GENETIC MODIFICATION OF RATS

FIELD

Non-human pluripotent, totipotent, and embryonic stem (ES) cells, in particular rat pluripotent, totipotent, and/or rat ES cells, and methods of making them. Methods for making rat pluripotent, totipotent, and ES cells are provided. Methods for targeting rat pluripotent, totipotent, and/or ES cells are provided. Methods for achieving germline transmission of a genetic modification in a rat cell are provided. Media for deriving, growing, and maintaining rat pluripotent, totipotent, and ES cells are provided.

REFERENCE TO A SEQUENCE LISTING SUBMITTED AS A TEXT FILE VIA EFS-WEB

The official copy of the sequence listing is submitted concurrently with the specification as a text file via EFS-Web, in compliance with the American Standard Code for Information Interchange (ASCII), with a file name of 441914seqlist.txt, a creation date of February 20, 2014 and a size of 2 Kb. The sequence listing filed via EFS-Web is part of the specification and is hereby incorporated in its entirety by reference herein.

BACKGROUND

The rat has been a valuable model for many applications, including, but not limited to, applications in drug discovery. The usefulness of the rat has been mitigated somewhat by difficulty in obtaining genetically modified rats, in particular, in developing methods for genetically modifying rats, and generating useful rat cells that can be used in genetic modification protocols, including but not limited to protocols that result in germline transmission of a genetic modification in a rat genome.

There is a need in the art for rat cells (e.g., embryonic stem cells) that can be genetically modified such that the genetic modification can be transmitted through the germline. There is a need in the art for improved frequency of germline transmission of genetic modifications in rats.

There is a need in the art for donor rat pluripotent, totipotent, and/or ES cells from various strains of rat that are capable of generating F0, or wholly donor cell-

derived, F0 rats. There is a need in the art for donor rat pluripotent, totipotent, and/or ES cells that are capable of generating rats that comprise a germline genetic modification.

SUMMARY

Compositions and methods are provided for making rat pluripotent and/or totipotent cells, including rat embryonic stem (ES) cells. Compositions and methods for improving efficiency or frequency of germline transmission of genetic modifications in rats are provided. In various aspects, the methods and compositions comprise an *in vitro* culture comprising a feeder cell layer and a population of rat ES cells or a rat ES cell line, wherein the *in vitro* culture conditions allow maintenance of pluripotency of the rat ES cell. Various methods of establishing rat ES cell lines are further provided. Method of selecting genetically modified rat ES cells are also provided, along with various methods to generate a transgenic rat from the genetically modified rat ES cells are provided herein. Various kits and articles of manufacture are further provided.

Non-limiting embodiments are as follows:

1. An isolated rat ES cell of a strain selected from ACI or DA, wherein the isolated rat ES cell is and capable of transmitting its genome through the germline.

2. The isolated rat ES cell of embodiment 1, wherein the cell is derived from an ACI rat.

3. The isolated rat ES cell of embodiment 1 or 2, wherein the cell is derived from a Dark Agouti (DA) rat.2.

4. The isolated rat ES cell of embodiment 1, 2, or 3, wherein the cell is euploid and capable of transmitting a targeted genetic modification through the germline.

5. The isolated rat ES cell of embodiment 4, wherein the rat ES cell comprises a germline transmission efficiency of the targeted genetic modification of at least 3%.

6. The isolated rat ES cell of embodiment 4, wherein the rat ES cell has a germline transmission efficiency of the targeted genetic modification of at least 60%.

7. The isolated rat ES cell of any one of embodiments 1-6, wherein the rat ES cell exhibits a targeting efficiency of homologous recombination of at least 2%.

8. The isolated rat ES cell of any one of embodiments 1-8, wherein the rat ES cell is capable of transmitting a targeted genetic modification into progeny following a successive round of electroporation.

9. The isolated rat ES cells of any one of embodiments 1-8, wherein the rat ES cell comprises one or more, two or more, or three or more targeted genetic modification.

10. The isolated rat ES cell of any one of embodiments 4-9, wherein the targeted genetic modification comprises an insertion, a deletion, a knockout, a knockin, a point mutation, or a combination thereof.

11. The isolated rat ES cell of embodiment 9, wherein the targeted genetic modification comprises at least one insertion of a heterologous polynucleotide into a genome of the cell.

12. The isolated rat ES cell of embodiment 11, wherein the heterologous polynucleotide comprises a selection marker.

13. The isolated rat ES cell of embodiment 12, wherein (a) the selection marker comprises a non-attenuated selection marker gene operably linked to a promoter; or (b) the rat ES cell comprises at least 2 copies of the polynucleotide encoding the selection marker.

14. The isolated rat ES cell of embodiment 12, wherein the selection marker has an increased activity compared to a wild type selection marker.

15. The isolated rat ES cell of any one of embodiments 1-14, wherein the rat ES cell forms a sphere-like colony when plated on a feeder cell layer in culture comprising a LIF polypeptide, a GSK3 inhibitor, and a MEK inhibitor.

16. The isolated rat ES cell of any one of embodiments 1-15, wherein the rat ES cell, when cultured *in vitro*, loosely adhere to the feeder cell layer.

17. The isolated rat ES cell of any one of embodiments 1-16, wherein the cell does not require paracrine LIF signaling for maintenance of pluripotency.

18. The isolated rat ES cell of any one of embodiments 1-17, wherein the cell is a male (XY) rat ES cell.

19. The isolated rat ES cell of any one of embodiments 1-19, wherein the cell is a female (XX) rat ES cell.

20. The isolated rat ES cell of any one of embodiments 1-19, wherein the rat ES cell can be passaged up to at least 11 times in a medium comprising a GSK3 inhibitor and a MEK inhibitor without decreasing its targeting efficiency or germline transmission efficiency of a targeted genetic modification.

21. The isolated rat ES cell of any one of embodiments 1-20, wherein the rat ES cells express at least one pluripotency marker selected from Dnmt3L, Eras, Err-beta, Fbxo15, Fgf4, Gdf3, Klf4, Lef1, LIF receptor, Lin28, Nanog, Oct4, Sox15, Sox2, Utf1, or a combination thereof.

22. The isolated rat ES cell of any one of embodiments 1-21, wherein the rat ES cells do not express one or more pluripotency markers selected from c-Myc, Ecat1, Rexo1, or a combination thereof.

23. The isolated rat ES cell of any one of embodiments 1-22, wherein the rat ES cells do not express one or more mesodermal markers selected from Brachyury, Bmpr2, or a combination thereof.

24. The isolated rat ES cell of any one of embodiments 1-23, wherein the rat ES cells do not express one or more endodermal markers selected from Gata6, Sox17, Sox7, or combination thereof;

25. The isolated rat ES cell of any one of embodiments 1-24, wherein the rat ES cells do not express one or more neural markers selected from Nestin, Pax6, or combination thereof.

26. The isolated rat ES cell of any one of embodiments 1-25, wherein the cell expresses a pluripotency marker comprising Oct-4, Sox2, alkaline phosphatase, or a combination thereof.

27. The isolated rat ES cell of any one of embodiments 1-26, wherein the rat ES cell is characterized by the expression of one or more of a rat ESC-specific gene selected from one or more of Adheres Junctions Associate Protein (Ajap1), Claudin 5 (Cldn5), Cdc42 guanine nucleotide exchange factor 9 (Arhgef9), Calcium/calmodulin-dependent protein kinase IV (Camk4), ephrin-A1 (Efna1), EPH receptor A4 (Epha4), gap junction protein beta 5 (Gjb5), Insulin-like growth factor binding protein-like 1 (Igfbpl1), Interleukin 36 beta(Il1f8), Interleukin 28 receptor, alpha (Il28ra), left-right determination factor 1 (Lefty1), Leukemia inhibitory factor receptor alpha (Lifr), Lysophosphatidic acid receptor 2 (Lpar2), Neuronal pentraxin receptor (Ntm), Protein tyrosine phosphatase non-receptor type 18 (Ptpn18), Caudal type homeobox 2 (Cdx2), Fibronectin type III and ankyrin repeat domains 1 (Fank1), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Lymphoid enhancer-binding factor 1 (Lef1), Sal-like 3 (Drosophila) (Sall3), SATB homeobox 1 (Satb1), miR-632, or a combination thereof.

28. An isolated population of rat ES cells, wherein at least 70% of the rat ES cells are euploid and form sphere-like colonies when plated on a feeder cell layer *in vitro*.

29. The isolated population of rat ES cells of embodiment 28, wherein the rat ES cells are derived from an ACI rat.

30. The isolated population of rat ES cells of embodiment 28, wherein the rat ES cells are derived from a Dark Agouti (DA) rat.

31. The isolated population of rat ES cells of any one of embodiments 28-30, wherein the rat ES cells are capable of transmitting their genome through the germline.

32. The isolated population of rat ES cells of any one of embodiments 28-31, wherein the rat ES cells have a germline transmission efficiency of the targeted genetic modification of at least 3%.

33. The isolated population of rat ES cells of any one of embodiments 28-31, wherein the rat ES cells have a germline transmission efficiency of the targeted genetic modification of at least 60%.

34. The isolated population of rat ES cells of any one of embodiments 28-31, wherein the rat ES cells exhibit a targeting efficiency of homologous recombination of at least 2%.

35. The isolated population of rat ES cells of any one of embodiments 28-34, wherein the rat ES cells are capable of transmitting a targeted genetic modification into progeny following a successive round of electroporation.

36. The isolated population of any one embodiments 28-35, wherein the rat ES cells comprise one or more, two or more, or three or more targeted genetic modification and can transmit the targeted genetic modification through the germline.

37. The isolated population of rat ES cells of embodiment 36, wherein the targeted genetic modification is at the rat Rosa26 locus.

38. The isolated population of rat ES cells of embodiment 36, wherein the targeted genetic modification comprises an insertion, a deletion, a knockout, a knockin, a point mutation, or a combination thereof.

39. The isolated population of rat ES cells of embodiment 36, wherein the targeted genetic modification comprises at least one insertion of a heterologous polynucleotide into a genome of the cell.

40. The isolated population of rat ES cells of embodiment 39, wherein the heterologous polynucleotide comprises a selection marker.

41. The isolated population of rat ES cells of embodiment 40, wherein

(a) the selection marker comprises a non-attenuated selection marker gene operably linked to a promoter; or

(b) the rat ES cell comprises at least 2 copies of the polynucleotide encoding the selection marker.

42. The isolated population of rat ES cells of embodiment 40, wherein the selection marker has an increased activity compared to a wild type selection marker

43. The isolated population of rat ES cells of any one of embodiments 28-42, wherein the cells form a sphere-like colony when plated on a feeder cell layer in culture comprising a LIF polypeptide, a GSK3 inhibitor, and a MEK inhibitor.

44. The isolated population rat ES cells of any one of embodiments 28-43, wherein the cells, when cultured *in vitro*, loosely adhere to the feeder cell layer.

45. The isolated population of rat ES cells of any one of embodiments 28-44, wherein the cells do not require paracrine LIF signaling for maintenance of pluripotency.

46. The isolated population of rat ES cells of any one of embodiments 28-44, wherein the rat ES cells are a male (XY) rat ES cells.

47. The isolated population of rat ES cells of any one of embodiments 28-44, wherein the rat ES cells are female (XX) rat ES cells.

48. The isolated population of rat ES cells of any one of embodiments 28-47, wherein the rat ES cells can be passaged up to at least 11 times in a medium comprising a GSK3 inhibitor and a MEK inhibitor without decreasing its targeting efficiency or germline transmission efficiency of a targeted genetic modification.

49. The isolated population of rat ES cells of any one of embodiments 28-48, wherein the rat ES cells express at least one pluripotency marker selected from Dnmt3L, Eras, Err-beta, Fbxo15, Fgf4, Gdf3, Klf4, Lef1, LIF receptor, Lin28, Nanog, Oct4, Sox15, Sox2, Utf1, or a combination thereof.

50. The isolated population of rat ES cells of any one of embodiments 28-49, wherein the rat ES cells do not express one or more pluripotency markers selected from c-Myc, Ecat1, Rexo1, or a combination thereof.

51. The isolated population of rat ES cells of any one of embodiments 28-50, wherein the rat ES cells do not express one or more mesodermal markers selected from Brachyury, Bmpr2, or a combination thereof.

52. The isolated population of rat ES cells of any one of embodiments 28-51, wherein the rat ES cells do not express one or more endodermal markers selected from Gata6, Sox17, Sox7, or combination thereof;

53. The isolated population of rat ES cells of any one of embodiments 28-52, wherein the rat ES cells do not express one or more neural markers selected from Nestin, Pax6, or combination thereof.

54. The isolated population of rat ES cells of any one of embodiment 28-53, wherein the rat ES cells expresses a pluripotency marker comprising Oct-4, Sox2, alkaline phosphatase, or a combination thereof.

55. The isolated population of rat ES cells of any one of embodiments 28-54, wherein the rat ES cells are characterized by the expression of one or more of a rat ESC-specific gene selected from one or more of Adheres Junctions Associate Protein (Ajap1), Claudin 5 (Cldn5), Cdc42 guanine nucleotide exchange factor 9 (Arhgef9), Calcium/calmodulin-dependent protein kinase IV (Camk4), ephrin-A1 (Efna1), EPH receptor A4 (Epha4), gap junction protein beta 5 (Gjb5), Insulin-like growth factor binding protein-like 1 (Igfbpl1), Interleukin 36 beta(Il1f8), Interleukin 28 receptor, alpha (Il28ra), left-right determination factor 1 (Lefty1), Leukemia inhibitory factor receptor alpha (Lifr), Lysophosphatidic acid receptor 2 (Lpar2), Neuronal pentraxin receptor (Ntm), Protein tyrosine phosphatase non-receptor type 18 (Ptpn18), Caudal type homeobox 2 (Cdx2), Fibronectin type III and ankyrin repeat domains 1 (Fank1), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Lymphoid enhancer-binding factor 1 (Lef1), Sal-like 3 (Drosophila) (Sall3), SATB homeobox 1 (Satb1), miR-632, or a combination thereof.

56. The isolated population of rat ES cells of any one of embodiments 28-55 , wherein the population comprises at least 10^4 cells.

57. The isolated population of rat ES cells of any one of embodiments 28-56, wherein the rat ES cells have one or more characteristic comprising:

a. at least 90% of the rat ES cells are euploid;

b. at least 70% of the rat ES cells express at least one pluripotency marker; wherein the at least one pluripotency marker comprises Oct-4, Sox2, alkaline phosphatase, or a combination thereof;

c. a cell from the rat ES cell population, when combined with a rat host embryo transmits the genome of the rat ES cell line into an offspring;

d. the rat ES cells when cultured *in vitro* loosely adhere to a feeder cell layer;

e. the rat ES cells form sphere-like colonies when plated on a feeder cell layer *in vitro*; (f) the rat ES cells maintain pluripotency when cultured *in vitro* in a media comprising an GSK3 inhibitor, a MEK inhibitor, LIF and a feeder cell layer that is not genetically modified to express LIF;

f. the rat ES cell exhibits a targeting efficiency of homologous recombination of at least 2%;

g. the rat ES cells maintain pluripotency *in vitro* without requiring paracrine LIF signaling;

h. at least 70% of the rat ES cells are euploid and form sphere-like colonies when plated on a feeder cell layer *in vitro*;

i. the rat ES cells express at least one pluripotency marker selected from Dnmt3L, Eras, Err-beta, Fbxo15, Fgf4, Gdf3, Klf4, Lef1, LIF receptor, Lin28, Nanog, Oct4, Sox15, Sox2, Utf1, or a combination thereof;

j. the rat ES cells do not express one or more differentiation markers selected from c-Myc, Ecat1, Rexo1;

k. the rat ES cells do not express one or more mesodermal markers selected from Brachyury, Bmpr2, or a combination thereof;

l. the rat ES cells do not express one or more endodermal markers selected from Gata6, Sox17, Sox7, or combination thereof; and/or

m. the rat ES cells do not express one ore more neural markers selected from Nestin, Pax6, or combination thereof.

58. The isolated population of rat ES cells of any one of embodiments 28-57, wherein (a) the rat ES cells is derived from a rat blastocyst; (b) the rat ES cell is derived from a rat morula stage embryo; and/or, (c) the rat ES cell line is derived from a superovulated rat.

59. An *in vitro* culture comprising a feeder cell layer, the population of rat embryonic stem (ES) cells, and a medium comprising a Leukemia Inhibitory Factor (LIF), GSK3 inhibitor, and a MEK inhibitor, wherein at least 70% of the rat ES cells are euploid and the rat ES cell forms a sphere-like colony.

60. The *in vitro* culture of embodiment 59 or 60, wherein the rat ES cell, loosely adhere to the feeder cell layer.

61. The *in vitro* culture of embodiment 59, 60, or 61, wherein the rat ES cells are capable of transmitting their genome through the germline.

62. The *in vitro* culture of embodiment 59, 60 or 61, wherein the rat ES cells are derived from an ACI rat.

63. The *in vitro* culture of embodiment 59, 60 or 61, wherein the rat ES cells are derived from a Dark Agouti (DA) rat.2.

64. The *in vitro* culture of any one of embodiments 59-63, wherein the rat ES cells are capable of transmitting a targeted genetic modification through the germline.

65. The *in vitro* culture of embodiment 64, wherein the rat ES cells comprise a germline transmission efficiency of the targeted genetic modification of at least 3%.

