GENERATION OF HUMAN iPS CELLS BY A SYNTHETIC SELF-REPLICATIVE RNA

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. Provisional Application Serial Nos. 61/649,876, filed May 21, 2012 and 61/798,229, filed March 15, 2013, each of which are hereby incorporated by reference in their entirety.

TECHNICAL FIELD

[0002] Provided are methods and compositions useful for producing and propagating stem cells from fibroblasts. The disclosure relates to the production of induced pluripotent stem cells (iPS) and methods of use thereof.

BACKGROUND

[0003] Stems cells are a potential source from which organs may be regenerated, tissues may be repaired, biological factors prepared or delivered and disease or disorders treated.

SUMMARY

[0003a] In one aspect, there is provided a purified synthetic RNA replicon comprising: at least one non-structural replicase domain from an alphavirus and at least one non-alphavirus heterologous sequence encoding one or more factors for inducing the generation of pluripotent stem cells when expressed in a somatic cell.

[0003b] In another aspect, there is provided a composition comprising human cells transfected with a replicon as described herein.

[0003c] In yet another aspect, there is provided an in vitro method comprising: transfecting a human somatic cell with a purified synthetic ectopic self-replicating RNA replicon derived from an alphavirus, comprising polynucleotides encoding at least four de-differentiation factors selected from the group consisting of a (i) KLF4, (ii) OCT4, (iii) SOX2, (iv) c-MYC or n-MYC or L-MYC, (v) GLIS1 and (vi) NANOG, culturing the somatic cell to express the de-differentiation factor; selecting cells that display a stem cell morphology and/or stem cell markers; and subculturing the cells to obtain a population of induced stem cells.

[0003d] In yet another aspect, there is provided a vector system for producing human stem cells, comprising: at least one purified synthetic self-replicating RNA replicon derived from an alphavirus, comprising one or more polynucleotides encoding de-differentiation factors selected from the group consisting of a KLF4, OCT4, SOX2, c-MYC or n-MYC or L-MYC, GLIS1, NANOG or any combination thereof.

[0003e] In yet another aspect, there is provided an isolated human somatic cell comprising a purified synthetic ectopic RNA replicon derived from an alphavirus comprising one or more de-differentiation polynucleotide sequences.

[0003f] In yet another aspect, there is provided a cell population comprising the cells as described herein.

[0004] Generation of induced Pluripotent Stem (iPS) cells from patients is important to use stem cells therapeutically. Generation of iPS cells requires expression of several pluripotent transcription factors or Reprogramming Factors (RFs), including Oct4, Sox2, Klf4, cMyc, Glis1 (and potentially Nanog and Lin28). However, due to concerns with integration of DNA vectors (viruses and naked DNA) into the genome during iPS cell generation excludes these approaches from being subsequently used in patients.

[0005] The disclosure describes an approach to generate induced Pluripotent Stem (iPS) cells by ectopically expressing RFs using a synthetic self-replicating RNA from a modified alphavirus (*e.g.*, Venezuelan Equine Encephalitis (VEE) virus). The alphavirus was designed to express, in one example, four RFs that resulted in the following advantages over mRNA transfection approaches: 1) utilized a single RNA species capable of self-replicating for a limited number cell divisions, thereby reducing the number of transfections; 2) is capable of encoding at one, two, three, four, or more RF open reading frames (ORFs); and 3) consistently expressed all the RF genes at high threshold levels over multiple

cellular divisions. By using the self-replicating backbone of an alphavirus (the structural genes being removed) to express the RFs requires only 3 to 4 transfections (and even only 1 or 2) into primary human fibroblasts to generate iPS cells. The generation of the alphavirus RF-RNA transcript utilizes a SP6 (or T7) *in vitro* transcription kit that does not require special conditions and thereby, further simplifies the approach for broad use. By expressing the four RFs at consistent, high levels over time in the same cell combined with replication of the alphavirus-RF RNA for a limited number of multiple cell generations, the alphavirus-RF RNA approach solves both of the major inefficiency problems associated with attempting to generate iPS cells by daily repeated daily transfections for >14 days of four individual RF mRNAs. The alphavirus-RF RNA is an ectopic approach that does not utilize a DNA intermediate and therefore, there is no opportunity for integrative mutation that can occur with DNA vector-based iPS cell approaches. Moreover, the timing of RNA replicon loss by degradation can be regulated by B18R withdrawal from the media. Using this approach, >100 independent iPS cell clones were generated from both OCT4/KLF4/SOX2/c-MYC and OCT4/KLF4/SOX2/GLIS1 alphavirus-RF RNA protocols from two independent parental human fibroblast populations. In addition, the approach can be engineered to express alternative RF combinations and/or insertion of additional RF ORFs into the RF-RNA backbone for refining iPS cell generation from specific cell types or for use in driving transdifferentiation.

