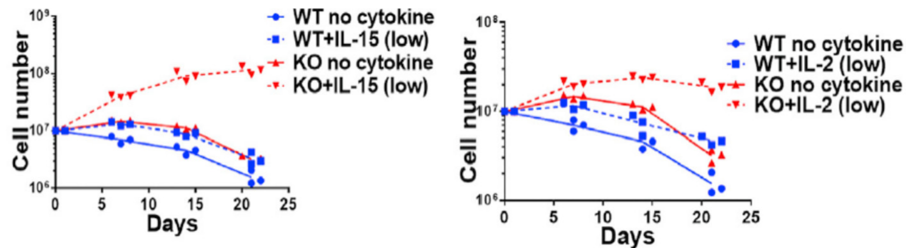
https://doi.org/10.1016/j.stem.2020.05.008

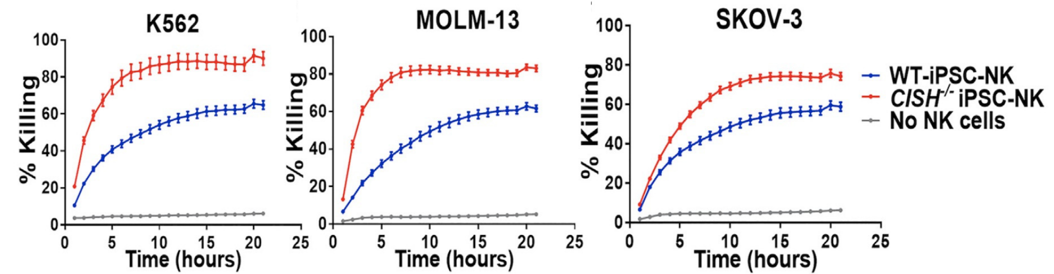
- **Deletion of CISH in human NK cells leads to improved antitumor activity and persistence**
- **CISH$^{-/-}$-NK cells demonstrate more efficient Glycolytic and OxPhos activity**
- **The improved metabolic profile is mediated by mTOR signaling**
- **CISH$^{-/-}$-NK cells more effectively treat AML in vivo with longer NK cell persistence**

Shoreline NK cells show superior expansion and function

iPSC-CISH$^{-/-}$-NK cells have better expansion and function *in vitro* compared with WT-iPSC-NK cells



*Cells grown in low IL-15 (1 ng/mL) or IL-2 (10 U/mL) for 3 weeks



*Cytotoxicity assay post-3-week culture in low IL-15 (1 ng/mL)

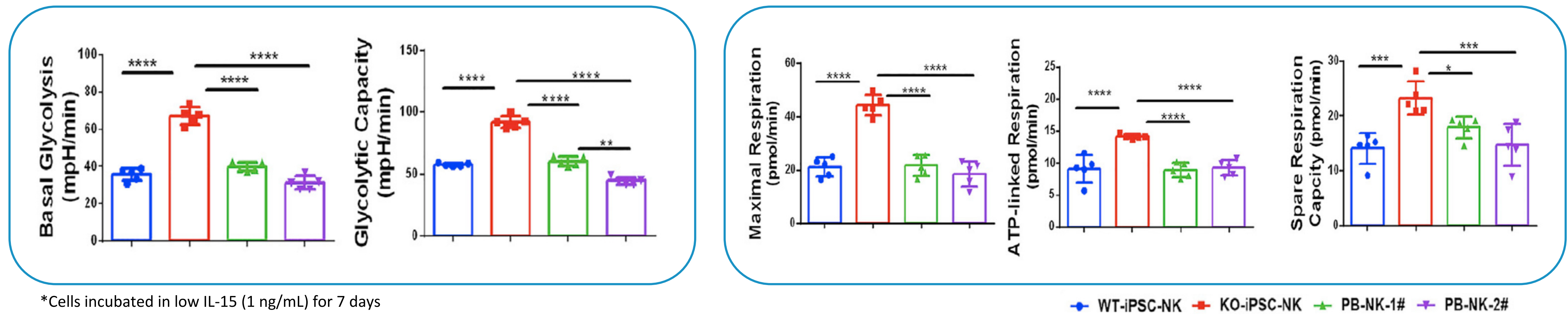
iPSC-CISH$^{-/-}$-NK cells exhibit improved expansion and increased cytotoxic activity against multiple tumor cell lines when maintained at low cytokine concentrations