66. The *in vitro* culture of embodiment 64, wherein the rat ES cells have a germline transmission efficiency of the targeted genetic modification of at least 60%.

67. The *in vitro* culture of any one of embodiments 59-66, wherein the rat ES cells exhibit a targeting efficiency of homologous recombination of at least 2%.

68. The *in vitro* culture of any one of embodiments 59-67, wherein the rat ES cell is capable of transmitting a targeted genetic modification into progeny following a successive round of electroporation.

69. The *in vitro* culture of any one of embodiments 59-68, wherein the rat ES cell comprises one or more, two or more, or three or more targeted genetic modification.

70. The *in vitro* culture of embodiment 69, wherein the targeted genetic modification comprises an insertion, a deletion, a knockout, a knockin, a point mutation, or a combination thereof.

71. The *in vitro* culture of embodiment 69, wherein the targeted genetic modification comprises at least one insertion of a heterologous polynucleotide into a genome of the cell.

72. The *in vitro* culture of embodiment 71, wherein the heterologous polynucleotide comprises a selection marker.

73. The *in vitro* culture of embodiment 72, wherein (a) the selection marker comprises a non-attenuated selection marker gene operably linked to a promoter; or (b) the rat ES cell comprises at least 2 copies of the polynucleotide encoding the selection marker.

74. The *in vitro* culture of embodiment 72, wherein the selection marker has an increased activity compared to a wild type selection marker.

75. The *in vitro* culture of any one of embodiments 59-74, wherein the cell does not require paracrine LIF signaling for maintenance of pluripotency.

76. The *in vitro* culture of embodiment of any one of embodiments 59-75, wherein the cell is a male (XY) rat ES cell.

77. The *in vitro* culture of embodiment of any one of embodiments 59-75, wherein the cell is a female (XX) rat ES cell.

78. The *in vitro* culture of embodiment of any one of embodiments 59-77, wherein the rat ES cell can be passaged up to at least 11 times in a medium comprising a GSK3 inhibitor and a MEK inhibitor without decreasing its targeting efficiency or germline transmission efficiency of a targeted genetic modification.

79. The *in vitro* culture of any one of embodiments 59-78, wherein the rat ES cells express at least one pluripotency marker selected from Dnmt3L, Eras, Err-beta, Fbxo15, Fgf4, Gdf3, Klf4, Lef1, LIF receptor, Lin28, Nanog, Oct4, Sox15, Sox2, Utf1, or a combination thereof.

80. The *in vitro* culture of any one of embodiments 59-79, wherein the rat ES cells do not express one or more pluripotency markers selected from c-Myc, Ecat1, Rexo1, or a combination thereof.

81. The *in vitro* culture of any one of embodiments 59-80, wherein the rat ES cells do not express one or more mesodermal markers selected from Brachyury, Bmpr2, or a combination thereof.

82. The *in vitro* culture of any one of embodiments 59-81, wherein the rat ES cells do not express one or more endodermal markers selected from Gata6, Sox17, Sox7, or combination thereof;

83. The *in vitro* culture of any one of embodiments 59-82, wherein the rat ES cells do not express one or more neural markers selected from Nestin, Pax6, or combination thereof.

84. The *in vitro* culture of any one of embodiments 59-83, wherein the cell expresses a pluripotency marker comprising Oct-4, Sox2, alkaline phosphatase, or a combination thereof.

85. The *in vitro* culture of any one of embodiments 59-84, wherein the rat ES cells are characterized by the expression of one or more of a rat ESC-specific gene selected from one or more of Adheres Junctions Associate Protein (Ajap1), Claudin 5 (Cldn5), Cdc42 guanine nucleotide exchange factor 9 (Arhgef9), Calcium/calmodulin-dependent protein kinase IV (Camk4), ephrin-A1 (Efna1), EPH receptor A4 (Epha4), gap junction protein beta 5 (Gjb5), Insulin-like growth factor binding protein-like 1 (Igfbpl1), Interleukin 36 beta(Il1f8), Interleukin 28 receptor, alpha (Il28ra), left-right

determination factor 1 (Lefty1), Leukemia inhibitory factor receptor alpha (Lifr), Lysophosphatidic acid receptor 2 (Lpar2), Neuronal pentraxin receptor (Ntm), Protein tyrosine phosphatase non-receptor type 18 (Ptpn18), Caudal type homeobox 2 (Cdx2), Fibronectin type III and ankyrin repeat domains 1 (Fank1), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Lymphoid enhancer-binding factor 1 (Lef1), Sal-like 3 (Drosophila) (Sall3), SATB homeobox 1 (Satb1), miR-632, or a combination thereof.

86. The *in vitro* culture of any one of embodiments 59-84, wherein the concentration of LIF is 50 U/ml to 150 U/ml.

87. The *in vitro* culture of any one of embodiments 59-85, wherein the concentration of LIF is 100 U/ml.

88. The *in vitro* culture of any one of embodiments 59-87, wherein the LIF is from mouse or comprises at least 92% sequence identity to SEQ ID NO: 1.

89. The *in vitro* culture of any one of embodiments 59-88, wherein the rat ES cell is capable of maintaining a pluripotency without requiring a paracrine LIF signaling.

90. The *in vitro* culture of any one of embodiments 59-89, wherein the feeder cell layer is not genetically modified to express LIF.

91. The *in vitro* culture of any one of embodiments 59-90, wherein the feeder cell layer comprises a monolayer of mitotically inactivated mouse embryonic fibroblasts (MEFs)

92. The *in vitro* culture of any one of embodiments 59-91, wherein the MEK inhibitor comprises PD0325901.

93. The *in vitro* culture of any one of embodiment 59-92, wherein the GSK-3 inhibitor comprises CHIR99021.

94. The *in vitro* culture of any one of embodiments 59-93, wherein the population of rat ES cells is derived from a rat blastocyst-stage embryo or a rat morula-stage embryo.

95. The *in vitro* culture of embodiment 94, wherein the blastocyst-stage or the morula-stage rat embryo further comprises an outgrowth of an amorphous undifferentiated mass of rat ES cells.

96. The *in vitro* culture of of embodiment 94, wherein the population of rat ES cells comprises an isolated outgrowth of an amorphous undifferentiated mass of rat ES cells.

97. A method for generating a rat embryonic stem (ES) cell line comprising: (a) culturing *in vitro* a first feeder cell layer and a morula or a blastocyst-stage rat embryo, wherein the zona pellucida of the morula or blastocyst-stage rat embryo has been removed, and wherein the culture conditions maintain pluripotency of a rat ES cell and comprise a medium having mouse leukemia inhibitor factor (LIF) or a sequence having at least 91% sequence identity to SEQ ID NO:1 and having LIF activity, and a GSK3 inhibitor, and a MEK inhibitor; and, (b) transferring an outgrowth of an amorphous undifferentiated mass of rat ES cells to an *in vitro* culture well comprising a second feeder cell layer and culturing the outgrowth under conditions comprising the medium having the mouse LIF or an active variant of the mouse LIF, and thereby maintaining pluripotency of the rat ES cells; and, establishing a rat ES cell line therefrom.

98. The method of embodiment 97, wherein the rat ES cell line is passaged at least 5 times.

99. The method of embodiment 97 or 98, wherein the rat ES cell line is passaged at least 10 times.

100. The method of embodiment 97, 98, or 99, wherein the medium comprises about 50 U/ml to about 150 U/ml of mouse LIF.

101. The method of any one of embodiments 97-100, wherein the medium comprises about 100 U/ml of mouse LIF.

102. The method of any one of embodiments 97-101, wherein the feeder cell layer is not genetically modified to express LIF.

103. The method of any one of embodiments 97-102, wherein the feeder cell layer comprises a monolayer of mitotically inactivated mouse embryonic fibroblasts (MEFs).

104. The method of any one of embodiments 97-103, wherein the MEK inhibitor comprises PD0325901.

105. The method of any one of embodiments 97-104, wherein the GSK-3 inhibitor comprises CHIR99021.

106. The method of any one of embodiment 97-105, wherein (a) the rat ES cell line is derived from an ACI rat or derived from a Dark Agouti (DA) rat; (b) the rat ES cell line is derived from a morula-stage or a blastocyst-stage rat embryo; and/or, (c) the rat ES cell line is derived from a morula-stage or a blastocyst-stage embryo from a superovulated rat.

107. The method of any one of embodiments 97-106, wherein the medium further comprises at least one of an FGF receptor inhibitor, a ROCK inhibitor, or an ALK inhibitor.

108. The method of embodiment 107, wherein the FGF receptor inhibitor comprises PD184352, the ROCK inhibitor comprises Y-27632, or the ALK inhibitor comprises A-83-01.

109. The method any one of embodiments 97-108, wherein at least one rat ES cell has a germline transmission efficiency of the targeted genetic modification is at least 3%.

110. The method of embodiment any one of embodiments 97-109, wherein the germline transmission efficiency of the targeted genetic modification is at least 60%.

111. A method of selecting a rat embryonic stem (ES) cells having stably incorporated into its genome a heterologous polynucleotide comprising: (a) providing an *in vitro* population of rat ES cells; (b) introducing into at least one rat ES cell a heterologous polynucleotide comprising a selection marker operably linked to a promoter active the rat ES cell; and, (c) culturing *in vitro* the rat ES cell population in an alternating first and second culture media, wherein the first culture medium comprises an effective amount of a selection agent for a first time period and the second culture medium does not comprise the selection agent, wherein the *in vitro* culture conditions are sufficient to maintain pluripotency; thereby selecting the rat ES cell having stably integrated into its genome the heterologous polynucleotide.

112. The method of embodiment 111, wherein the first and second culture media are alternated every 24 hours.

113. The method of embodiment 111 or 112, wherein the selection marker imparts resistance to an antibiotic.

114. The method of any one of embodiments 111-113, wherein the antibiotic comprises G418.

115. The method of any one of embodiment 111-114, wherein the selection marker comprises neomycin phosphotransferase (neo^r), hygromycin B phosphotransferase (hyg^r), puromycin-N-acetyltransferase ($puro^r$) , blasticidin S deaminase (bsr^r), xanthine/guanine phosphoribosyl transferase (gpt), and herpes simplex virus thymidine kinase (HSV-k), or a combination thereof.

116. The method of any one of embodiments 111-115, wherein (a) the selection marker has an increased activity compared to the wild type selection marker;

and/or (b) multiple copies of the selection marker are stably incorporated into the genome of the rat ES cell.

117. The method of embodiment 116, wherein the selection marker is a non-attenuated selection marker.

118. A method for genetically modifying an isolated rat embryonic stem (ES) cell comprising introducing into the genome of an isolated rat ES cell of any one of embodiment 1-58 a heterologous polynucleotide to form a genetically modified rat ES cell.

119. A method of making a genetically modified rat comprising:

(a) introducing into the genome of the isolated rat embryonic stem (ES) cell of any one of embodiments 1-58, a heterologous polynucleotide to form a rat ES cell having a genetic modification;

(b) introducing at least one of the rat ES cells comprising the targeted genetic modification into a rat host embryo to produce an F0 embryo;

(c) implanting the F0 embryo into a surrogate mother;

(d) gestating the F0 embryo in the surrogate mother to term; and, (e) identifying an F0 rat having the targeted genetic modification.

120. The method of embodiment 119, further comprising breeding a male F0 rat with a wild type female rat to produce an F1 progeny that is heterozygous for the targeted genetic modification.

121. The method of embodiment 120, further comprising breeding a male F0 rat with a wild type female rat to produce an F1 progeny that is heterozygous for the targeted genetic modification.

122. The method of embodiment 119, further comprising breeding a male rat of the F1 progeny with a female rat of the F1 progeny to obtain an F2 progeny that is homozygous for the genetic modification.

123. The method of any one of embodiments 119-122, wherein at least 3% of the F0 rats having the genetic modification transmit the genetic modification to the F1 progeny.

124. The method of any one of embodiments 119-123, wherein at least 10% of the F0 rats having the genetic modification transmit the genetic modification to the F1 progeny.

125. The method of any one of embodiments 119-124, wherein at least 60% of the F0 rats having the genetic modification transmit the genetic modification to the F1 progeny.

126. The method of any one of embodiments 119-125, wherein the genetically modified rat ES cell is from the same rat strain as the rat host embryo.

127. The method of any one of embodiments 119-127, wherein the genetically modified rat ES cell is from a different rat strain as the rat host embryo.

128. The isolated population of rat ES cells of any of the preceding claims, the in vitro culture of any of the preceding claims, or the method of any of the preceding claims, wherein the rat ES cells in the population comprise:

(a) at least 90% of the rat ES cells are euploid;

(b) at least 70% of the rat ES cells express at least one pluripotency marker; wherein the at least one pluripotency marker comprises Oct-4, Sox2, alkaline phosphatase, or a combination thereof;

(c) a cell from the rat ES cell population, when combined with a rat host embryo transmits the genome of the rat ES cell line into an offspring;

(d) the rat ES cells when cultured *in vitro* loosely adhere to a feeder cell layer;

(e) the rat ES cells form sphere-like colonies when plated on a feeder cell layer *in vitro*;

(f) the rat ES cells maintain pluripotency when cultured *in vitro* in a media comprising an GSK3 inhibitor, a MEK inhibitor, LIF and a feeder cell layer that is not genetically modified to express LIF;

(g) the rat ES cell exhibits a targeting efficiency of homologous recombination of at least 2%;

(h) the rat ES cells maintain pluripotency *in vitro* without requiring paracrine LIF signaling;

(i) at least 70% of the rat ES cells are euploid and form sphere-like colonies when plated on a feeder cell layer *in vitro*;

(j) the rat ES cells express at least one pluripotency marker selected from Dnmt3L, Eras, Err-beta, Fbxo15, Fgf4, Gdf3, Klf4, Lef1, LIF receptor, Lin28, Nanog, Oct4, Sox15, Sox2, Utf1, or a combination thereof;

(k) the rat ES cells do not express one or more differentiation markers selected from c-Myc, Ecat1, Rexo1.

(l) the rat ES cells do not express one or more mesodermal markers selected from Brachyury, Bmpr2, or a combination thereof;

(m) the rat ES cells do not express one or more endomermal markers selected from Gata6, Sox17, Sox7, or combination thereof; and/or

(n) the rat ES cells do not express one or more neural markers selected from Nestin, Pax6, or combination thereof.

BRIEF DESCRIPTION OF THE FIGURES

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

FIG. 1 depicts rESCs, which grow as compact spherical colonies that routinely detach and float in the dish.

FIG. 2A through D depicts various pluripotency markers expressed by rESCs: A depicts Oct-4 (green); B depicts Sox-2 (red); C depicts DAPI (blue); D depicts an overlay of pluripotency markers expressed by rESCs.

FIG. 3 depicts that the rESCs express light levels of alkaline phosphatase (a pluripotency marker) (left), and the karyotype for line DA.2B is 42X,Y (right). Karyotyping was done because rESCs often become tetraploid; lines were thus pre-screened by counting metaphase chromosome spreads, and lines with mostly normal counts were then formally karyotyped.

FIG. 4 depicts a closer view of a rESC of FIG. 1.

FIG. 5 depicts production of chimeras by blastocyst injection and transmission of the rESC genome through the germline; chimeras produced by blastocyst injection using parental ACI.G1 rESC; high percentage chimeras usually have albino snouts.

FIG. 6 depicts F1 agouti pups with albino littermates, sired by ACI/SD chimera labeled with an asterisk (*) in FIG. 5.

FIG. 7 Panel A depicts targeting of the rat Rosa 26 locus, which lies between the Setd5 and Thumpd3 genes as in mouse, with the same spacing. Panel A shows the structure of the mouse Rosa 26 locus. mRosa26 transcripts consist of 2 or 3 exons. Panel B depicts the structure of the rRosa26 locus; the rat locus contains a second exon 1 (Ex1b) in addition to the homologous exon to mouse exon1 (Ex1a); no third exon has been identified in rat. Panel C depicts a targeted rRosa26 allele; homology arms of 5kb

each were cloned by PCR using genomic DNA from DA rESC; the targeted allele contains a SA-lacZ-hUB-neo cassette replacing a 117bp deletion in the rRosa26 intron.

FIG. 8A depicts a control brain of a 14-week-old wild type rat, which was treated with X-gal. The control brain showed a low level of background staining for LacZ (dorsal view).

FIG. 8B depicts LacZ expression in the brain of an rRosa26 heterozygous rat (14-week old). The lacZ reporter was expressed ubiquitously throughout the brain of the rRosa26 heterozygote.

FIG. 8C depicts a control heart and thymus (inset) of a 14-week-old wild type rat, which were treated with X-gal. The control heart and thymus showed a low level of background staining for LacZ.

FIG. 8D depicts LacZ expression in the heart and thymus (inset) of a 14-week-old rRosa26 heterozygous rat. The lacZ reporter was expressed ubiquitously throughout the heart and thymus of the rROSA26 heterozygote.

FIG. 8E depicts a control lung of a 14-week-old wild type rat, which were treated with X-gal. The control lung showed a low level of background staining for LacZ.

FIG. 8F depicts LacZ expression in the lung of a 14-week-old rRosa26 heterozygote rat. The lacZ reporter was expressed ubiquitously throughout the lung of the rRosa26 heterozygote.

FIG. 8G and H depict LacZ expression in e12.5 embryos. In contrast to the wild-type control embryo (H), which shows a low level of background LacZ staining, the rRosa26 heterozygous embryo exhibited ubiquitous expression of the LacZ reporter throughout the embryo.