[0006] The disclosure provides an alphavirus replicon RNA comprising at least one non-structural replicase domain from an alphavirus and at least one non-alphavirus heterologous sequence encoding factors for inducing the generation of pluripotent stem cells when expressed in a somatic cell. In one example, the replicon comprises sequences obtained from an alphavirus selected from the group consisting of Eastern Equine Encephalitis virus (EEE), Venezuelan Equine Encephalitis virus (VEE), Everglades virus, Mucambo virus, Pixuna virus and Western Equine Encephalitis virus (WEE). In another example, the replicon comprises sequences obtained from an alphavirus selected from the group

consisting of Sindbis virus, Semliki Forest virus, Middelburg virus, Chikungunya virus, O'nyong-nyong virus, Ross River virus, Barmah Forest virus, Getah virus, Sagiyama virus, Bebaru virus, Mayaro virus, Una virus, Aura virus, Whataroa virus, Babanki virus, Kyzylagach virus, Highlands J virus, Fort Morgan virus, Ndumu virus and Buggy Creek virus. In yet another example, the at least one non-alphavirus heterologous sequence comprises at least 2, 3, 4 or 5 non-alphavirus heterologous sequences. In yet another example, of any of the foregoing the non-alphavirus heterologous sequence is selected from a polynucleotide encoding a KLF polypeptide, a SOX-2 polypeptide, a OCT-3/4 polypeptide, a c-MYC or n-MYC or L-MYC polypeptide, a GLIS1 polypeptide, a NANOG polypeptide and any combination thereof. In a further example, the polynucleotide encoding the KLF polypeptide encodes a KLF polypeptide having at least 95% identity to a sequence of SEQ ID NO:8. In another example, the polynucleotide encoding the KLF polypeptide encodes a KLF polypeptide having a sequence of SEQ ID NO:8. In yet another example, the polynucleotide encoding the KLF polypeptide comprises a sequence as set forth in SEQ ID NO:7, wherein "T" is "U". In another example, the polynucleotide encoding the SOX-2 polypeptide encodes a SOX-2 polypeptide having at least 95% identity to a sequence of SEQ ID NO:6. In another example, the polynucleotide encoding the SOX-2 polypeptide encodes a SOX-2 polypeptide having a sequence of SEQ ID NO:6. In yet another example, the polynucleotide encoding the Sox-2 polypeptide comprises a sequence as set forth in SEQ ID NO:5, wherein "T" is "U". In another example, the polynucleotide encoding the OCT-4 polypeptide encodes a OCT-4 polypeptide having at least 95% identity to a sequence of SEQ ID NO:4. In a further example, the polynucleotide encoding the OCT-4 polypeptide encodes a OCT-4 polypeptide having a sequence of SEQ ID NO:4. In a further example, the polynucleotide encoding the OCT-4 polypeptide comprises a sequence as set forth in SEQ ID NO:3, wherein "T" is "U". In another example, the polynucleotide encoding the c-MYC polypeptide encodes a c-MYC polypeptide having at least 95% identity to a sequence of SEQ ID NO:10. In a further example, the polynucleotide encoding the c-MYC polypeptide