Zhu, et al. *Cell Stem Cell* 2020

Exhibit I
Page 156

Shoreline NK cells display improved ImmunoMetabolism

iPSC-CISH$^{-/-}$-NK cells are metabolically reprogrammed, resulting in enhanced glycolysis and oxidative phosphorylation



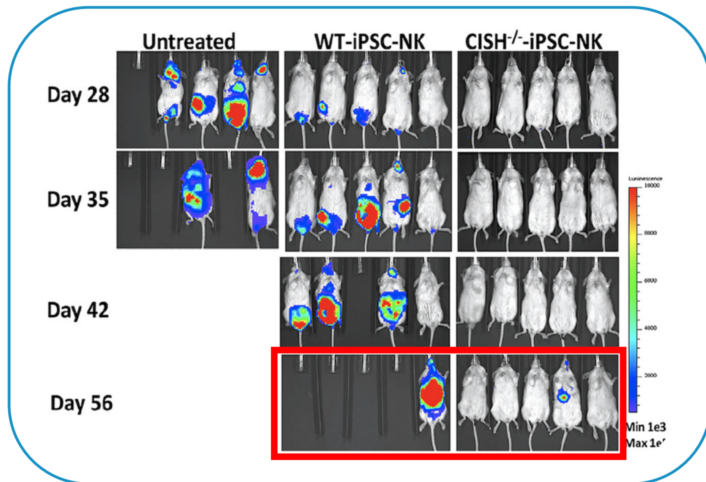
*Cells incubated in low IL-15 (1 ng/mL) for 7 days

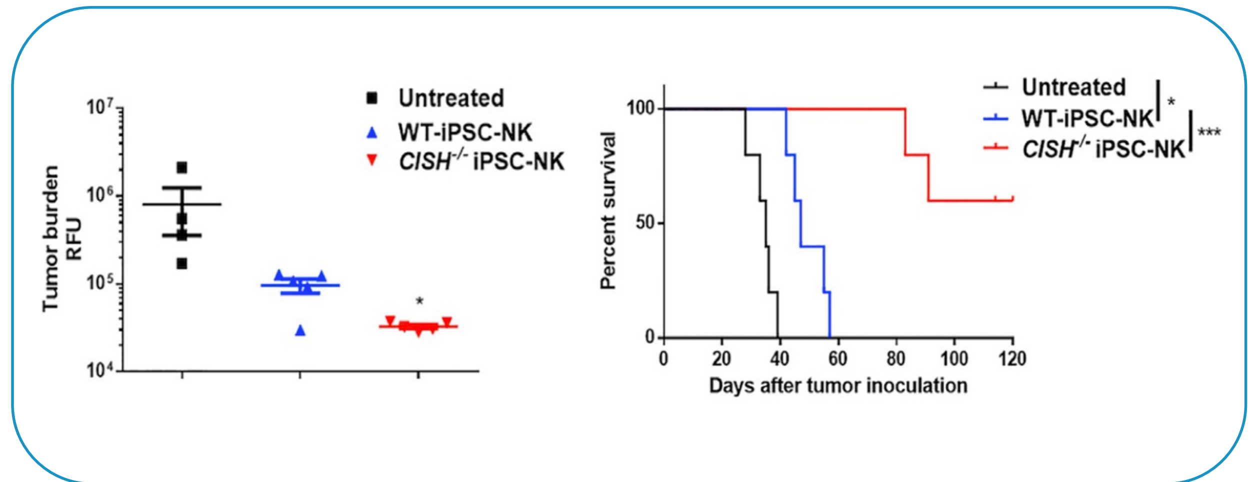
iPSC-CISH$^{-/-}$-NK cells demonstrate an improved metabolic fitness that mediates the enhanced persistence and anti-tumor activity seen *in vitro* and *in vivo*