FIG. 8I and J depict LacZ expression in e14.5 embryos. In contrast to the wild-type control embryo (J), which shows a low level of background LacZ staining, the rRosa26 heterozygous rat embryo exhibited ubiquitous expression of the LacZ reporter throughout the embryo.

Figure 9A-B provides a photograph showing the analysis of the chromosome number of the ACI.G1 rat ES cell line.

Figure 10A-B provides a photograph showing the analysis of the chromosome number of the DA.2B rat ES cell line.

Figure 11A-B provides a photograph showing the analysis of the chromosome number of the DA.C2 rat ES cell line.

DETAILED DESCRIPTION

The present methods and compositions now will be described more fully hereinafter with reference to the accompanying drawings, in which some, but not all embodiments of the methods and compositions are shown. Indeed, these methods and compositions may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements. Like numbers refer to like elements throughout.

Many modifications and other embodiments of the methods and compositions set forth herein will come to mind to one skilled in the art to which this methods and compositions pertains having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the methods and compositions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

I. Overview

The rat has long been the preferred rodent model organism for several fields of biomedical research such as cardiovascular disease, metabolism, toxicology, neurobiology and behavior. Hundreds of strains of rat have been developed; some are excellent models for complex human diseases such as hypertension, diabetes and cancer. However, progress in understanding the genetics of these models has been severely hampered by the difficulty of modifying the rat genome in a controlled manner. Through the use of site-specific endonucleases it is possible to produce mutations in a gene of interest, but this method remains imprecise and expensive. Targeting and germline transmission of rat ES cells remains a difficult task to achieve.

Isolation of rat ES cells (rESC) from two inbred strains of rat is described herein. rESC from the DA and ACI strains were derived. These cells express pluripotency markers and exhibit a normal 42X,Y karyotype. High percentage chimeras have been produced, by microinjection into SD host embryos at the blastocyst stage, and transmission of the rESC genome has been demonstrated through the germline for both strains. Using plasmid targeting vectors, we have produced targeted mutations in the rat equivalent of the ROSA26 locus, and we have achieved germline transmission of the

targeted allele in both strains. These heterozygous animals express lacZ in all tissues at all stages examined.

In various aspects, ES cells were derived from the ACI strain in order to obtain a favorable number of male progeny from ACI donor ES cells. In one embodiment, the amount of male progeny is about 50%.

In various aspects, ES cells were derived from the DA strain in order to obtain primarily female progeny.

II. Rat Embryonic Stem (ES) Cells

Various compositions and methods are provided herein which comprise embryonic stem (ES) cells from rat. Stem cells are a cell population possessing the capability to self-renew indefinitely and are pluripotent. An "embryonic stem cell" or an “ES cell” comprises a stem cell obtained from an embryo or a fetus. The various rat ES cells provided herein can have one or more of any of the following properties:

(a) have germ-line competency, meaning when the rat ES cell is implanted into a rat host embryo, the genome of the rat ES cell line is transmitted into an offspring;

(b) have germ-line competency following at least one targeted genetic modification, meaning when the rat ES cell having the targeted genetic modification is implanted into a rat host embryo, the targeted genetic modification within the genome of the rat ES cell line is transmitted into an offspring;

(c) have pluripotency *in vitro*;

(d) have totipotency *in vitro*;

(e) when cultured *in vitro* loosely adhere to a feeder cell layer;

(f) when cultured *in vitro* form sphere-like colonies when plated on a feeder cell layer *in vitro*;

(g) maintain pluripotency when cultured *in vitro* under conditions comprising a feeder cell layer that is not genetically modified to express leukemia inhibitor factor (LIF), wherein the culture media comprises a sufficient concentration of LIF;

(h) maintain pluripotency when cultured *in vitro* under conditions comprising a feeder cell layer, wherein the culture media comprises mouse LIF or an active variant or fragment thereof;

(i) comprise a molecular signature that is characterized by

i) the expression of one or more of rat ES cell-specific genes comprising Adheres Junctions Associate Protein (Ajap1), Claudin 5 (Cldn5), Cdc42 guanine nucleotide exchange factor 9 (Arhgef9), Calcium/calmodulin-dependent protein kinase IV (Camk4), ephrin-A1 (Efna1), EPH receptor A4 (Epha4), gap junction protein beta 5 (Gjb5), Insulin-like growth factor binding protein-like 1 (Igfbpl1), Interleulin 36 beta(Il1f8), Interleukin 28 receptor, alpha (Il28ra), left-right determination factor 1 (Lefty1), Leukemia inhibitory factor receptor alpha (Lifr), Lysophosphatidic acid receptor 2 (Lpar2), Neuronal pentraxin receptor (Ntm), Protein tyrosine phosphatase non-receptor type 18 (Ptpn18), Caudal type homeobox 2 (Cdx2), Fibronectin type III and ankyrin repeat domains 1 (Fank1), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Lymphoid enhancer-binding factor 1 (Lef1), Sal-like 3 (Drosophila) (Sall3), SATB homeobox 1 (Satb1), miR-632, or a combination thereof;

ii) the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more of the rat ES cell-specific genes comprising Adheres Junctions Associate Protein (Ajap1), Claudin 5 (Cldn5), Cdc42 guanine nucleotide exchange factor 9 (Arhgef9), Calcium/calmodulin-dependent protein kinase IV (Camk4), ephrin-A1 (Efna1), EPH receptor A4 (Epha4), gap junction protein beta 5 (Gjb5), Insulin-like growth factor binding protein-like 1 (Igfbpl1), Interleulin 36 beta(Il1f8), Interleukin 28 receptor, alpha (Il28ra), left-right determination factor 1 (Lefty1), Leukemia inhibitory factor receptor alpha (Lifr), Lysophosphatidic acid receptor 2 (Lpar2), Neuronal pentraxin receptor (Ntm), Protein tyrosine phosphatase non-receptor type 18 (Ptpn18), Caudal type homeobox 2 (Cdx2), Fibronectin type III and ankyrin repeat domains 1 (Fank1), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Lymphoid enhancer-binding factor 1 (Lef1), Sal-like 3 (Drosophila) (Sall3), SATB homeobox 1 (Satb1), miR-632, or a combination thereof;

iii) at least a 20-fold increase in the expression of one or more of the rat ES cell-specific genes as set forth in Table 14 when compared to a F1H4 mouse ES cell;

iv) at least a 20-fold increase in the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more of the rat ES cell-specific genes as set forth in Table 14 when compared to a F1H4 mouse ES cell;

v) the expression of one or more of rat ES cell-specific genes as set forth in Table 13;

vi) the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50 or more of the rat ES cell-specific genes as set forth in Table 13;

vii) at least a 20-fold increase in the expression of one or more of the rat ES cell-specific genes as set forth in Table 13 when compared to a F1H4 mouse ES cell;

viii) at least a 20-fold increase in the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50 or more of the rat ES cell-specific genes as set forth in Table 13 when compared to a F1H4 mouse ES cell;

ix) at least a 20-fold decrease in the expression of one or more of the rat ES cell-specific genes as set forth in Table 12 when compared to a F1H4 mouse ES cell; and/or

x) at least a 20-fold decrease in the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50 or more of the rat ES cell-specific genes as set forth in Table 12 when compared to a F1H4 mouse ES cell;

xi) any combination of expression of the rat ES cell-specific genes of parts (i)-(x);

xii) a relative expression level of pluripotency markers as shown in Table 15 for at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 or 18 of the listed pluripotency markers. See, pluripotency ranking column of Table 15 for relative expression levels;

xiii) a relative expression level of the mesodermal markers as shown in Table 15 for at least 2, 3, or 4 of the listed mesodermal markers. See, mesodermal ranking column in Table 15 for relative expression levels;

xiv) a relative expression level of endodermal markers as shown in Table 15 for at least 2, 3, 4, 5, or 6 of the listed endodermal markers. See, endodermal ranking column in Table 15 for relative expression levels;

xv) a relative expression level of neural markers as shown in Table 15 for at least 2 and 3 of the listed neural markers. See, neural ranking column in table 15 for relative expression levels;

xvi) a relative expression level of trophectoderm markers as shown in Table 15 for the listed trophectoderm markers. See, trophectoderm ranking column in table 15 for relative expression levels;

xvii) any relative expression level of one or more (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23,2 4, 25, 26, 27, 28, 29 or 30) of the pluripotency markers, mesodermal markers, endodermal markers, neural markers and/or trophectoderm markers set forth in Table 15;

xviii) the relative expression level of each of the markers set forth in table 15;

xix) any combination of the signatures set forth in xii-xiix; and/or

xx) any combination of the signature set forth in i-xiix;

(j) have the ability to produce a F0 rat;

(k) are capable of being subcultured and maintaining the undifferentiated state;

(l) have the same number of chromosomes as a normal rat cell;

(m) maintain pluripotency *in vitro* without requiring paracrine LIF signaling; and/or

(n) have self renewal, meaning they divide indefinitely while maintaining pluripotency.

One or more of the characteristics outlined in (a)-(n) can be present in an isolated rat ES cell, a rat ES cell population or a rat ES cell line provided herein, wherein the rat ES cells have not undergone a targeted genetic modification. In other embodiments, one or more of the characteristics outlined in (a)-(n) can be present in an isolated rat ES cell, a rat ES cell population or a rat ES cell line provided herein that has one or more targeted genetic modifications. A targeted genetic modification comprises an alteration in the genome of the rat ES cell and includes, for example, an insertion, a deletion, a knockout, a knockin, a mutation, or a combination thereof. In other instances, the targeted genetic modification comprises at least one insertion of a heterologous polynucleotide into the genome of the rat ES cell. A further description of such targeted genetic modifications are discussed elsewhere herein.

In specific embodiments, the various rat ES cells and cell lines provided herein are germ-line competent, meaning when the rat ES cell is implanted into a rat host embryo, the genome of the rat ES cell is transmitted into an offspring. Such transmission into the offspring (i.e., the F1 population) can occur when the rat ES cell has not undergone a targeted genetic modification. In addition, a rat ES cell having a targeted genetic modification are also germ-line competent, meaning when the rat ES cell having the targeted genetic modification is implanted into a rat host embryo, the targeted genetic modification of the rat ES cell is transmitted to the offspring (i.e., the F1 population.) Thus, in various aspects, the rat ES cells and methods described herein are employed to obtain a high frequency, or high efficiency, of germline transmission of a a rat cell genome from both rat ES cells that have not undergone a targeted genetic modification and also from rat ES cells that have undergone a targeted genetic modification. In various embodiments, the frequency of germline transmission is greater than 1:600, greater than 1:500, greater than 1:400, greater than 1:300, greater than 1:200, and greater than 1:100. In various embodiments, the frequency of germline transmission is greater than 1%, greater than 2%, greater than 3%, greater than 4%, greater than 5%, greater than 6%, greater than 7%, greater than 8%, greater than 9%, greater than 10%, up to about 16%, greater than 25%, greater than 50%, greater than 60%, greater than 65%, greater than 70%, greater than 75% or greater. In various embodiments, the frequency of germline transmission ranges from 9% to 16%. In various aspects, percent of donor rESC-derived progeny in the F1 generation is 1% or more, 2% or more, 3% or more, 10% or more, 20% or more, 30% or more, 40% or more, 50% or more, 60% or more, from 3% to about 10% or more; from 3% or more to about 63%, from about 10% to about 30%, from about 10% to about 50%, from about 30% to about 70%, from about 30% to about 60%, from about 20% to about 40%, from about 20% to 65%, or from about 40% to 70%. Thus, a rat ES cell provided herein that has not undergone a targeted genetic modification or, alternatively, a rat ES cell that has a targeted genetic modification have the ability to transmit their genome into the F1 population.

A rat ES cell that has not undergone a targeted genetic modification or a rat ES cell that has a targeted genetic modification can be pluripotent and/or totipotent. “Pluripotent” or “pluripotency” refers to a stem cell that has the potential to differentiate into any of the three germ layers: endoderm, mesoderm, or ectoderm. Cell potency is a general term which describes a cell's ability to differentiate into other cell types. See, for example, Hans et al. (2007). "The Potential of Stem Cells: An Inventory". *Human*

biotechnology as Social Challenge. Ashgate Publishing, Ltd. p. 28, herein incorporate by reference. The term "totipotency" or "totipotent" is the ability of a single cell to divide and produce all of the differentiated cells in an organism. See, for example, Western P (2009). *Int. J. Dev. Biol.* 53 (2–3): 393–409, herein incorporated by reference. In specific embodiments, the various ES cells disclosed herein can be either pluripotent and/or totipotent.

Various methods can be used to determine if a rat ES cell is pluripotent. For example, the ES cell can be assayed for the expression of various pluripotent markers including, but not limited to, Oct-4, Sox2, alkaline phosphatase, or a combination thereof. See, for example, Okamoto, K. et al., *Cell*, 60: 461-472 (1990), Scholer, H. R. et al., *EMBO J.* 9: 2185-2195 (1990)) and Nanog (Mitsui, K. et al., *Cell*, 113: 631-642 (2003), Chambers, I. et al., *Cell*, 113: 643-655 (2003) for various methods of assaying for the presence or the level of such markers. See, also Figures 2 and 3 provided herein. Other pluripotency markers include, for example, the presence of at least 1, 2, 3, 4, or 5 pluripotency marker comprising Nanog, Klf4, Dppa2, Fgf4, Rex1, Eras, Err-beta and/or Sall3. Other pluripotency markers include, for example, the absence of at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 pluripotency marker comprising T/Brachyury, Flk1, Nodal, Bmp4, Bmp2, Gata6, Sox17, Hhex1, Sox7, and/or Pax6.

In specific embodiments, the expression and/or the level of expression of these markers can be determined using RT-PCR. Various kits are available to determine the level and/or presence of alkaline phosphatase, including, for example, an ALP tissue staining kit (Sigma) and Vector Red Alkaline Phosphatase Substrate Kit I (Funakoshi) and the like. Additional assays include in situ hybridization, immunohistochemistry, immunofluorescence. In specific embodiments, the rat ES cell is characterized by expression of at least one pluripotency marker, including for example expression of Oct-4, Sox2, alkaline phosphatase, or a combination thereof, and preferably all three of these markers.

The various rat ES cell provided herein (i.e. rat ES cells that have not undergone a targeted genetic modification and/or rat ES cells that have a targeted genetic modification) are capable of maintaining pluripotency and/or totipotency while being maintained in *in vitro* culturing conditions. Thus, the various rat ES cells provide herein can, in some embodiments, be subcultured while still maintaining the undifferentiated state. Various methods of culturing the rat ES cells are discussed in further detail elsewhere herein.

The rat embryonic stem cells provided herein have been isolated from the rat embryo employing various isolation, purification, and culture expansion techniques which are discussed in detail elsewhere herein. The term "cell" as used herein refers to individual cells, cell lines, or cultures derived from such cells. An “isolated” rat ES cell or rat embryo has been removed from its natural environment. The term “isolated” can mean free from 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% of the constituents with which a component is found in its natural state. As used herein, a rat ES “cell line” comprises a population of isolated rat cells that were developed from a single rat ES cell and therefore the population of cells within a given cell line have a uniform genetic makeup other than for mutations or karyotypic changes occurring during propagation or during targeted genetic modifications. For example, as indicated elsewhere, the disclosed rat ES cells are characterized by a high level of euploidy. Nevertheless, in some cell lines the level of euploidy is less than 100% due to karyotypic changes in propagation of the line from a single cell. Moreover, a given population of rat ES cells can comprise at least 10 exp 3, 10 exp4, $10x10^4$, $10x10^5$, $10x10^6$, $10x10^7$, $10x10^8$, $10x10^9$, or $10x10^{10}$ cells or greater. Some cell populations have sufficient cells to permit selection of a desired modified cell but not an excessively greater number so as to reduce the possibility of mutations or karyotypic changes developing in the cell line. For example, some cell populations have 10exp3 to 10exp6 cells.

As discussed elsewhere herein, various methods are provided for the targeted genetic modification of a rat ES cell line. When such methods are carried out, at least one cell within a rat ES cell line contains the targeted genetic modification. Through various culturing and/or selection techniques rat ES cell lines having one or more desired targeted genetic modifications are produced.

In specific embodiments, a rat ES cell, a population of rat ES cell or a rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) are euploid, and thus have a chromosome number that is an exact multiple of the haploid number. In further embodiment, a rat ES cell, a population of rat ES cells or a rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) are diploid, and thus have two haploid sets of homologous chromosomes. When referring to a rat ES cell population or a population of cells from a given population of rat ES cells or a rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification), at least about 50%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%,

92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the cells with the given population or euploid and/or diploid. In other instances, when referring to a rat ES cell population or a population of cells from a given rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification), at least about 50% to 95%, about 60% to 90%, about 60% to 95%, about 60% to 85%, about 60% to 80%, about 70% to 80%, about 70% to 85%, about 70% to about 90%, about 70% to about 95%, about 70% to about 100%, about 80% to about 100%, about 80% to about 95%, about 80% to about 90%, about 90% to about 100%, about 90% to about 99%, about 90% to about 98%, about 90% to about 97%, about 90% to about 95% of the cells within the given population are euploid and/or diploid.