encodes a c-MYC polypeptide having a sequence of SEQ ID NO:10. In yet a further example, the polynucleotide encoding the c-MYC polypeptide comprises a sequence as set forth in SEQ ID NO:9, wherein "T" is "U". In another example, the polynucleotide encoding the GLIS1 polypeptide encodes a GLIS1 polypeptide having at least 95% identity to a sequence of SEQ ID NO:34. In a further example, the polynucleotide encoding the GLIS1 polypeptide encodes a GLIS1 polypeptide having a sequence of SEQ ID NO:34. In yet a further example, the polynucleotide encoding the GLIS1 polypeptide comprises a sequence as set forth in SEQ ID NO:33, wherein "T" is "U". In another example, the polynucleotide encoding the NANOG polypeptide encodes a NANOG polypeptide having at least 95% identity to a sequence of SEQ ID NO:2. In a further example, the polynucleotide encoding the NANOG polypeptide encodes a NANOG polypeptide having a sequence of SEQ ID NO:2. In yet a further example, the polynucleotide encoding the NANOG polypeptide comprises a sequence as set forth in SEQ ID NO:1, wherein "T" is "U". In one embodimentexample of any of the foregoing, the replicon comprises from 5′ to 3′: (VEE RNA replicases)-(promoter)-(RF_1)-(self cleaving peptide)-(RF_2)-(self cleaving peptide)-(RF_3)-(IRES or core promoter)-(RF_4)-(IRES or optional promoter)-(optional selectable marker)-(VEE 3′UTR and polyA tail)-(optional selectable marker)-promoter; wherein RF_{1-4} are factors that induce de-differentiation of a somatic cell to a pluripotent cells, wherein RF_{2-3} are optional, RF_{3-4} are optional, or RF_4 is optional; wherein RF_{1-4} are selected from the group consisting of Oct-4, Klf4, Sox-2, c-Myc, Nanog, and Glis1. In another example, the replicon comprise a sequence that is 90%, 95%, 98%, 99% or 100% identical to SEQ ID NO:29, 30, 31, or 32 from about position 1 to about position 7561 wherein "T" of the sequence is substituted with "U", followed by one or more RFs, followed by a 3′UTR and polyA tail, wherein the one or more RFs are selected from the group consisting of Oct-3/4, Sox-2, Klf4, c-Myc, Nanog, and Glis1; wherein when more than one RF is present, the coding sequences may be separated by an internal ribosome entry site (IRES) or a small promoter. In a further example, the replicon comprise a sequence that is at least 95%,

98%, 99% or 100% identical to a sequence selected from the group consisting of SEQ ID NO:29, 30, 31, or 32, wherein "T" is "U".

[0007] The disclosure also provides a composition comprising human cells transformed with a replicon as described in any of the foregoing examples and examples further described herein. In one example, the composition further comprises B18R conditioned media. In another example, the human cells are somatic cells. In a further example, the human cells are fibroblasts.

[0008] The disclosure also provides a method of making stem cells comprising culturing the composition described above and elsewhere herein, for at least 30 days under conditions to express the coding sequences of the replicon and isolating stem cells.

[0009] The disclosure also provides a method of making stem cells comprising transforming somatic cells with a replicon of the disclosure, culturing the somatic cells under conditions to promote expression of the replicon and isolating stem cells. In one example, the culturing comprise culturing the cells in media conditioned with B18R. In another example, the B18R conditioned media is produced by transfection of B18R mRNA into primary human fibroblasts.

[0010] The disclosure also provides isolated stem cells obtained from the methods described herein, wherein the stem cells are retroviral DNA- or RNA-free.

[0011] The disclosure also provides a method comprising contacting a human somatic cell with an ectopic self-replicating RNA replicon comprising polynucleotides encoding at least four de-differentiation factors selected from the group consisting of a (i) KLF4, (ii) OCT4, (iii) SOX2, (iv) c-MYC or n-MYC or L-MYC, (v) GLIS1 and (vi) NANOG; culturing the somatic cell to express the de-differentiation factor; selecting cells that display a stem cell morphology and/or stem cell markers; and subculturing the cells to obtain a population of induced stem cells. In one embodiment, the cells are selected by detecting expression of a Tumor Rejection Antigen 1-60 and/or 1-81.

[0012] The disclosure also provides a vector system for producing human stem cells, comprising at least one self-replicating RNA replicon comprising one or more polynucleotides encoding de-

differentiation factors selected from the group consisting of a KLF4, OCT4, SOX2, c-MYC or n-MYC or L-MYC, GLIS1, and NANOG. In one embodiment, the replicon comprises (a)Oct4, Sox2, Klf4, and c-Myc, or (b)Oct4, Sox2, Klf4, and Glis1. In another example, the at least one self-replicating RNA vector is derived from an alphavirus. In a further example, the alphavirus is VEE.

[0013] The disclosure also provides an isolated human somatic cell comprising an ectopic RNA replicon comprising one or more de-differentiation polynucleotide sequences. In a further example, wherein upon culture conditions to express the de-differentiation polynucleotides in the ectopic RNA replicon, the somatic cell de-differentiates.

[0014] The disclosure also provides a cell population comprising the human somatic cell containing an ectopic RNA replicon comprising one or more de-differentiation polynucleotide sequences.