Zhu, et al. *Cell Stem Cell* 2020

Shoreline NK cells show superior *in vivo* efficacy

CISH-/- iPSC-NK cells display significant anti-tumor activity in AML *in vivo* model compared to WT-iPSC-NK cells



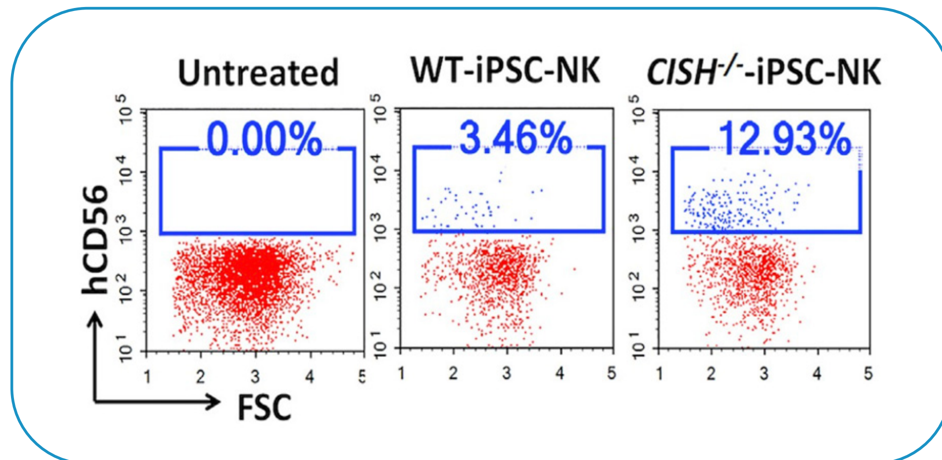


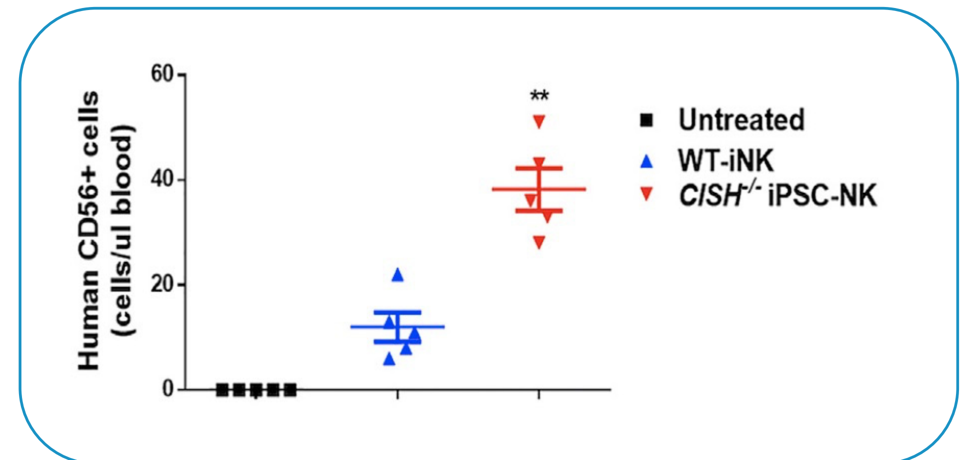
Three out of five mice treated with iPSC-CISH$^{-/-}$-NK cells had complete tumor clearance and long-term (>100 days) survival versus zero of five mice treated with WT iPSC-NK cells

Zhu, et al. *Cell Stem Cell* 2020

Shoreline NK cells show superior *in vivo* persistence

CISH-/- iPSC-NK cells display significantly improved *in vivo* persistence compared to WT-iPSC-NK cells





- Flow cytometry plot of human CD56+ cells in population from mouse peripheral blood 7 days after NK cell treatment
- Overall, studies demonstrate that iPSC-CISH$^{-/-}$-NK cells have improved anti-tumor activity and persistence *in vivo*