In still further embodiments, a rat ES cell, a population of rat ES cells or a rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) have 42 chromosomes. When referring to a rat ES cell population or a population of cells from a given rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) at least about 50%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the cells with the given population have 42 chromosomes. In other instances, when referring to a rat ES cell population or a population of cells from a given rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) at least about 50% to 95%, about 60% to 90%, about 60% to 95%, about 60% to 85%, about 60% to 80%, about 70% to 80%, about 70% to 85%, about 70% to about 90%, about 70% to about 95%, about 70% to about 100%, about 80% to about 100%, about 80% to about 95%, about 80% to about 90%, about 90% to about 100%, about 90% to about 99%, about 90% to about 98%, about 90% to about 97%, about 90% to about 95% of the cells within the given population have 42 chromosomes.

In further embodiments, a rat ES cell, a population of rat ES cells or a rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) provided herein form sphere-like colonies when plated on a feeder cell layer *in vitro.* The "sphere-like" morphology refers to the shape of rat ES cell colonies in culture, rather than the shape of individual ES cells. The rat ES cell colonies are spherical-like. Colonies which are loosely attached to the feeder cells appear circular (have a circular-like morphology). Free-floating colonies are spherical-like. The rat ES cell colonies are spherical-like and very compact, meaning: the boundaries between cells

are very hard to see. The edge of the colony appears bright and sharp. Individual nuclei are difficult to distinguish because the cells are very small (so that the nucleus takes up most of the volume of the cell). Mouse ES Cells form elongated colonies and attach strongly to feeder cells. mESC morphology can vary with strain; e.g. B6 colonies are rounder and more domed than F1H4 colonies but are still more elongated than rESC. Human ES cell colonies are flatter and more spread out than mESC colonies. The instant rat ES colonies are not flat and do not resemble human ES cell colonies.

In still further embodiments, a rat ES cell, a population of rat ES cells or a rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) have a circular morphology. A morphology scale for a circle is provided below, where a score of a 10 represents a perfect circle and a score of a 1 represents an ellipse.

Morphology scale of a circle:

10=A circle with a structure having a longitudinal axis and a vertical axis that run through the center of the structure and are of equal length.

9 = A structure having a longitudinal axis and vertical axis that run through the center of the structure, wherein one of the axis is between 0.9999 to 0.9357 the length of the other axis.

8 = A structure having a longitudinal axis and vertical axis that run through the center of the structure, wherein one of the axis is between 0.9357 to 0.875 the length of the other axis.

7 = A structure having a longitudinal axis and vertical axis that run through the center of the structure, wherein one of the axis is between 0.875 to about 0.8125 the length of the other axis.

6 = A structure having a longitudinal axis and vertical axis that run through the center of the structure, wherein one of the axis is between 0.8125 to 0.750 the length of the other axis.

5 = A structure having a longitudinal axis and vertical axis that run through the center of the structure, wherein one of the axis is between 0.750 to 0.6875 the length of the other axis.

4 =A structure having a longitudinal axis and vertical axis that run through the center of the structure, wherein one of the axis is between 0.6875 to 0.625 the length of the other axis.

3 =A structure having a longitudinal axis and vertical axis that run through the center of the structure, wherein one of the axis is between 0.625 to 0.5625 the length of the other axis.

2= A structure having a longitudinal axis and vertical axis that run through the center of the circle, wherein one of the axis is between 0.5625 to 0.523 the length of the other axis.

1= An ellipse is defined as having a longitudinal axis and vertical axis that run through the center of the structure, wherein one of the axis is between 0.523 to 0.500 the length of the other axis.

In one non-limiting embodiment, the rat ES cell population or a population of cells from a given rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) have at least about 50%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the cells with the given population have a circular morphology score of a 10, 9 or 8. In other embodiments, the rat ES cell population or a population of cells from a given rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) have at least about 50% to 95%, about 60% to 90%, about 60% to 95%, about 60% to 85%, about 60% to 80%, about 70% to 80%, about 70% to 85%, about 70% to about 90%, about 70% to about 95%, about 70% to about 100%, about 80% to about 100%, about 80% to about 95%, about 80% to about 90%, about 90% to about 100%, about 90% to about 99%, about 90% to about 98%, about 90% to about 97%, about 90% to about 95% of the cells within the given population have a circular morphology score of a 10, 9, or 8.

In another non-limiting embodiment, the rat ES cell population or a population of cells from a given rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) have at least about 50%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the cells with the given population have a circular morphology score of a 7, 6, 5, 4 or 3. In other non-limiting embodiments, the rat ES cell population or a population of cells from a given rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) have at least about 50% to 95%, about 60% to 90%, about 60% to 95%, about 60% to 85%, about 60% to 80%, about 70% to 80%, about 70% to 85%, about 70% to about 90%, about 70% to about 95%, about 70% to about 100%, about 80% to about 100%, about 80% to

about 95%, about 80% to about 90%, about 90% to about 100%, about 90% to about 99%, about 90% to about 98%, about 90% to about 97%, about 90% to about 95% of the cells within the given population have a circular morphology score of a 7, 6, 5, 4, or 3.

In still further embodiments, sphere-like colonies form when the rat ES cells (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) are plated on a feeder cell layer *in vitro*. A morphology scale for a sphere is provided below, where a score of a 10 represents a perfect sphere and a score of a 1 represents a three dimensional elliptical structure.

Morphology scale of a sphere-like structure:

10=A sphere is a structure having an X-axis and a Y-axis and a Z-axis each of which runs through the center of the structure and are of equal length.

9 = A structure having an X axis and a Y-axis and a Z-axis that run through the center of the structure, wherein one of the axis is between 0.9999 to 0.9357 the length of at least one of the other axes.

8 = A structure having an X axis and a Y-axis and a Z-axis that run through the center of the structure, wherein one of the axis is between 0.9357 to 0.875 the length of at least one or both of the other axes.

7 = A structure having an X axis and a Y-axis and a Z-axis that run through the center of the structure, wherein one of the axis is between 0.875 to 0.8125 the length of at least one or both of the other axes.

6 =A structure having an X axis and a Y-axis and a Z-axis that run through the center of the structure, wherein one of the axis is between 0.8125 to 0.750 the length of at least one or both of the other axes.

5 = A structure having an X axis and a Y-axis and a Z-axis that run through the center of the structure, wherein one of the axis is 0.750 to 0.6875 the length of at least one or both of the other axes.

4 = A structure having an X axis and a Y-axis and a Z-axis that run through the center of the structure, wherein one of the axis is 0.6875 to 0.625 the length of at least one or both of the other axes.

3 =A structure having an X axis and a Y-axis and a Z-axis that run through the center of the structure, wherein one of the axis is between 0.625 to 0.5625 the length of at least one or both of the other axes.

2= A structure having an X axis and a Y-axis and a Z-axis that run through the center of the structure, wherein one of the axis is between 0.5625 to 0.523 the length of at least one or both of the other axes.

1= A structure having an X axis and a Y-axis and a Z-axis that run through the center of the structure, wherein one of the axis is between 0.523 to 0.500 the length of at least one or both of the other axes.

In one non-limiting embodiment, the rat ES cell population or a population of cells from a given rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) have at least about 50%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the colonies that form when the cells are plated on a feeder cell layer *in vitro* have a sphere-like morphology of a 10, 9 or 8. In other embodiments, the rat ES cell population or a population of cells from a given rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) have at least about 50% to 95%, about 60% to 90%, about 60% to 95%, about 60% to 85%, about 60% to 80%, about 70% to 80%, about 70% to 85%, about 70% to about 90%, about 70% to about 95%, about 70% to about 100%, about 80% to about 100%, about 80% to about 95%, about 80% to about 90%, about 90% to about 100%, about 90% to about 99%, about 90% to about 98%, about 90% to about 97%, about 90% to about 95% of the colonies that form when the cells are plated on a feeder cell layer *in vitro* have a sphere-like morphology of a 10, 9 or 8.

In another non-limiting embodiment, the rat ES cell population or a population of cells from a given rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) have at least about 50%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the colonies that form when the cells are plated on a feeder cell layer *in vitro* have a sphere-like morphology of a 7, 6, 5, 4, or 3. In other embodiments, the rat ES cell population or a population of cells from a given rat ES cell line (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) have at least about 50% to 95%, about 60% to 90%, about 60% to 95%, about 60% to 85%, about 60% to 80%, about 70% to 80%, about 70% to 85%, about 70% to about 90%, about 70% to about 95%, about 70% to about 100%, about 80% to about 100%, about 80% to about 95%, about 80% to about 90%, about 90% to about 100%, about 90% to about 99%, about 90% to about 98%, about 90% to about

97%, about 90% to about 95% of the colonies that form when the cells are plated on a feeder cell layer *in vitro* have a sphere-like morphology of a 7, 6, 5, 4, or 3.

A given rat ES cell, a population of rat ES cells or a rat ES cell line provided herein can be a male (XY) rat ES cell, a male (XY) population of rat ES cells, or a male (XY) rat ES cell line. In other embodiments, a population of rat ES cells or a rat ES cell line provided herein can be a female (XX) rat ES cell, a female (XX) population of rat ES cells, or a female (XX) rat ES cell line. Any such rat ES cell, population of rat ES cells or rat ES cell line can comprise the euploidy and/or diploidy as described above.

The various rat ES cell provided herein can be from any rat strain, including but not limited to, an ACI rat strain, a Dark Agouti (DA) rat strain, a Wistar rat strain, a LEA rat strain, a Sprague Dawley (SD) rat strain, or a Fischer rat strain such as Fisher F344 or Fisher F6. The various rat ES cells can also be obtained from a strain derived from a mix of two or more strains recited above. In one embodiment, the rat ES cell is derived from a strain selected from a DA strain and an ACI strain. In a specific embodiment, the rat ES cell is derived from an ACI strain. The ACI rat strain is are characterized as having black agouti, with white belly and feet and an $RT1^{av1}$ haplotype. Such strains are available from a variety of sources including Harlan Laboratories. In other embodiments, the various rat ES cells are from a Dark Agouti (DA) rat strain which is characterized as having an agouti coat and an $RT1^{av1}$ haplotype. Such rats are available from a variety of source including Charles River and Harlan Laboratories. In a further embodiment, the various rat ES cells provided herein are from an inbred rat strain.

In specific embodiments the rat ES cell line is from an ACI rat and comprises the ACI.G1 rat ES cell as described herein. In another embodiment, the rat ES cell line is from a DA rat and comprises the DA.2B rat ES cell line or the DA.2C rat ES cell line as described herein.

A given rat ES cell provided herein can be obtained from a rat embryo at any stage of rat embryo development. Representative stages of rat embryo development are outline below in Table 1. The rat embryos employed to derive the rat ES cells can be a morula-stage embryo, a blastocyst-stage embryo, or a rat embryo at a developmental stage between a morula-stage embryo and a blastocyst-stage embryo. Thus, in specific embodiments, the rat embryo employed is at or between the Witschi stages of 5 and 7. In other embodiments, the rat embryo employed is at the Witschi stage 5, 6, or 7.

In one embodiment, the rat ES cell is obtained from a rat blastocyst. In other embodiments, the rat ES cell is obtained from a blastocyst from a superovulated rat. In

other embodiments, the rat ES cells are obtained from an 8-cell stage embryo which is then cultured *in vitro* until it develops into a morula-stage, blastocyst stage, an embryo between the Witschi stages 5 and 7, or into an embryo at the Witschi stage 5, 6, or 7. At which time the embryos are then plated. Morula-stage embryos comprise a compact ball of cells with no internal cavity. Blastocyst-stage embryos have a visible internal cavity (the blastocoel) and contain an inner cell mass (ICM). The ICM cells form ES cells.

Table 1. Stages of Rat Embryo Development

Standard Stages (Witschi)	**Age** (days)	**Identification of Stages**
		Cleavage and Blastula
1	1	1 cell (in oviduct)
2	2	2 cells (in oviduct)
3	3	4 cells (in oviduct)
4	3.25	8-12 cells (in oviduct)
5	3.5	Morula (in uterus)
6	4	Early blastocyst (in uterus)
7	5	Free blastocyst (in uterus)
		Gastrula
8	6	Implanting blastocyst, with trophoblastic cone and inner cell mass; outgrowth of endoderm (hypoblast)
9	6.75	Diplotrophoblast; inner cell mass (pendant), covered with endoderm
10	7.25	Near complete implantation; pendant begins differentiation into embryonic and extra-embryonic parts
11	7.75	Completion of implantation; primary amniotic cyst; ectoplacental cone

Further provided are various rat ES cells (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) which are characterized by

The rat ES cell is characterized by:

i) the expression of one or more of rat ES cell-specific genes comprising Adheres Junctions Associate Protein (Ajap1), Claudin 5 (Cldn5), Cdc42 guanine nucleotide exchange factor 9 (Arhgef9), Calcium/calmodulin-dependent protein kinase IV (Camk4), ephrin-A1 (Efna1), EPH receptor A4 (Epha4), gap junction protein beta 5 (Gjb5), Insulin-like growth factor binding protein-like 1 (Igfbpl1), Interleulin 36

beta(Il1f8), Interleukin 28 receptor, alpha (Il28ra), left-right determination factor 1 (Lefty1), Leukemia inhibitory factor receptor alpha (Lifr), Lysophosphatidic acid receptor 2 (Lpar2), Neuronal pentraxin receptor (Ntm), Protein tyrosine phosphatase non-receptor type 18 (Ptpn18), Caudal type homeobox 2 (Cdx2), Fibronectin type III and ankyrin repeat domains 1 (Fank1), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Lymphoid enhancer-binding factor 1 (Lef1), Sal-like 3 (Drosophila) (Sall3), SATB homeobox 1 (Satb1), miR-632, or a combination thereof;

ii) the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more of the rat ES cell-specific genes comprising Adheres Junctions Associate Protein (Ajap1), Claudin 5 (Cldn5), Cdc42 guanine nucleotide exchange factor 9 (Arhgef9), Calcium/calmodulin-dependent protein kinase IV (Camk4), ephrin-A1 (Efna1), EPH receptor A4 (Epha4), gap junction protein beta 5 (Gjb5), Insulin-like growth factor binding protein-like 1 (Igfbpl1), Interleulin 36 beta(Il1f8), Interleukin 28 receptor, alpha (Il28ra), left-right determination factor 1 (Lefty1), Leukemia inhibitory factor receptor alpha (Lifr), Lysophosphatidic acid receptor 2 (Lpar2), Neuronal pentraxin receptor (Ntm), Protein tyrosine phosphatase non-receptor type 18 (Ptpn18), Caudal type homeobox 2 (Cdx2), Fibronectin type III and ankyrin repeat domains 1 (Fank1), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Lymphoid enhancer-binding factor 1 (Lef1), Sal-like 3 (Drosophila) (Sall3), SATB homeobox 1 (Satb1), miR-632, or a combination thereof;

iii) at least a 20-fold increase in the expression of one or more of the rat ES cell-specific genes as set forth in Table 14 when compared to a F1H4 mouse ES cell;

iv) at least a 20-fold increase in the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more of the rat ES cell-specific genes as set forth in Table 14 when compared to a F1H4 mouse ES cell;

v) the expression of one or more of rat ES cell-specific genes as set forth in Table 13;

vi) the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50 or more of the rat ES cell-specific genes as set forth in Table 13;

vii) at least a 20-fold increase in the expression of one or more of the rat ES cell-specific genes as set forth in Table 13 when compared to a F1H4 mouse ES cell;

viii) at least a 20-fold increase in the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50 or more of the rat ES cell-specific genes as set forth in Table 13 when compared to a F1H4 mouse ES cell;

ix) at least a 20-fold decrease in the expression of one or more of the rat ES cell-specific genes as set forth in Table 12 when compared to a F1H4 mouse ES cell; and/or

x) at least a 20-fold decrease in the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50 or more of the rat ES cell-specific genes as set forth in Table 12 when compared to a F1H4 mouse ES cell;

xi) any combination of expression of the rat ES cell-specific genes of parts (i)-(x);

xii) a relative expression level of pluripotency markers as shown in Table 15 for at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 or 18 of the listed pluripotency markers. See, pluripotency ranking column of Table 15 for relative expression levels;

xiii) a relative expression level of the mesodermal markers as shown in Table 15 for at least 2, 3, or 4 of the listed mesodermal markers. See, mesodermal ranking column in Table 15 for relative expression levels;

xiv) a relative expression level of endodermal markers as shown in Table 15 for at least 2, 3, 4, 5, or 6 of the listed endodermal markers. See, endodermal ranking column in Table 15 for relative expression levels;

xv) a relative expression level of neural markers as shown in Table 15 for at least 2 and 3 of the listed neural markers. See, neural ranking column in table 15 for relative expression levels;

xvi) a relative expression level of trophectoderm markers as shown in Table 15 for the listed trophectoderm markers. See, trophectoderm ranking column in table 15 for relative expression levels;

xvii) any relative expression level of one or more (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23,2 4, 25, 26, 27, 28, 29 or 30) of the pluripotency markers, mesodermal markers, endodermal markers, neural markers and/or trophectoderm markers set forth in Table 15;

xviii) the relative expression level of each of the markers set forth in table 15;

xix) any combination of the signatures set forth in xii-xiix; and/or

xx) any combination of the signature set forth in i-xiix.

In one embodiment, when transplanted into a pre-morula stage rat embryo, the rat ES cell (that have not undergone a targeted genetic modification and/or have a targeted genetic modification) can contribute to at least 90% of the cells in an F0 generation, contribute to at least 95% of the cells in an F0 generation, contribute to at least 96% of the cells in an F0 generation, contribute to at least 97% of the cells in an F0 generation, contribute to at least 98% of the cells in an F0 generation, or contribute to at least 99% of the cells in an F0 generation.