[0015] The disclosure also provides a cell population obtained by contacting a human somatic cell with an ectopic self-replicating RNA replicon comprising polynucleotides encoding at least four de-differentiation factors selected from the group consisting of a (i) KLF4, (ii) OCT4, (iii) SOX2, (iv) c-MYC or n-MYC or L-MYC, (v) GLIS1 and (vi) NANOG; culturing the somatic cell to express the de-differentiation factor; selecting cells that display a stem cell morphology and/or stem cell markers; and subculturing the cells to obtain a population of induced stem cells. In one example, the cells are selected by detecting expression of a Tumor Rejection Antigen 1-60 and/or 1-81.

[0016] The disclosure also provides a recombinant human fibroblast cells containing an ectopic RNA molecule encoding B18R. In one example, the RNA encoding B18R comprise SEQ ID NO:39, wherein "T" is replaced with "U". In another example, the RNA encodes a polypeptide comprising the sequence set forth in SEQ ID NO:40.

[0017] The disclosure also provides a method of making B18R conditioned media comprising culturing a human fibroblast cell transformed with RNA encoding B18R under conditions that

WHAT IS CLAIMED IS:

1. A purified synthetic RNA replicon comprising:

at least one non-structural replicase domain from an alphavirus and at least one non-alphavirus heterologous sequence encoding one or more factors for inducing the generation of pluripotent stem cells when expressed in a somatic cell.

2. The purified synthetic RNA replicon of claim 1, wherein the replicon comprises sequences obtained from an alphavirus selected from the group consisting of Eastern Equine Encephalitis virus (EEE), Venezuelan Equine Encephalitis virus (VEE), Everglades virus, Mucambo virus, Pixuna virus and Western Equine Encephalitis virus (WEE).

3. The purified synthetic RNA replicon of claim 1, wherein the replicon comprises sequences obtained from an alphavirus selected from the group consisting of Sindbis virus, Semliki Forest virus, Middelburg virus, Chikungunya virus, O'nyong-nyong virus, Ross River virus, Barmah Forest virus, Getah virus, Sagiyama virus, Bebaru virus, Mayaro virus, Una virus, Aura virus, Whataroa virus, Babanki virus, Kyzylagach virus, Highlands J virus, Fort Morgan virus, Ndumu virus and Buggy Creek virus.

4. The purified synthetic RNA replicon of claim 1, wherein the at least one non-alphavirus heterologous sequence comprises at least 2, 3, 4 or 5 non-alphavirus heterologous sequences.

5. The purified synthetic RNA replicon of any one of claims 1-4, wherein the non-alphavirus heterologous sequence is selected from a polynucleotide encoding a KLF polypeptide, a SOX-2 polypeptide, a OCT-3/4 polypeptide, a c-MYC or n-MYC or L-MYC polypeptide, a GLIS1 polypeptide, a NANOG polypeptide and any combination thereof.

6. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the KLF polypeptide encodes a KLF

polypeptide having at least 95% identity to a sequence of SEQ ID NO:8.

7. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the KLF polypeptide encodes a KLF polypeptide having a sequence of SEQ ID NO:8.

8. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the KLF polypeptide comprises a sequence as set forth in SEQ ID NO:7, wherein "T" is "U".

9. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the SOX-2 polypeptide encodes a SOX-2 polypeptide having at least 95% identity to a sequence of SEQ ID NO:6.

10. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the SOX-2 polypeptide encodes a SOX-2 polypeptide having a sequence of SEQ ID NO:6.

11. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the Sox-2 polypeptide comprises a sequence as set forth in SEQ ID NO:5, wherein "T" is "U".

12. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the OCT-4 polypeptide encodes a OCT-4 polypeptide having at least 95% identity to a sequence of SEQ ID NO:4.

13. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the OCT-4 polypeptide encodes a OCT-4 polypeptide having a sequence of SEQ ID NO:4.

14. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the OCT-4 polypeptide comprises a sequence as set forth in SEQ ID NO:3, wherein "T" is "U".

15. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the c-MYC polypeptide encodes a c-MYC polypeptide having at least 95% identity to a sequence of SEQ ID NO:10.

16. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the c-MYC polypeptide encodes a c-MYC polypeptide having a sequence of SEQ ID NO:10.

17. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the c-MYC polypeptide comprises a sequence as set forth in SEQ ID NO:9, wherein "T" is "U".

18. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the GLIS1 polypeptide encodes a GLIS1 polypeptide having at least 95% identity to a sequence of SEQ ID NO:34.

19. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the GLIS1 polypeptide encodes a GLIS1 polypeptide having a sequence of SEQ ID NO:34.

20. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the GLIS1 polypeptide comprises a sequence as set forth in SEQ ID NO:33, wherein "T" is "U".

21. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the NANOG polypeptide encodes a NANOG polypeptide having at least 95% identity to a sequence of SEQ ID NO:2.

22. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the NANOG polypeptide encodes a NANOG polypeptide having a sequence of SEQ ID NO:2.

23. The purified synthetic RNA replicon of claim 5, wherein the polynucleotide encoding the NANOG polypeptide comprises a sequence as set forth in SEQ ID NO:1, wherein "T" is "U".

24. The purified synthetic RNA replicon of claim 1, wherein the replicon comprises from 5' to 3': (VEE RNA replicases)-(promoter)-(RF_1)-(self cleaving peptide)-(RF_2)-(self cleaving peptide)-(RF_3)-(IRES or core promoter)-(RF_4)-(IRES or optional promoter)-(optional selectable marker)-(VEE 3'UTR and polyA tail)-(optional selectable marker)-promoter; wherein RF_{1-4} are factors that induce de-differentiation of a somatic cell to a pluripotent cells, wherein RF_{2-3} are optional, RF_{3-4} are optional, or RF_4 is optional; wherein RF_{1-4} are selected from the group consisting of Oct-4, Klf4, Sox-2, c-Myc, Nanog, and Glis1.

25. The purified synthetic RNA replicon of claim 1 or 24, wherein the replicon comprise a sequence that is 90%, 95%, 98%, 99% or 100% identical to SEQ ID NO:29, 30, 31, or 32 from about position 1 to about position 7561 wherein "T" of the sequence is substituted with "U", followed by one or more RFs, followed by a 3'UTR and polyA tail, wherein the one or more RFs are selected from the group consisting of Oct-3/4, Sox-2, Klf4, c-Myc, Nanog, and Glis1; wherein when more than one RF is present, the coding sequences may be separated by an internal ribosome entry site (IRES) or a small promoter.

26. The purified synthetic RNA replicon of claim 25, wherein the replicon comprises a sequence that is at least 95%, 98%, 99% or 100% identical to a sequence selected from the group consisting of SEQ ID NO:29, 30, 31, or 32, wherein "T" is "U".

27. A composition comprising human cells transfected with a replicon of any one of claims 1-26.

28. The composition of claim 27, further comprising B18R conditioned media.

29. The composition of claim 27, wherein the human cells are somatic cells.

30. The composition of claim 29, wherein the human cells are fibroblasts.

31. An *in vitro* method comprising:

transfecting a human somatic cell with a purified synthetic ectopic self-replicating RNA replicon derived from an alphavirus, comprising polynucleotides encoding at least four de-differentiation factors selected from the group consisting of a (i) KLF4, (ii) OCT4, (iii) SOX2, (iv) c-MYC or n-MYC or L-MYC, (v) GLIS1 and (vi) NANOG,

culturing the somatic cell to express the de-differentiation factor;

selecting cells that display a stem cell morphology and/or stem cell markers; and

subculturing the cells to obtain a population of induced stem cells.

32. The method of claim 31, wherein the cells are selected by detecting expression of a Tumor Rejection Antigen 1-60 and/or 1-81.

33. A vector system for producing human stem cells, comprising:

at least one purified synthetic self-replicating RNA replicon derived from an alphavirus, comprising one or more polynucleotides encoding de-differentiation factors selected from the group consisting of a KLF4, OCT4, SOX2, c-MYC or n-MYC or L-MYC, GLIS1, NANOG or any combination thereof.

34. The vector system of claim 33, wherein the replicon comprises:

(a)Oct4, Sox2, Klf4, and c-Myc, or

(b)Oct4, Sox2, Klf4, and Glis1.

35. The vector system of claim 33, wherein the one self-replicating RNA vector is derived from an alphavirus.

36. The vector system of claim 35, wherein the alphavirus is VEE.

37. An isolated human somatic cell comprising a purified synthetic ectopic RNA replicon derived from an alphavirus comprising one or more de-differentiation polynucleotide sequences.

38. The human somatic cell of claim 37, wherein the somatic cell de-differentiates when cultured under conditions to express the de-differentiation polynucleotides in the ectopic RNA replicon.

39. A cell population comprising the cells of claim 38.

40. A cell population of claims 39 or method of claims 40 or claim 31, wherein the cells or method comprise culturing the cells in a medium conditioned with B18R so that the degradation of the ectopic RNA replicon is inhibited.

41. The cell population or method of claim 40, wherein the B18R conditioned media is produced by transfection of B18R mRNA into primary human fibroblasts.