Zhu, et al. *Cell Stem Cell* 2020

Shoreline's iPSC-Macrophage Cell Platform

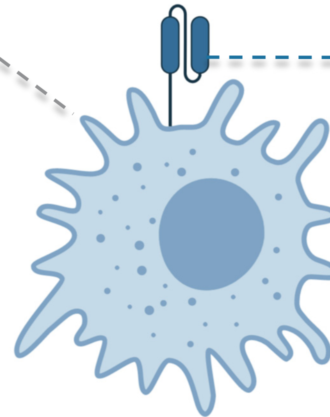
Major Pillars of Development

Off-the-Shelf Production

Development of First-in-Class iPSC derived CAR-Macrophages via proprietary technology

Intelligently Designed CARs

Macrophage-specific CARs for activity against solid tumors and non-oncology indications

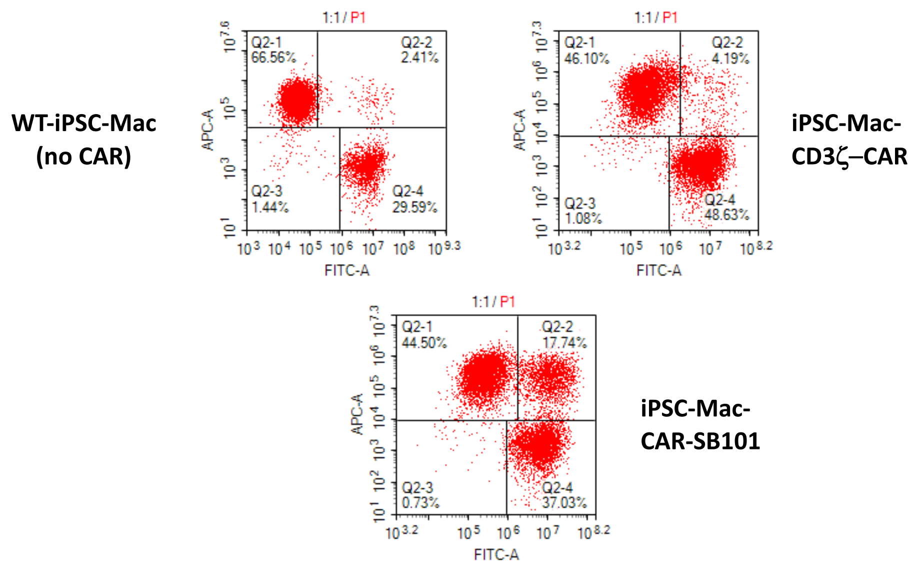


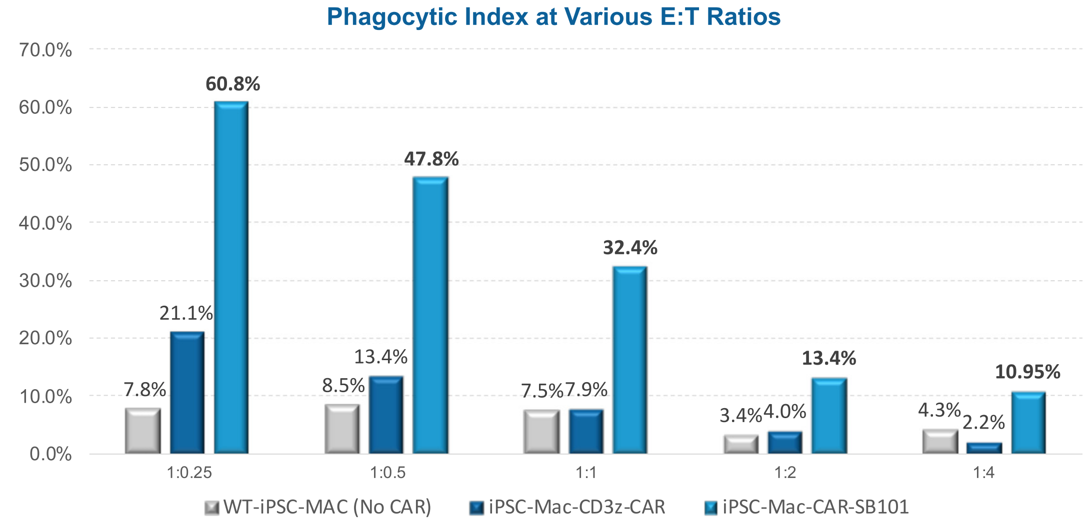
Optimized iPSC-Mϕ Activity and Persistence