III. Derivation and Propagation of Rat Embryonic Stem (ES) Cells

Various methods are provided for obtaining the rat ES cells disclosed herein. In specific embodiments, such methods comprise (a) providing an *in vitro* culture comprising a feeder cell layer and a population of isolated rat embryonic stem (ES) cells; (b) culturing *in vitro* under conditions which are sufficient to maintain pluipotency and/or totipotency of the isolated rat ES cell. Such methods thereby allow for the propagation of a rat ES cell population and/or a rat ES cell line.

In one embodiment, a method for culturing a rat embryonic stem cell line is provided. Such methods comprises culturing *in vitro* a feeder cell layer and a rat ES cell line, wherein the culture conditions maintain pluripotency of the rat ES cells and comprise a media having mouse leukemia inhibitor factor (LIF) or an active variant or fragment thereof. The various methods further comprise passaging and culturing *in vitro* the cells of the rat ES cell line, wherein each subsequent *in vitro* culturing comprises culturing the rat ES cells on the feeder cell layer under conditions that maintain pluripotency of the rat ES cells and comprises a media having mouse LIF or an active variant or fragment thereof.

i. Culture Conditions

The culture media employed in the various methods and compositions will maintain the rat ES cells. The terms "maintaining" and "maintenance" refer to the stable preservation of at least one or more of the characteristics or phenotypes of the rat ES cells outline herein. Such phenotypes can include maintaining pluripotency and/or totipotency, cell morphology, gene expression profiles and the other functional characteristics of the rat stem cells described herein. The term "maintain" can also

encompass the propagation of stem cells, or an increase in the number of stem cells being cultured. The term further contemplates culture conditions that permit the stem cells to remain pluripotent, while the stem cells may or may not continue to divide and increase in number.

The term "feeder cell" or "feeder cell layer" refers to a culture of cells that grow *in vitro* and secrete at least one factor into the culture medium that is used to support the growth of another cell of interest in the culture. The feeder cells employed herein aid in maintaining the pluripotency of the rat ES cells, and in specific embodiments, one or more of the other characteristics or phenotypes described herein. Various feeder cells can be used including, for example, mouse embryonic fibroblasts, including mouse embryonic fibroblasts obtained between the 12^{th} and 16^{th} day of pregnancy. In specific embodiments, feeder cell layer comprises a monolayer of mitotically inactivated mouse embryonic fibroblasts (MEFs).

The *in vitro* cultures of the rat ES cells further comprise an effective amount of Leukemia Inhibitor Factor (LIF) or an active variant or fragment thereof. Leukemia inhibitory factor (LIF) belongs to the IL-6 receptor family. LIF binds to a heterodimeric membrane receptor made up of a LIF-specific subunit, gp190 or LIFR, and the subunit gp130, which is shared with the other members of the IL-6 family. LIF inhibits the differentiation of embryonic stem cells in mice and contribute to stem cell self-renewal. Human and mouse LIF share 79% sequence homology and exhibit cross-species activity. Rat LIF (rtLIF) is a 22.1 kDa protein containing 202 amino acid residues that exhibits 91% amino acid sequence identity with murine LIF (Takahama *et al.* 1998). There are six possible asparagine-linked glycosylation (N-glycosylation) sites which are conserved among the LIF polypeptide from the various species and an additional site of Asn150 which is specific for rat LIF. The tertiary structure of the mouse LIF and its function is described in further detail in Aikawa *et al.* (1998) *Biosci. Biotechnol. Biochem.* 62 1318-1325 and Senturk *et al.* (2005) *Immunology of Pregnancy*, editor Gil Mor., US Patent No. 5,750,654 and D P Gearing (1987) *EMBO Journal* 1987-12-20, each of which is herein incorporated by reference in their entirety. A partial mouse LIF sequence is reported on the SwissProt website under the accession number P09056.

Mouse LIF activity is assessed by its ability to induce differentiation of M1 myeloid leukemia cells. The specific activity is 1×10^6 units/ml (Cat. No. 03-0011 from Stemgent) and 1×10^7 units/ml (Cat. No. 03-0011-100 from Stemgent), where 50 units is defined as the amount of mouse LIF required to induce differentiation in 50% of the M1

colonies in 1 ml of medium. See, also, Williams, R.L. et al. (1988) Nature 336: 684-687.; Metcalf, D. et al. (1988) Leukemia 2: 216-221; Niwa, H. et al. (2009) Nature 460: 118-122; Xu, J. *et al.* (2010) *Cell Biol Int.* 34: 791-797; Fukunaga, N. et al. (2010) *Cell Reprogram.* 12: 369-376; and, Metcalf D. (2003) *Stem Cells* 21: 5-14, each of which is herein incorporated by reference in their entirety. An "effective amount of LIF" comprises a concentration of LIF that allows the rat ES cells of an *in vitro* culture to remain in an undifferentiated pluripotent state. Various markers that can be used to assay for the cells remaining in a pluripotent state are discussed elsewhere herein.

The LIF polypeptide employed in the various methods and compositions provided herein can be from any organism, including from a mammal, a rodent, a human, a rat or a mouse. In one embodiment, the LIF polypeptide is from a mouse. In still further embodiments, the mouse LIF polypeptide comprises the amino acid sequence set forth in SwissProt Accesion number: P09056, which is herein incorporated by reference in its entirety and is also set forth in SEQ ID NO: 1.

In other embodiments, an active variant or fragment of the mouse LIF polypeptide as set forth in SEQ ID NO: 1 or in SwissProt Accesion number: P09056 can be used. Such active variants and fragments (including active variants having at least 75%, 80%, 85% 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 1) are discussed in further detail elsewhere herein.

LIF polypeptide or the active variant or fragment thereof can be provided to the *in vitro* culture in a variety of ways. In one embodiment, the effective amount of the LIF polypeptide or the active variant or fragment thereof is added to the culture media. In other embodiments, the feeder cells have been genetically modified to overexpress the LIF polypeptide or the active variant or fragment thereof. Such feeder cells include feeder cells prepared from gamma-irradiated or mitomycin-C treated DIA-M mouse fibroblasts that express matrix-associated LIF. Method of generating and using such genetically modified feeder cells can be found, for example, in See, Buehr *et al.* (2003) *Biol Reprod* 68:222-229, Rathjen *et al.* (1990) *Cell* 62 1105-1115, and Buehr *et al.* (2008) *Cell* 135:1287-1298, each of which is herein incorporated by reference. The heterologous LIF expressed in the feeder cells can be from the same organism as the feeder cells or from an organism that is different from that of the feeder cell. In addition, the heterologous LIF expressed in the feeder cells can be from the same or from a different organism than the ES cells the feeder layer is supporting.

In still other embodiments, the feeder cells employed in the various methods disclosed herein are not genetically modified to express a heterologous LIF polypeptide or an active variant or fragment thereof. Thus, in particular embodiments, the monolayer of mitotically inactivated mouse embryonic fibroblast employed in the methods has not been genetically modified to express a heterologous LIF polypeptide.

In other embodiments, the LIF polypeptide or the active variant or fragment thereof is added to the culture media. When LIF is added to the culture media, the LIF can be from any organism, including from a mammal, a rodent, a human, a rat or a mouse. In one embodiment, the LIF present in the culture media is from a mouse. In still further embodiments, the mouse LIF polypeptide comprises the amino acid sequence set forth in SEQ ID NO:1. In other embodiments, an active variant or fragment of the mouse LIF polypeptide as set forth in SEQ ID NO:1 can be used. Such active variants and fragments (including active variants having at least 75%, 80%, 85% 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 1) are discussed in further detail elsewhere herein.

In specific embodiments, the rat ES cells and rat ES cell lines provided herein maintain pluripotency *in vitro* without requiring paracrine LIF signaling.

In specific embodiments, LIF or an active variant or fragment thereof is present in the culture media at any concentration that maintains the rat ES cells. LIF polypeptide or active variant or fragment thereof is present in the culture media at about 25U/ml to about 50U/ml, at about 50U/ml to about 100U/ml, at about 100U/ml to about 125U/ml, at about 125U/ml to about 150U/ml, at about 150U/ml to about 175U/ml, at about 175U/ml to about 200U/ml, at about 200U/ml to about 225U/ml, at about 225U/ml to about 250U/ml, at about 250U/ml to about 300U/ml, to about 300U/ml to about 325U/ml, at about 325U/ml to about 350U/ml, at about 350U/ml to about 400U/ml, at about 400U/ml to about 425U/ml, at about 425U/ml to about 450U/ml, at about 450U/ml to about 475U/ml, at about 475U/ml to about 500U/ml, at about 75U/ml to about 500U/ml or greater. In other embodiments, LIF polypeptide or active variant or fragment thereof is present in the culture media at about 25U/ml to about 50U/ml, at about 25U/ml to about 100U/ml, at about 75U/ml to about 125U/ml, at about 50U/ml to about 150U/ml, at about 90U/ml to about 125U/ml, at about 90U/ml to about 110U/ml, at about 80U/ml to about 150U/ml, at about 80U/ml to about 125U/ml. In a specific embodiment, LIF polypeptide or active variant or fragment thereof is present in the culture media at about 100U/ml.

When mouse LIF is employed, the mouse LIF polypeptide or active variant or fragment thereof is present in the culture media at any concentration that maintains the rat ES cells. Mouse LIF polypeptide or active variant or fragment thereof is present at about 25U/ml to about 50U/ml, at about 50U/ml to about 100U/ml, at about 100U/ml to about 125U/ml, at about 125U/ml to about 150U/ml, at about 150U/ml to about 175U/ml, at about 175U/ml to about 200U/ml, at about 200U/ml to about 225U/ml, at about 225U/ml to about 250U/ml, at about 250U/ml to about 300U/ml, to about 300U/ml to about 325U/ml, at about 325U/ml to about 350U/ml, at about 350U/ml to about 400U/ml, at about 400U/ml to about 425U/ml, at about 425U/ml to about 450U/ml, at about 450U/ml to about 475U/ml, at about 475U/ml to about 500U/ml, at about 75U/ml to about 500U/ml or greater. In other embodiments, mouse LIF polypeptide or active variant or fragment thereof is present at about 25U/ml to about 50U/ml, at about 25U/ml to about 100U/ml, at about 75U/ml to about 125U/ml, at about 50U/ml to about 150U/ml, at about 90U/ml to about 125U/ml, at about 90U/ml to about 110U/ml, at about 80U/ml to about 150U/ml, at about 80U/ml to about 125U/ml. In a specific embodiment, mouse LIF polypeptide or active variant or fragment thereof is present in the culture media at about 100U/ml.

The culture media employed maintains rat ES cells. As such, in specific embodiments, the culture media employed in the various method and compositions will maintain the pluripotency of all or most of (i.e., over 50%) of the rat ES cells in a cell line for a period of a at least 5, 10 or 15 passages. In one embodiment, the culture media comprises one or more compounds that assist in maintaining pluripotency. In one embodiment, the culture media comprises a MEK pathway inhibitor and a glycogen synthase kinase-3 (GSK-3) inhibitor. The media can further comprise additional components that aid in maintaining the ES cells, including for example, FGF receptor inhibitors, ROCK inhibitors, and/or ALK (TGFb receptor) inhibitors. A non-limiting example of an FGF receptor inhibitors includes PD184352. A non-limiting example of a ROCK inhibitor includes Y-27632, and non-limiting example of an ALK (TGFb receptor) inhibitor includes A-83-01. In specific embodiments, 2i media (table 2) is used with 10 uM ROCKi when thawing cryopreserved rESC or when re-plating rESC after dissociation with trypsin.

In other embodiments, the media comprises a combination of inhibitors consisting of a MEK pathway inhibitor and a glycogen synthase kinase-3 (GSK-3) inhibitor.

In one non-limiting embodiment, the culture media comprises a GSK-3 inhibitor comprising CHIR99021 and/or comprises a MEK inhibitor comprising PD0325901. In other embodiments, the media comprises a combination of inhibitors consisting of CHIR99021 and PD0325901. Either of these compounds can be obtained, for example, from Stemgent. In specific embodiments, CHIR99021 is present in the culture media at a concentration of about 0.5μ to about 3 μM, about 0.5μ to about 3.5 μM, about 0.5μM to about 4 μM, about 0.5μM to about 1 μM, about 1μM to about 1.5μM, about 1.5μM to about 2 μM, about 2 μM to about 2.5 μM , about 2.5 to about 3 μM, 3 μM to about 3.5 μM. In further embodiments, CHIR99021 is present in the culture media at a concentration of about 3 μM. In other embodiments, PD0325901 is present in the culture media at a concentration of about 0.4 μM to about 1uM, about 0.4 μM to about 1.5 uM, about 0.4 μM to about 2 μM, about 0.4 μM to about 0.8 μM, 0.8μM to about 1.2 μM, about 1.2 to about 1.5 μM. In further embodiments, PD0325901 is present in the culture media at a concentration of about 1μM. In specific embodiments, CHIR99021 is present in the culture media at a concentration of about 3 μM and PD0325901 is present at a concentration of about 1μM.

In one non-limiting embodiment, the culture media employed in the various methods and compositions disclosed herein is set forth in Table 2. Within the context of this application, the media described in Table 2 is referred to as 2i media.

Table 2: Non-limiting rat ES culture media.

Reagent	Concentration
DMEM/F12 basal media	1x (50%)
Neurobasal media	1x (50%)
Penicillin/streptomycin	1%
L-Glutamine	4 mM
2-Mercaptoethanol	0.1 mM
N2 supplement	1x
B27 supplement	1x
LIF	100 U/ml
PD0325901 (MEK inhibitor).	1μM
CHIR99021 (GSK inhibitor).	3 μM

Additional media that can be employed include those disclosed in Li *et al.* (2008) *Cell* 135:1299-1310, Yamamoto *et al.* (2012) *Transgenic Rats* 21:743-755, Ueda *et al.* (2008) *PLoS ONE* 3(6):e2800, Meek *et al.* (2010) *PLoS ONE* 4 (12): e14225; Tong *et al.*

(2010) *Nature* 467:211-213; US Patent Publication 2012/0142092, Buehr *et al.* (2008) Cell 135:1287-1298, Li *et al.* (135) *Cell* 1299-1310, each of which is herein incorporated by reference in their entirety. When employing such media, the concentration and the source of LIF can be modified as outlined herein. In specific embodiments, the various culture medias are used in combination with mouse LIF or an active variant or fragment thereof, and in even further embodiments, the various culture medias comprise a mouse LIF or an active variant or fragment thereof at a concentration of about 50U/ml to about 100U/ml, about 50U/ml to about 150U/ml, or about 100U/ml.

The temperature of the cultures of rat ES cells, both for the production of the ES cell line and for the culturing and maintaining of the ES line it typically carried out at about 35°C to about 37.5° C. In specific embodiment, the temperature is 37.0°C. The culture is typically carried out at 7.5% CO_2.

ii. *Establishing a rat ES Cell Line*

Methods for generating a rat embryonic stem (ES) cell line are provided. Such methods comprises (a) culturing *in vitro* a first feeder cell layer and a morula-stage embryo, a blastocyst-stage embryo, or a rat embryo at a developmental stage between a morula-stage embryo and a blastocyst-stage embryo, wherein the zona pellucida of the rat embryo has been removed and wherein the culture conditions maintain pluripotency of the rat ES cell and comprises a media having mouse leukemia inhibitor factor (LIF) or an active variant or fragment thereof; and, (b) transferring an outgrowth of an amorphous undifferentiated mass of rat ES cells to an *in vitro* culture well comprising a second feeder cell layer and culturing the outgrowth under conditions that maintain pluripotency of the rat ES cells and comprises a media having mouse LIF or an active variant or fragment thereof, and thereby establishing a rat ES cell line. The various methods further comprise passaging and culturing *in vitro* the cells of the rat ES cell line, wherein each subsequent *in vitro* culturing comprises culturing the rat ES cells on the feeder cell layer under conditions that maintain pluripotency of the rat ES cells and comprises a media having mouse LIF or an active variant or fragment thereof. Rat ES cell lines made by such methods are also provided.

Non-limiting examples of methods to establish a rat ES cell line having the various characteristics discussed herein are set forth in Example 3. Briefly, a rat embryo (i.e., a morula-stage embryo, a blastocyst-stage embryo, or a rat embryo at a developmental stage between a morula-stage embryo and a blastocyst-stage embryo) is

flushed from the uteruses of a female rat. In specific embodiments, a blastocyst or an 8 cell embryo is obtained. The zona pellucida is removed and the rat embryos are cultured on feeder cells (as discussed elsewhere herein) which, in specific embodiments, comprise a monolayer of mitotically inactivated mouse embryonic fibroblasts (MEFs). The cells of the morula-stage embryo, the blastocyst-stage embryo, or the rat embryo at a developmental stage between a morula-stage embryo and a blastocyst-stage embryo are cultured *in vitro* under conditions that maintain the ES rat cells and thereby are sufficient to maintain pluripotency and/or totipotency of the ES cells. Various medias can be employed at this stage, including any of the various medias discussed above which have LIF, including mouse LIF or an active variant or fragment thereof, in the media.