Directed R&D effort to establish potent and persistent iPSC-Mϕ therapies

Novel iPSC-CAR-Macrophages display superior phagocytic activity compared to traditional CD3ζ–CAR

in vitro **Phagocytosis Assay: Test of novel iPSC-derived CAR-expressing Macrophages against A1847 Ovarian cancer cells**





Compared to traditional CD3ζ-CAR, **CAR-SB101** displays enhanced phagocytic activity against ovarian cancer cells

SHORELINE biosciences

Shoreline Biosciences Manufacturing Capabilities

Advance Cell Therapy Laboratory

- Located in La Jolla, CA
- Leads transition from R&D to compliant and clinically-relevant manufacture of cell therapy products for IND-enabling studies or Phase I/II trials
- Equipped with two manufacturing suites to enable product segregation and multi-lot production
- Nitrogen storage freezers suitable for long-term storage of cellular products in vapor-phase

- Established partnership with **UCSD's Advanced Cell Therapy Laboratory**, providing access to a state-of-the-art GMP and GLP facility to accelerate product development
- Able to bring in-house GMP grade iPSCs to bank and rapidly initiate preclinical development and IND-enabling studies
- Shoreline to leverage extensive network of CMC/Manufacturing professionals with 20+ years of experience for manufacturing and development plans and strategies for cell therapies

Shoreline's platform addresses many of the shortfalls in current treatment options

	DONOR-FREE MANUFACTURE	TARGETED	LOW RISK OF CRS, GVHD	APPLIED TO MACROPHAGES
NKarta (donor derived CAR-NKs)	✗	✓	✓	✗
Artiva (donor derived NKs)	✗	✗	✓	✗
FATE Tx (iPSC derived CAR-NKs)	✓	✓	✓	✗
Editas/BlueRock (iPSC derived NKs)	✓	✗	✓	✓
Allogene (iPSC derived CAR-Ts)	✓	✓	✗	✗
Takeda (iPSC derived CAR-Ts)	✓	✓	✗	✗
Century Tx (iPSC derived T and NKs)	✓	TBD	TBD	✗
Carisma (CAR-Macrophages)	✗	✓	TBD	✓
Shoreline Biosciences	✓	✓	✓	✓

Shoreline Biosciences Approach:

Intelligently engineered iPSC derived allogeneic cell therapies

- Pipeline of **proprietary technology and know-how** from founder and iPSC-cell therapy pioneer, Dan Kaufman MD, PhD
- Near-term focus on **proof of concept of CISH$^{-/-}$ technology** for improved CAR-NK persistence and anti-tumor activity
- Development of **first-in-class iPSC-derived CAR-Macrophages** for solid tumors and non-oncology indications
- Immediate access to **GMP Manufacturing facility** speeds process development and provides rapid entry into the clinic

Intelligently Engineered iPSC Derived
Allogeneic Cell–based Therapies

August 2020

Non-Confidential Corporate Presentation

EXHIBIT J

We are partnered with the Advanced Cell Therapy Laboratory (ACTL) of UC San Diego

ACTL is a well-recognized manufacturing facility.
Their state-of-the-art GMP and GLP systems and facilities allow for accelerated product development from research and development to compliant and clinically relevant manufacturing of cell therapy products.
ACTL is equipped with manufacturing suites to enable product segregation and multi-lot production and has nitrogen storage freezers suitable for long-term storage of cellular products in vapor-phase.
This partnership allows us to bring in-house GMP grade iPSCs to bank and rapidly initiate preclinical development and IND-enabling studies.
We also have an extensive network of CMC/Manufacturing professionals with over 20 years of experience that we leverage for manufacturing and development plans and strategies for cell therapies.

EXHIBIT K

Partnered with Advanced Cell Therapy Manufacturing

We are partnered with the Advanced Cell Therapy Laboratory (ACTL) of UC San Diego, a state-of-the-art GMP and GLP facilities that allows us to rapidly drive our innovation. We also leverage an extensive network of leading CMC professionals to guide our manufacturing agenda.