The cultures are monitored for the presence of an outgrowth (an amorphous undifferentiated mass of cells). Once the outgrowth reaches an appropriate size, a given outgrowth is transferred to new feeder plate and cultured. Transfer is accompanied by enzymatic dissociation using trypsin in order to produce multiple colonies. This transfer is commonly referred to as "passage 1". The speed at which each line expands varies. The media is changed as needed in order to maintain the pluripotency or totipotency of the rat ES cells. The culture is monitored for the presence of colonies having embryonic stem cell morphology. Such morphology includes one or more of the following characteristics: (a) round, circular mounds that rise above the monolayer of feeder cells; (b) cells that are packed tightly together such that cell boarders are difficult to see; (c) smaller cell size; (d) small amount of cytoplasm and enlarged nucleus, (e) form sphere-like colonies when plated on feeder cells *in vitro*. Once such colonies appear, the culturing can continue until reaching approximately 50% confluency. The colonies are then transferred to a new feeder plate. Transfer is accompanied by enzymatic dissociation using trypsin in order to expand the number of colonies. This is referred to as "passage 2". The cells are continued to be cultured with feeder cells until they reach approximately 50% confluent, at which point the cells can undergo further passages to maintain the cell lines or the lines can be frozen. See, also Tong *et al.* (2010) *Nature* 467 (9):211-215; Li *et al.* (2008) *Cell* 135:1299-1310, and Buehr *et al.* (2008) *Cell* 135:1287-1298, each of which is herein incorporated by reference. Thus, in specific embodiments, the various rat ES cells, cell lines and cell populations disclosed herein are capable of being subcultured and maintaining the undifferentiated state.

In one non-limiting embodiment, the derivation of the rat ES cells occurs as follows. At day 0, female rats are euthanized and the oviducts and uterine horns are

dissected out and place into a tissue culture dish containing warm N2B27 media. Media is flushed through the uterine horns and oviducts to eject blastocysts into the media. The blastocysts are collected and transfer to embryo culture dish containing KSOM + 2i (1μMPD0325901, 3 μM CHIR99021). KSOM can be purchased from Millipore, catalog number is MR-106-D. The 2i media referred to herein comprises the media set forth in Table 2. The cells are culture overnight at 37° at 7.5% CO_2.

In other non-limiting embodiments, the rat ES cells are derived from 8-cell embryos or frozen 8-cell embryos. The embryos are cultured in M2 medium for 10 minutes at room temperature and are then transfer to KSOM + 2i and culture overnight.

A non-limiting example for derivation of the rat ES cells is as follows: on Day 1, transferring cavitated embryos to 2i medium (Table 2) and culture overnight. The culturing is continued un-cavitated in KSOM +2i. On day 2, all remaining embryos are transferred to 2i medium, whether or not they have cavitated. Culture continues overnight in 2i medium. On day 3, embryos are incubated with acid tyrodes to remove the zona pellucida and washed 3 times in 2i medium to remove the acid tyrodes. Each embryo is deposited into a separate well feeder plate, in which each well contains a monolayer of mitotically inactivated mouse embryonic fibroblasts (MEFs). The cells are cultured overnight in 2i medium. On day 4 and 5, the cells plated embryos are monitored for the presence of an outgrowth, an amorphous undifferentiated mass of cells. Outgrowths are ready for transfer when they are approximately twice the size of the plated embryo. Each day, spent media is removed and replaced with fresh 2i media. The outgrowths are transferred to new feeder wells, and again spent media is removed and the wells are washed with PBS. The PBS is removed and trypsin is added and incubated for about 10 minutes. The trypsin reaction is stopped by the addition of 30μl 2i media and 10% FBS. The cells are gently dissociated and the entire content is transferred to a well in a feeder plate. This is referred to as Passage 1 (P1). The cells are cultured overnight in 2i medium. On day 5-8, depending on how fast each line expands, the 2i media is changed each day and culture is monitored for the presence of colonies with an ESC morphology. Such ESC morphology is discussed in detail elsewhere herein. Culturing continues until colonies expand to about 50% confluency. The colonies are then trypsinzied and passaged as before into feeder wells. This is referred to as passage 2. Feeding and monitoring each line is continued until they are approximately 50% confluent. The cells are trypsinized as usual. The trypsin is stopped with 2i media

+ 10% FBS. The cells are pelleted by centrifugation, and the cells in 400 μl Freezing Medium (70% 2i, 20% FBS, 10% DMSO). The cells can then be frozen. This is referred to passage 3.

iii. Maintaining and Passaging a rat ES cell Line

Further provided are methods for maintaining or culturing a rat embryonic stem cell line. The method comprises culturing *in vitro* a feeder cell layer and a rat ES cell line, wherein the culture conditions maintain pluripotency of a rat embryonic stem (ES) cell and comprise a media having mouse leukemia inhibitor factor (LIF) or an active variant or fragment thereof. Such methods employ the culture media and feeder cell layer as outlined above. In one embodiment, the rat ES cell line can be passaged at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 40, 50 or more times. In specific embodiments, the rat ES cells can be passaged up to at least 11 times in a medium comprising a GSK3 inhibitor and a MEK inhibitor without decreasing its targeting efficiency or germline transmission efficiency of a targeted genetic modification.

The rat ES cell lines are fed and monitored. In specific embodiments, passage occurs when the culture is approximately 30%, 40%, 50%, or 60% confluent. In other embodiments, passage occurs when the culture is 50% confluent. Depending on how fast each line expands, the passages can occur every 24, 25, 26, 27, 28, 29, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 90, 95 or 100 hours. In other embodiments, the time between passages ranges between 24 hours and 96 hours, between about 30 and 50 hours, between about 25 and 75 hours, between about 50 and 96 hours, between about 25 and 75 hours, between about 35 and 85 hours, or between about 40 and 70 hours. In one embodiment, the rat ES cell, cell line or cell population as disclosed herein has a doubling time ranging from about 24 hours to about 36 hours. In one embodiment, the rat ES cell has a doubling time of 25 hours.

The various rat ES cell lines when derived and maintained as outlined herein can have one or more any the following properties:

(a) have germ-line competency, meaning when the rat ES cell is implanted into a rat host embryo, the genome of the rat ES cell line is transmitted into an offspring;

(b) have germ-line competency following targeted genetic modification, meaning when the rat ES cell is implanted into a rat host embryo, the targeted genetic modification within the genome of the rat ES cell line, is transmitted into an offspring;

(c) have pluripotency *in vitro*;

(d) have totipotency *in vitro*;

(e) when cultured *in vitro* loosely adhere to a feeder cell layer;

(f) when cultured *in vitro* form sphere-like colonies when plated on a feeder cell layer *in vitro*;

(g) maintain pluripotency when cultured *in vitro* under conditions comprising a feeder cell layer that is not genetically modified to express leukemia inhibitor factor (LIF), wherein the culture media comprises a sufficient concentration of LIF;

(h) maintain pluripotency when cultured *in vitro* under conditions comprising a feeder cell layer, wherein the culture media comprises mouse LIF or an active variant or fragment thereof;

(i) comprise a molecular signature characterized by

i) the expression of one or more of rat ES cell-specific genes comprising Adheres Junctions Associate Protein (Ajap1), Claudin 5 (Cldn5), Cdc42 guanine nucleotide exchange factor 9 (Arhgef9), Calcium/calmodulin-dependent protein kinase IV (Camk4), ephrin-A1 (Efna1), EPH receptor A4 (Epha4), gap junction protein beta 5 (Gjb5), Insulin-like growth factor binding protein-like 1 (Igfbpl1), Interleulin 36 beta(Il1f8), Interleukin 28 receptor, alpha (Il28ra), left-right determination factor 1 (Lefty1), Leukemia inhibitory factor receptor alpha (Lifr), Lysophosphatidic acid receptor 2 (Lpar2), Neuronal pentraxin receptor (Ntm), Protein tyrosine phosphatase non-receptor type 18 (Ptpn18), Caudal type homeobox 2 (Cdx2), Fibronectin type III and ankyrin repeat domains 1 (Fank1), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Lymphoid enhancer-binding factor 1 (Lef1), Sal-like 3 (Drosophila) (Sall3), SATB homeobox 1 (Satb1), miR-632, or a combination thereof;

ii) the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more of the rat ES cell-specific genes comprising Adheres Junctions Associate Protein (Ajap1), Claudin 5 (Cldn5), Cdc42 guanine nucleotide exchange factor 9 (Arhgef9), Calcium/calmodulin-dependent protein kinase IV (Camk4), ephrin-A1 (Efna1), EPH receptor A4 (Epha4), gap junction protein beta 5

(Gjb5), Insulin-like growth factor binding protein-like 1 (Igfbpl1), Interleulin 36 beta(Il1f8), Interleukin 28 receptor, alpha (Il28ra), left-right determination factor 1 (Lefty1), Leukemia inhibitory factor receptor alpha (Lifr), Lysophosphatidic acid receptor 2 (Lpar2), Neuronal pentraxin receptor (Ntm), Protein tyrosine phosphatase non-receptor type 18 (Ptpn18), Caudal type homeobox 2 (Cdx2), Fibronectin type III and ankyrin repeat domains 1 (Fank1), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Lymphoid enhancer-binding factor 1 (Lef1), Sal-like 3 (Drosophila) (Sall3), SATB homeobox 1 (Satb1), miR-632, or a combination thereof;

iii) at least a 20-fold increase in the expression of one or more of the rat ES cell-specific genes as set forth in Table 14 when compared to a F1H4 mouse ES cell;

iv) at least a 20-fold increase in the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more of the rat ES cell-specific genes as set forth in Table 14 when compared to a F1H4 mouse ES cell;

v) the expression of one or more of rat ES cell-specific genes as set forth in Table 13;

vi) the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50 or more of the rat ES cell-specific genes as set forth in Table 13;

vii) at least a 20-fold increase in the expression of one or more of the rat ES cell-specific genes as set forth in Table 13 when compared to a F1H4 mouse ES cell;

viii) at least a 20-fold increase in the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50 or more of the rat ES cell-specific genes as set forth in Table 13 when compared to a F1H4 mouse ES cell;

ix) at least a 20-fold decrease in the expression of one or more of the rat ES cell-specific genes as set forth in Table 12 when compared to a F1H4 mouse ES cell; and/or

x) at least a 20-fold decrease in the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50 or more of the rat ES cell-specific genes as set forth in Table 12 when compared to a F1H4 mouse ES cell;

xi) any combination of expression of the rat ES cell-specific genes of parts (i)-(x);

xii) a relative expression level of pluripotency markers as shown in Table 15 for at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 or 18 of the listed pluripotency markers. See, pluripotency ranking column of Table 15 for relative expression levels;

xiii) a relative expression level of the mesodermal markers as shown in Table 15 for at least 2, 3, or 4 of the listed mesodermal markers. See, mesodermal ranking column in Table 15 for relative expression levels;

xiv) a relative expression level of endodermal markers as shown in Table 15 for at least 2, 3, 4, 5, or 6 of the listed endodermal markers. See, endodermal ranking column in Table 15 for relative expression levels;

xv) a relative expression level of neural markers as shown in Table 15 for at least 2 and 3 of the listed neural markers. See, neural ranking column in table 15 for relative expression levels;

xvi) a relative expression level of trophectoderm markers as shown in Table 15 for the listed trophectoderm markers. See, trophectoderm ranking column in table 15 for relative expression levels;

xvii) any relative expression level of one or more (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23,2 4, 25, 26, 27, 28, 29 or 30) of the pluripotency markers, mesodermal markers, endodermal markers, neural markers and/or trophectoderm markers set forth in Table 15;

xviii) the relative expression level of each of the markers set forth in table 15;

xix) any combination of the signatures set forth in xii-xiix; and/or

xx) any combination of the signature set forth in i-xiix;

(j) have the ability to produce a F0 rat;

(k) capable of being subcultured and maintaining the undifferentiated state;

(l) have the same number of chromosomes a normal rat cell; and/or

(m) maintain pluripotency *in vitro* without requiring paracrine LIF signaling.

(n) have self renewal, meaning they divide indefinitely while maintaining pluripotency.

Such properties of a given rat ES cell line can be present at any one of the passage, including at passages 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21,

22, 23, 24, 25, 30, 40, 50 or later.

Thus, in one non-limiting embodiment, an *in vitro* culture comprising a feeder cell layer and a population of rat ES cells is provided, wherein the *in vitro* culture conditions maintain pluripotency of the rat ES cells and comprises a media having mouse leukemia inhibitor factor (LIF) or an active variant or fragment thereof. In specific embodiments, the rat ES cells cultured under such conditions maintains pluripotency of at least 50% of cell population over a period of at least 10 passages, maintains pluripotency of at least 60% of cell population over a period of at least 10 passages, maintains pluripotency of at least 70% of cell population over a period of at least 10 passages, maintains pluripotency of at least 75% of cell population over a period of at least 10 passages, maintains pluripotency of at least 80% of cell population over a period of at least 10 passages, maintains pluripotency of at least 85% of cell population over a period of at least 10 passages, maintains pluripotency of at least 90% of cell population over a period of at least 10 passages, or maintains pluripotency of at least 95% of cell population over a period of at least 10 passages.

Further provided herein are *in vitro* cultures comprising the various rat ES cells, cell populations and cell lines disclosure herein, as well as, culturing kits for these various ES cells. For example, as discussed above, in specific embodiments, the various rat ES cells provided herein have a one or more of the following characteristics: (1) when cultured *in vitro* loosely adhere to a feeder cell layer; (2) when cultured *in vitro* they form sphere-like colonies when plated on a feeder cell layer *in vitro*; (3) they maintain pluipotency when cultured *in vitro* under conditions comprising a feeder cell layer that is not genetically modified to express leukemia inhibitor factor (LIF), wherein the culture media comprises a sufficient concentration of LIF; and/or (4) they capable of being subcultured and maintaining the undifferentiated state. Moreover, the rat ES cell populations of any of these *in vitro* cultures can comprise, for example, a population of cells in which at least 70%, 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the cells within the population are euploid, are diploid and/or have 42 chromosomes.

One method of culturing a rat embryonic stem cells *in vitro* comprises (a) providing an *in vitro* culture comprising a feeder cell layer and a population of isolated rat embryonic stem (ES) cells; and (b) culturing the *in vitro* culture under conditions which are sufficient to maintain pluipotency or totipotency of the isolated rat embryonic stem (ES) cell, and wherein the rat ES cells form colonies that loosely adhere to the

feeder cell layer. "Loose adherence" or "adhere loosely" means that, if the culture dish is undisturbed for a period of time (minimum 8 hours), some colonies will adhere to the feeders such that they can maintain adherence if the dish is gently moved. In smaller wells (where the media moves less), loose adherence can happen faster. In either case, these colonies can be dislodged by either a) swirling the media in the dish or by gently pipetting media across the surface of the feeders. The morphology of these loosely adherent colonies is still spherical. In such instances the rat ES cells form sphere-like colonies when plated on a feeder cell layer *in vitro*. Such sphere-like colonies are shown for example in Figure 1.

iv. Kits and In Vitro Cultures

The rat ES cells and rat ES cell lines provided herein can be contained within a kit or an article of manufacture. In specific embodiments, the kit or article of manufacture comprises any of the rat ES cell lines or populations disclosed herein. The kit can further comprise any culture media that maintains the rat ES cell, including media that maintains the pluripotency of the rat ES cells. Such media can comprise culture media having mouse LIF or an active variant or fragment thereof, as discussed in greater detail elsewhere herein. The media within the kit can further comprise a MEK inhibitor and a GSK-3 inhibitor, or alternatively, the media within the kit can further comprise a combination of inhibitors consisting of a MEK inhibitor and a GSK-3 inhibitor. In specific embodiments, the media in the kit comprises a MEK inhibitor comprising PD0325901 and/or a GSK-3 inhibitor comprising CHIR99021. Any of the various medias discussed herein, can be contained within the kit.

Further provided is a kit or article of manufacture that comprises any of the rat ES cell lines or populations disclosed herein, any of the various media disclosed herein, and a population of feeder cells. In one embodiment, the feeder cells in the kit or article of manufacture are not genetically modified to express LIF and/or the feeder cells comprises mitotically inactivated mouse embryonic fibroblasts (MEFs). Any of the other feeder cells disclosed herein can be employed in the kit or article of manufacture.

IV. Genetic Modification of Rat Embryonic Stem (ES) Cells

The various rat ES cells and cell lines disclosed herein can be modified to contain at least one targeted genetic modification. Thus, various methods are provided for genetically modifying an isolated rat embryonic stem (ES) cell as disclosed herein. The

method comprises introducing into the genome of an isolated rat ES cell disclosed herein a targeted genetic modification to form a genetically modified rat ES cell. The targeted genetic modification can comprise any modification to the genome of rat ES including, for example, an insertion, a deletion, a knockout, a knockin, a mutation, or a combination thereof. In one embodiment, the targeted genetic modification comprises insertion of a heterologous polynucleotide into the genome of the rat ES cell. As used herein, "heterologous" in reference to a sequence is a sequence that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention.

In one aspect, an isolated rat ES cell or rat ES cell line is provided that is capable of sustaining pluripotency following one or more genetic modifications *in vitro* and that is capable of transmitting a genetically modified genome to a germline of an F1 generation. Thus, the rat ES cell maintains its pluripotency to develop into a plurality of cell types following the one or more serial genetic modifications *in vitro* (*e.g.*, two, three, four, five, or six or more serial genetic modifications). In other embodiments, multiple targeted genetic modifications are made in a given rat ES cell, including, for example, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 14, 15 or more. As such, multiple heterologous polynucleotides can also be integrated into the genome, including for example, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 14, 15 or more.

In one embodiment, following any one of the one to 15 serial genetic modifications, the genetically modified rat ES cells upon exposure to differentiation medium are capable of differentiation into a plurality of cell types. In one embodiment, following any one of the one to 15 serial genetic modifications, the genetically modified rat ES cells are capable of being maintained in an undifferentiated state in culture. In one embodiment, the genetically modified and cultured rat ES cells in the undifferentiated state, when employed as donor cells in a rat host embryo, populate the embryo and form a blastocyst comprising the one to fifteen genetic modifications. In one embodiment, the blastocyst, when implanted into a surrogate mother under conditions suitable for gestation, develops into an F0 rat progeny that comprises the one to 15 genetic modifications.

Various methods for making targeted genetic modifications within a genome of a rat ES cell can be used. For example, in one instance, the targeted genetic modification employs a system that will generate a targeted genetic modification via a homologous recombination event. In other instances, the rat ES cells can be modified using nuclease

agents that generate a single or double strand break at a targeted genomic location. The single or double-strand break is then repaired by the non-homologous end joining pathway (NHEJ). Such systems find use, for example, in generating targeted loss of function genetic modifications. See, for example, Tesson et al. (2011) Nature Biotechnology 29:695-696, herein incorporated by reference. Such agents include, Transcription Activator-Like Effector Nuclease (TALEN) (WO 2010/079430; Morbitzer *et al.* (2010) *PNAS* 10.1073/pnas.1013133107; Scholze & Boch (2010) *Virulence* 1:428-432; Christian *et al. Genetics* (2010) 186:757-761; Li *et al.* (2010) *Nuc. Acids Res.* (2010) doi:10.1093/nar/gkq704; and Miller *et al.* (2011) *Nature Biotechnology* 29:143–148; US Patent Application No. 2011/0239315 A1, 2011/0269234 A1, 2011/0145940 A1, 2003/0232410 A1, 2005/0208489 A1, 2005/0026157 A1, 2005/0064474 A1, 2006/0188987 A1, and 2006/0063231 A1 (each hereby incorporated by reference); a zinc-finger nuclease (ZFN) (US20060246567; US20080182332; US20020081614; US20030021776; WO/2002/057308A2; US20130123484; US20100291048; and, WO/2011/017293A2, each of which is herein incorporated by reference); a meganuclease (see, Epinat *et al.*, (2003) *Nucleic Acids Res* 31:2952-62; Chevalier *et al.*, (2002) *Mol Cell* 10:895-905; Gimble *et al.*, (2003) *Mol Biol* 334:993-1008; Seligman *et al.*, (2002) *Nucleic Acids Res* 30:3870-9; Sussman *et al.*, (2004) *J Mol Biol* 342:31-41; Rosen *et al.*, (2006) *Nucleic Acids Res* 34:4791-800; Chames *et al.*, (2005) *Nucleic Acids Res* 33:e178; Smith *et al.*, (2006) *Nucleic Acids Res* 34:e149; Gruen *et al.*, (2002) *Nucleic Acids Res* 30:e29; Chen and Zhao, (2005) *Nucleic Acids Res* 33:e154; WO2005105989; WO2003078619; WO2006097854; WO2006097853; WO2006097784; and WO2004031346); and a CAS/CRISPER system (Mali P et al. (2013) *Science* 2013 Feb 15;339(6121):823-6; Jinek M et al. *Science* 2012 Aug 17;337(6096):816-21; Hwang WY et al. *Nat Biotechnol* 2013 Mar;31(3):227-9; Jiang W et al. *Nat Biotechnol* 2013 Mar;31(3):233-9; and, Cong L et al. *Science* 2013 Feb 15;339(6121):819-23, each of which is herein incorporated by reference).

In other embodiments, the targeted genomic modification can be made by employing a homologous recombination targeting vector. In such instances, the targeting vector comprises the insert polynucleotide and further comprises an upstream and a downstream homology arm which flank the insert polynucleotide. The homology arms which flank the insert polynucleotide correspond to genomic regions within the targeted genomic locus. For ease of reference, the corresponding genomic regions within the targeted genomic locus are referred to herein as “target sites”. Thus, in one example,

a targeting vector can comprise a first insert polynucleotide flanked by a first and a second homology arm corresponding to a first and a second target site located at the targeted genomic locus. The targeting vector thereby aids in the integration of the insert polynucleotide into the targeted genomic locus through a homologous recombination event that occurs between the homology arms and the corresponding target sites within the genome of the cell.

As used herein, a homology arm and a target site "correspond" or are "corresponding" to one another when the two regions share a sufficient level of sequence identity to one another to act as substrates for a homologous recombination reaction. By "homology" is meant DNA sequences that are either identical or share sequence identity to a corresponding sequence. The sequence identity between a given target site and the corresponding homology arm found on the targeting vector can be any degree of sequence identity that allows for homologous recombination to occur. For example, the amount of sequence identity shared by the homology arm of the targeting vector (or a fragment thereof) and the target site (or a fragment thereof) can be at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.9% or 100% sequence identity, such that the sequences undergo homologous recombination. Moreover, a corresponding region of homology between the homology arm and the corresponding target site can be of any length that is sufficient to promote homologous recombination at the cleaved recognition site.

In specific embodiments, the isolated rat ES cell, cell line or cell population exhibits a homologous recombination efficiency of at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 11%, at least 12%, at least 13%, at least 14%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, or at least 80%. In one embodiment, the homologous recombination efficiency employing the rat ES cell is greater than 4%.

In specific embodiments, a selection marker is employed when generating a targeted genetic modification in a rat ES cell. The polynucleotide encoding the selection marker can be present on the targeting vector which is designed to introduce the targeted genetic modification into the genome, or it can be found on a separate plasmid or vector. The polynucleotide encoding the selection marker can be contained within in expression

cassette. The various components of such expression cassettes are discussed in further detail elsewhere herein.

Various selection markers can be used in the methods and compositions disclosed herein. Such selection markers can, for example, impart resistance to an antibiotic such as G418, hygromycin, blastocidin, puromycin or neomycin. Such selection markers include neomycin phosphotransferase (neo^r), hygromycin B phosphotransferase (hyg^r), puromycin *N*-acetyl-transferase and blasticidin S deaminase (bsr^r). In still other embodiments, the selection marker is operably linked to an inducible promoter and the expression of the selection marker is toxic to the cell. Non-limiting examples of such selection markers include xanthine/guanine phosphoribosyl transferase (gpt), hahypoxanthine-guanine phosphoribosyltransferase (HGPRT) or herpes simplex virus thymidine kinase (HSV-TK). See, for example, Santerre *et al.* (1984) *Gene* 30:147-56; Joyner (1999) *The Practical Approach Series*, 293; Santerre *et al.* (1984) Gene 30:147-56; Bernard *et al.* (1985) *Exp Cell Res* 158:237-43; Giordano and McAllister (1990) *Gene*, 88:285-8; Izumi *et al.* (1991) *Exp Cell Res* 197:229-33), each of which is herein incorporated by reference in their entirety. In specific embodiments, the neoR selectable marker is the neomycin phosphotransferase (*neo*) gene of Beck *et al.* (1982) Gene, 19:327-36, which is herein incorporated by reference. The neoR selection maker is that used in US Patent No, 7,205,148 or 6,596,541, each of which are herein incorporated by reference.

In specific embodiments, the selection marker employed is a non-attenuated selection marker. A "non-attenuated selection marker" comprises a selection marker that retains the activity of the native polypeptide or the selection marker has an increased activity when compared to the native form of the polypeptide. An increased in activity of a selection marker can comprise any statistically significant increase in activity including, for example, an increase of at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70% 80% or higher. Thus, a non-attenuated selection marker when expressed in a host cell will allow for a higher percentage of the host cells to survive in the presence of a higher concentration of the selection agent than when employing an attenuated selection marker. Non-attenuated selection markers include, for example, a neomycin non-attenuate selection marker. See, for example, Beck *et al.* (1982) Gene, 19:327-36 or in US Patent No, 7,205,148 or 6,596,541, each of which are herein incorporated by reference.

In other instances, the increased activity of a selection marker when compared to an attenuated selection marker and/or the wild type (native) selection marker can result from increasing the copy number of either the non-attenuated, attenuated or native selection marker within the genome of the rat ES cell. As such, a given rat ES cell can comprise within its genome at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more copies of a given selection marker (i.e., a non-attenuated selection marker, an attenuated selection marker or a native (wildtype) selection marker).

The various selection markers employed are encoded by a polynucleotide operably linked to a promoter active in the rat ES cell. In specific embodiments, the increase in the activity of the selection marker can result from an increase in expression of the selection marker. Thus, a promoter can be employed to elevate the expression levels of a given selection marker. Promoters of interest include, but are not limited to, the CMV promoter, the PGK promoter and the CAG promoter. In one embodiment, the human Ubiquitin (hUb) promoter is used to express the selection marker. See, Valenzuela *et al.* (2003) *Nature Biotechnology* 21:652-659, herein incorporate by reference in its entirety.

In one embodiment, the rat ES cell maintains its pluripotency to develop into a plurality of cell types following a single round of electroporation with an exogenous nucleic acid. In another embodiment, the rat ES cell maintains its pluripotency to develop into a plurality of cell types following a second round of electroporation with an exogenous nucleic acid, following a third round of electroporation with an exogenous nucleic acid, following a fourth round of electroporation with an exogenous nucleic acid, following a fifth round of electroporation with an exogenous nucleic acid, following a sixth round of electroporation with an exogenous nucleic acid, following a seventh round of electroporation with an exogenous nucleic acid, following an eighth round of electroporation with an exogenous nucleic acid, following a ninth round of electroporation with an exogenous nucleic acid, following a tenth round of electroporation with an exogenous nucleic acid, following an eleventh round of electroporation with an exogenous nucleic acid, following a twelfth round of electroporation with an exogenous nucleic acid, following a thirteenth round of electroporation with an exogenous nucleic acid, following a fourteenth round of electroporation with an exogenous nucleic acid and/or following a fifteenth round of electroporation with an exogenous nucleic acid. In other embodiments, the rat ES cell is capable of transmitting a targeted genetic modification into progeny following a

successive round of electroporation (i.e., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more rounds of electroporation).

i. Introducing Sequences into a Rat Embryonic Stem Cell

The methods provided herein comprise introducing into a cell with one or more polynucleotides or polypeptide constructs comprising the various components needed to make the targeted genomic modification. "Introducing" means presenting to the cell the sequence (polypeptide or polynucleotide) in such a manner that the sequence gains access to the interior of the cell. The methods provided herein do not depend on a particular method for introducing any component of the targeted genomic integration system into the cell, only that the polynucleotide gains access to the interior of a least one cell. Methods for introducing polynucleotides into various cell types are known in the art and include, but are not limited to, stable transfection methods, transient transfection methods, and virus-mediated methods. Such method include, but are not limited to, electroporation, intracytoplasmic injection, viral infection (including adenovirus, lentivirus, and retrovirus vectors), transfection, lipid-mediated transfection and/or Nucleofaction™. See, for example, Stadtfeld *et al.* (2009) *Nature Methods* 6(5):329-330; Yusa *et al.* (2009) *Nat. Methods* 6:363-369; Woltjen *et al*. (2009) Nature 458, 766-770. Such methods include, but are not limited to, direct delivery of DNA such as by ex vivo transfection (Wilson et al., Science, 244:1344-1346, 1989, Nabel and Baltimore, Nature 326:711-713, 1987), optionally with Fugene6 (Roche) or Lipofectamine (Invitrogen), by injection (U.S. Pat. Nos. 5,994,624, 5,981,274, 5,945,100, 5,780,448, 5,736,524, 5,702,932, 5,656,610, 5,589,466 and 5,580,859, each incorporated herein by reference), including microinjection (Harland and Weintraub, J. Cell Biol., 101:1094-1099, 1985; U.S. Pat. No. 5,789,215, incorporated herein by reference); by electroporation (U.S. Pat. No. 5,384,253, incorporated herein by reference; Tur-Kaspa et al., Mol. Cell Biol., 6:716-718, 1986; Potter et al., Proc. Nat'l Acad. Sci. USA, 81:7161-7165, 1984); by calcium phosphate precipitation (Graham and Van Der Eb, Virology, 52:456-467, 1973; Chen and Okayama, Mol. Cell Biol., 7(8):2745-2752, 1987; Rippe et al., Mol. Cell Biol., 10:689-695, 1990); by using DEAE-dextran followed by polyethylene glycol (Gopal, Mol. Cell Biol., 5:1188-1190, 1985); by direct sonic loading (Fechheimer et al., Proc. Nat'l Acad. Sci. USA, 84:8463-8467, 1987); by liposome mediated transfection (Nicolau and Sene, Biochim. Biophys. Acta, 721:185-190, 1982; Fraley et al., Proc. Nat'l Acad. Sci. USA, 76:3348-3352, 1979; Nicolau et al.,

Methods Enzymol., 149:157-176, 1987; Wong et al., Gene, 10:87-94, 1980; Kaneda et al., Science, 243:375-378, 1989; Kato et al., Biol. Chem., 266:3361-3364, 1991) and receptor-mediated transfection (Wu and Wu, Biochemistry, 27:887-892, 1988; Wu and Wu, J. Biol. Chem., 262:4429-4432, 1987); and any combination of such methods, each of which is incorporated herein by reference.

In some embodiments, the cells employed in the methods and compositions have a DNA construct stably incorporated into their genome. "Stably incorporated" or "stably introduced" means the introduction of a polynucleotide into the cell such that the nucleotide sequence integrates into the genome of the cell and is capable of being inherited by progeny thereof. Any protocol may be used for the stable incorporation of the DNA constructs or the various components employed to generate the targeted genomic modification.

Transfection protocols as well as protocols for introducing polypeptides or polynucleotide sequences into cells may vary. Non-limiting transfection methods include chemical-based transfection methods include the use of liposomes; nanoparticles; calcium phosphate (Graham et al. (1973). *Virology* 52 (2): 456–67, Bacchetti et al. (1977) *Proc Natl Acad Sci USA* 74 (4): 1590–4 and, Kriegler, M (1991). *Transfer and Expression: A Laboratory Manual*. New York: W. H. Freeman and Company. pp. 96–97); dendrimers; or cationic polymers such as DEAE-dextran or polyethylenimine. Non chemical methods include electroporation; Sono-poration; and optical transfection . Particle-based transfection include the use of a gene gun, magnet assisted transfection (Bertram, J. (2006) *Current Pharmaceutical Biotechnology* 7, 277–28). Viral methods can also be used for transfection.

A non-limiting example, of a method of introduction a heterologous polynucleotide into a rat ES cell follows. The rat ES cells, as described herein, are passaged for about 24 to about 48 prior to electroporation. About 24 hours prior to electroporation the media is changed to RVG2i + ROCKi (10μM Y-27632). The rat ES cells are trypsinized and the rat ES cells are isolated. The rat ES cells are suspended to achieve a final cell concentration of about 2 x 10^6 to about 10 x 10^6 cells per 75 ul. About 75λ of rat ES cells are added to about 50λ DNA comprising the heterologous polynucleotide and about 125λ EP buffer is added. In one non-limiting embodiment, the electroporation is carried out with the following parameters: 400V; 400V; Ω; 100 μF.

The cells are then cultured in RVG2i and 10μM ROCKi and can be transferred onto feeder cells.

ii. Selecting Rat Embryonic Stem Cells Having a Targeted Genomic Modification

Various method are provided for selecting and maintaining rat ES cells having stably incorporated into their genome a targeted genetic modification. In one non-limiting example, when introducing a heterologous polynucleotide into a rat ES cell, the method can comprise (a) providing an *in vitro* population of rat ES cells; (b) introducing into at least one rat ES cell a heterologous polynucleotide comprising a selection marker operably linked to a promoter active the rat ES cell; and, (c) culturing *in vitro* the rat ES cell population in an alternating first and second culture media wherein the first culture media comprises an effective amount of a selection agent for a first time period and the second culture media do not comprise the selection agent, wherein the *in vitro* culture conditions maintain pluripotency or totipotency; and thereby selecting the rat ES cell having stably integrated into its genome the heterologous polynucleotide. The various methods by which the rat ES cell having the targeted genetic modification can be selected in a given population can employ an *in vitro* culture system which allows the rat ES cells to maintain pluripotency. Thus, any of the *in vitro* culture medias and feeder cells discussed herein can be employed.

In specific embodiments, the first and the second culture media are alternated about every 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40 hours or more. In a specific embodiment, the first and second culture media are alternated every 24 hours.

Any appropriate selection marker can be used and the corresponding selection agent will be present at an effective concentration with the culture media. Such selection markers include any of the native, attenuated or non-attenuated selection marker discussed herein. In one embodiment, the selection marker employed imparts resistance to an antibiotic, including for example, G418. Non-limiting selection markers comprise neomycin phosphotransferase II (*npt*II) or hygromycin phosphotransferase (*hpt*).

The concentration of the selection agent is such as to allow for the selection of a rat ES cell having the selection marker while maintaining the pluipotency of the rat ES cells that are present within the culture. When employing, for example, G418, the concentration of the G418 in the selection media can range from about 50 ug/ml to about

125 ug/ml, about 60 ug/ml to about 125ug/ml, about 70 ug/ml to about 125ug/ml, about 80ug/ml to about 125ug/ml, about 90ug/ml to about 125ug/ml, about 100ug/ml to about 125 ug/ml, about 110 ug/ml to about 125ug/ml, about 80ug/ml to about 100ug/ml, about 65ug/ml to about 85ug/ml, about 70ug/ml to about 80ug/ml. In one embodiment, the concentration of G418 in the culture is 75 μg/ml.

The media employed in the selection allows the rat embryonic stem cells to retain pluripotency. Such media is described in detail elsewhere herein.

The selection protocol can be initiated at any time following the introduction of the polynucleotide encoding the selection marker into the genome of the rat ES cell. In specific embodiments, the selection protocol begins 10, 15, 20, 24, 30, 35, 40, 50, 60 or more hours after the introduction of the selection marker into the rat ES cell. In one embodiment, the selection protocol beings about 2 days following the introduction of the polynucleotide encoding the selection marker.

A non-limiting selection protocol employing G418 is as follows. Day 2, (2^{nd} day after the introduction of the polynucleotide encoding the selection marker) the population of rat ES cells is incubated cells in 2i media and G418 at 75 μg/ml. At day 3, the population of rat ES cells are incubated cells in 2i media without G418. At day 4, the population of rat ES cells are incubated in 2i media and G418 at 75 μg/ml. At day 5, the population of rat ES cells are incubated cells in 2i media without G418. At day 6, the population of rat ES cells are incubated in 2i media and G418 at 75 μg/ml. At day 7, the population of rat ES cells is incubated in 2i media without G418. At day 8, the population of rat ES cells is incubated in in 2i media without G418 and 75 μg/ml. At day 9, the population of rat ES cells is incubated cells in 2i media without G418. At day 10, the population of rat ES cells is incubated cells in 2i media and G418 at 75 μg/ml. At day 11, the population of rat ES cells is incubated cells in 2i media without G418. At day 12, colonies are picked for expansion and screening.

Following the selection of the rat ES cells having the selection marker, the colonies can be expanded. In specific embodiments, the period for expansion can be about 1, 2, 3, 4, 5, or more days in a culture condition that maintains the pluripotency of the cells. In one non-limiting embodiment, the selected colonies are expanded for 3 days. In a further embodiment, the media employed is a 2i media. Each clone can then be passed and further expanded.

The rat ES cells and cell lines having one or more of the targeted genetic modifications can have one or more any the following properties:

(a) have germ-line competency following targeted genetic modification, meaning when the rat ES cell is implanted into a rat host embryo, the targeted genetic modification within the genome of the rat ES cell line, is transmitted into an offspring;

(b) have pluripotency *in vitro*;

(c) have totipotency *in vitro*;

(d) when cultured *in vitro* loosely adhere to a feeder cell layer;

(e) when cultured *in vitro* form sphere-like colonies when plated on a feeder cell layer *in vitro*;

(f) maintain pluripotency when cultured *in vitro* under conditions comprising a feeder cell layer that is not genetically modified to express leukemia inhibitor factor (LIF), wherein the culture media comprises a sufficient concentration of LIF;

(g) maintain pluripotency when cultured *in vitro* under conditions comprising a feeder cell layer, wherein the culture media comprises mouse LIF or an active variant or fragment thereof;

(h) comprise a molecular signature characterized by

i) the expression of one or more of rat ES cell-specific genes comprising Adheres Junctions Associate Protein (Ajap1), Claudin 5 (Cldn5), Cdc42 guanine nucleotide exchange factor 9 (Arhgef9), Calcium/calmodulin-dependent protein kinase IV (Camk4), ephrin-A1 (Efna1), EPH receptor A4 (Epha4), gap junction protein beta 5 (Gjb5), Insulin-like growth factor binding protein-like 1 (Igfbpl1), Interleulin 36 beta(Il1f8), Interleukin 28 receptor, alpha (Il28ra), left-right determination factor 1 (Lefty1), Leukemia inhibitory factor receptor alpha (Lifr), Lysophosphatidic acid receptor 2 (Lpar2), Neuronal pentraxin receptor (Ntm), Protein tyrosine phosphatase non-receptor type 18 (Ptpn18), Caudal type homeobox 2 (Cdx2), Fibronectin type III and ankyrin repeat domains 1 (Fank1), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Lymphoid enhancer-binding factor 1 (Lef1), Sal-like 3 (Drosophila) (Sall3), SATB homeobox 1 (Satb1), miR-632, or a combination thereof;

ii) the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more of the rat ES cell-specific genes comprising Adheres Junctions Associate Protein (Ajap1), Claudin 5 (Cldn5), Cdc42 guanine

nucleotide exchange factor 9 (Arhgef9), Calcium/calmodulin-dependent protein kinase IV (Camk4), ephrin-A1 (Efna1), EPH receptor A4 (Epha4), gap junction protein beta 5 (Gjb5), Insulin-like growth factor binding protein-like 1 (Igfbpl1), Interleulin 36 beta(Il1f8), Interleukin 28 receptor, alpha (Il28ra), left-right determination factor 1 (Lefty1), Leukemia inhibitory factor receptor alpha (Lifr), Lysophosphatidic acid receptor 2 (Lpar2), Neuronal pentraxin receptor (Ntm), Protein tyrosine phosphatase non-receptor type 18 (Ptpn18), Caudal type homeobox 2 (Cdx2), Fibronectin type III and ankyrin repeat domains 1 (Fank1), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Lymphoid enhancer-binding factor 1 (Lef1), Sal-like 3 (Drosophila) (Sall3), SATB homeobox 1 (Satb1), miR-632, or a combination thereof;

iii) at least a 20-fold increase in the expression of one or more of the rat ES cell-specific genes as set forth in Table 14 when compared to a F1H4 mouse ES cell;

iv) at least a 20-fold increase in the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more of the rat ES cell-specific genes as set forth in Table 14 when compared to a F1H4 mouse ES cell;

v) the expression of one or more of rat ES cell-specific genes as set forth in Table 13;

vi) the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50 or more of the rat ES cell-specific genes as set forth in Table 13;

vii) at least a 20-fold increase in the expression of one or more of the rat ES cell-specific genes as set forth in Table 13 when compared to a F1H4 mouse ES cell;

viii) at least a 20-fold increase in the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50 or more of the rat ES cell-specific genes as set forth in Table 13 when compared to a F1H4 mouse ES cell;

ix) at least a 20-fold decrease in the expression of one or more of the rat ES cell-specific genes as set forth in Table 12 when compared to a F1H4 mouse ES cell; and/or

x) at least a 20-fold decrease in the expression of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50 or

more of the rat ES cell-specific genes as set forth in Table 12 when compared to a F1H4 mouse ES cell;

xi) any combination of expression of the rat ES cell-specific genes of parts (i)-(x);

xii) a relative expression level of pluripotency markers as shown in Table 15 for at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 or 18 of the listed pluripotency markers. See, pluripotency ranking column of Table 15 for relative expression levels;

xiii) a relative expression level of the mesodermal markers as shown in Table 15 for at least 2, 3, or 4 of the listed mesodermal markers. See, mesodermal ranking column in Table 15 for relative expression levels;

xiv) a relative expression level of endodermal markers as shown in Table 15 for at least 2, 3, 4, 5, or 6 of the listed endodermal markers. See, endodermal ranking column in Table 15 for relative expression levels;

xv) a relative expression level of neural markers as shown in Table 15 for at least 2 and 3 of the listed neural markers. See, neural ranking column in table 15 for relative expression levels;

xvi) a relative expression level of trophectoderm markers as shown in Table 15 for the listed trophectoderm markers. See, trophectoderm ranking column in table 15 for relative expression levels;

xvii) any relative expression level of one or more (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23,2 4, 25, 26, 27, 28, 29 or 30) of the pluripotency markers, mesodermal markers, endodermal markers, neural markers and/or trophectoderm markers set forth in Table 15;

xviii) the relative expression level of each of the markers set forth in table 15;

xix) any combination of the signatures set forth in xii-xiix; and/or

xx) any combination of the signature set forth in i-xiix.

(i) have the ability to produce a F0 rat;

(j) capable of being subcultured and maintaining the undifferentiated state;

(k) having the same number of chromosomes a normal rat cell;

(l) maintain pluripotency *in vitro* without requiring paracrine LIF signaling; and/or

(m) have self renewal, meaning they divide indefinitely while maintaining pluripotency.

iii. Expression Cassettes

The terms “polynucleotide,” “polynucleotide sequence,” “nucleic acid sequence,” and “nucleic acid fragment” are used interchangeably herein. These terms encompass nucleotide sequences and the like. A polynucleotide may be a polymer of RNA or DNA that is single- or double-stranded, that optionally contains synthetic, non-natural or altered nucleotide bases. A polynucleotide in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA, synthetic DNA, or mixtures thereof. Polynucleotides can comprise deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues, and any combination these. The polynucleotides provided herein also encompass all forms of sequences including, but not limited to, single-stranded forms, double-stranded forms, hairpins, stem-and-loop structures, and the like.

Further provided are recombinant polynucleotides. The terms “recombinant polynucleotide” and “recombinant DNA construct” are used interchangeably herein. A recombinant construct comprises an artificial or heterologous combination of nucleic acid sequences, e.g., regulatory and coding sequences that are not found together in nature. In other embodiments, a recombinant construct may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. Such a construct may be used by itself or may be used in conjunction with a vector. If a vector is used, then the choice of vector is dependent upon the method that is used to transform the host cells as is well known to those skilled in the art. For example, a plasmid vector can be used. Genetic elements required to successfully transform, select and propagate host cells and comprising any of the isolated nucleic acid fragments provided herein. Screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, immunoblotting analysis of protein expression, or phenotypic analysis, among others.

In specific embodiments, one or more of the components described herein can be provided in an expression cassette for expression in a rat cell. The cassette can include 5' and 3' regulatory sequences operably linked to a polynucleotide provided herein. “Operably linked” means a functional linkage between two or more elements. For

example, an operable linkage between a polynucleotide of interest and a regulatory sequence (i.e., a promoter) is a functional link that allows for expression of the polynucleotide of interest. Operably linked elements may be contiguous or non-contiguous. When used to refer to the joining of two protein coding regions, operably linked means that the coding regions are in the same reading frame. In another instance, a nucleic acid sequence encoding a protein may be operably linked to regulatory sequences (*e.g.*, promoter, enhancer, silencer sequence, *etc*.) so as to retain proper transcriptional regulation. The cassette may additionally contain at least one additional polynucleotide of interest to be co-introduced into the rat ES cell. Alternatively, the additional polynucleotide of interest can be provided on multiple expression cassettes. Such an expression cassette is provided with a plurality of restriction sites and/or recombination sites for insertion of a recombinant polynucleotide to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selection marker genes.

The expression cassette can include in the 5'-3' direction of transcription, a transcriptional and translational initiation region (i.e., a promoter), a recombinant polynucleotide provided herein, and a transcriptional and translational termination region (i.e., termination region) functional in mammalian cell or a host cell of interest. The regulatory regions (i.e., promoters, transcriptional regulatory regions, and translational termination regions) and/or a polynucleotide provided herein may be native/analogous to the host cell or to each other. Alternatively, the regulatory regions and/or a polynucleotide provided herein may be heterologous to the host cell or to each other. For example, a promoter operably linked to a heterologous polynucleotide is from a species different from the species from which the polynucleotide was derived, or, if from the same/analogous species, one or both are substantially modified from their original form and/or genomic locus, or the promoter is not the native promoter for the operably linked polynucleotide. Alternatively, the regulatory regions and/or a recombinant polynucleotide provided herein may be entirely synthetic.

The termination region may be native with the transcriptional initiation region, may be native with the operably linked recombinant polynucleotide, may be native with the host cell, or may be derived from another source (i.e., foreign or heterologous) to the promoter, the recombinant polynucleotide, the host cell, or any combination thereof.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation. Toward

this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

A number of promoters can be used in the expression cassettes provided herein. The promoters can be selected based on the desired outcome. It is recognized that different applications can be enhanced by the use of different promoters in the expression cassettes to modulate the timing, location and/or level of expression of the polynucleotide of interest. Such expression constructs may also contain, if desired, a promoter regulatory region (e.g., one conferring inducible, constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific/selective expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

iv. Generating F0 Rat Embryos and F1 Progeny Having the Targeted Genetic Modification

The various methods and compositions provided herein can be used to generate a genetically modified rat. Such methods generally comprise (a) introducing into the genome of an isolated rat ES cell disclosed herein a targeted genetic modification to form a rat ES cell having a genetic modification; (b) implanting at least one of the genetically modified rat ES cells having the genetic modification into a rat host embryo to produce a F0 embryo; (c) implanting the F0 embryo into a surrogate mother; (d) gestating the F0 embryo in the surrogate mother to term; and, (e) identifying a F0 rat having the genetic modification.

The genetically modified rat ES cells having the genetic modification can be implanted into a rat host embryo that is from the same rat strain or from a different rat strain. For example, a genetically modified DA rat ES cell can be implanted into a DA rat host embryo or it can be implanted into an SD host embryo, ACI host embryo or other heterologous rat host embryo. Similarly, a genetically modified ACI rat ES cell can be introduced into an ACI rat host embryo or it can be introduced into an SD host embryo, DA host embryo or other heterologous rat host embryo. Likewise, the surrogate mother can be from the same rat strain as the genetically modified rat cell and/or the rat host embryo or the surrogate mother can be from a different rat strain as the genetically

modified rat cell and/or the rat host embryo. In one non-limiting embodiment, the genetically modified rat cell is from a DA strain, the host rat embryo is from an SD host embryo and the surrogate mother is from a DA strain. In another non-limiting embodiment, the genetically modified rat cell is from an ACI strain, the host rat embryo is from an SD strain, and the surrogate mother is from a DA strain.

In still further embodiments, the chimeric rat (F0) can be breed to produce an F1 progeny that is heterozygous for the targeted genetic modification. In addition, the male rat of the F1 progeny can be breed with a female rat of the F1 progeny to obtain an F2 progeny that is homozygous for the genetic modification.

The methods and compositions provided herein allow for at least 1%, 3%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% or greater of the F0 F0 rats having the genetic modification to transmit the genetic modification to the F1 progeny.

In some embodiments, the rat ES cells having the targeted genetic modification are introduced into a pre-morula stage embryo from a corresponding organism, *e.g.*, an 8-cell stage mouse embryo. See, *e.g.*, US 7,576,259, US 7,659,442, US 7,294,754, and US 2008-0078000 A1, all of which are incorporated by reference herein in their entireties. In other embodiments, the For, the donor rat ES cells may be implanted at 4 cell stage, 8 cell stage of the host embryo.

The rat embryos comprising the genetically modified rat ES cells is incubated until the blastocyst stage and then implanted into a surrogate mother to produce an F0. Rats bearing the genetically modified genomic locus can be identified via modification of allele (MOA) assay as described herein. The resulting F0 generation derived from the genetically modified rat ES cells is crossed to a wild-type rat to obtain F1 generation offspring. Following genotyping with specific primers and/or probes, F1 rats that are heterozygous for the genetically modified genomic locus are crossed to each other to produce a rat that is homozygous for the genetically modified genomic locus.

Further provided is a F0 rat embryo comprising an inner cell mass having at least one heterologous stem cell comprising any one of the rat ES cells provided herein. In other embodiments, progeny of a rat F0 embryo are provided wherein at least 50%, 60%, 70% or more of the F0 progeny are derived from a genetically modified rat ES cell of as disclosed herein.

In one aspect, a method for making a rat ES cell is provided, comprising deriving from a morula-stage rat embryo, a blastocyst-stage rat embryo, or a rat embryo at a

developmental stage between a morula-stage embryo and a blastocyst-stage embryo a rat cell, and culturing the rat cell from the rat embryo under conditions sufficient to maintain pluripotency and/or totipotency. In on embodiment, the conditions sufficient to maintain pluripotency and/or totipotency include 2i media.

In one aspect, a method for making a genetically modified rat is provided, comprising a step of modifying a rat ES cell genome with a nucleic acid sequence of interest to form a modified rat ES cell, and employing the modified rat ES cell as a donor rat ES cell, combining the rat donor ES cell with a rat host embryo, culturing the donor ES cell and rat host embryo, and employing the cultured host embryo to make a genetically modified rat.

In one aspect, a method for making genetically modified rat F1 progeny is provided, comprising a step of modifying a rat ES cell genome with a nucleic acid sequence of interest to form a modified rat ES cell, and employing the modified rat ES cell as a donor rat ES cell, combining the rat donor ES cell with a rat host embryo, culturing the donor ES cell and rat host embryo, and employing the cultured host embryo to make a genetically modified rat, wherein the progeny are about 3%, about 10% or more, or about 63% or more derived from the genetically modified donor rat ES cell.

In one embodiment, the cultured host embryo is implanted into a surrogate rat mother, and the cultured host embryo is gestated in the surrogate mother.

In one aspect, a method of transmitting a genetic modification from a rat pluripotent cell to a rat progeny with high frequency is provided, comprising genetically modifying a pluripotent rat cell with a nucleic acid sequence of interest on a bacterial artificial chromosome to form a genetically modified rat pluripotent cell, and employing the genetically modified rat pluripotent cell with a rat host embryo in a rat surrogate mother to generate a progeny comprising the genetic modification and, optionally, breeding the progeny.

In one aspect, a method for making a rat ES cell is provided, wherein the method comprises culturing a frozen 8-cell stage embryo to a blastocyst stage, and deriving from the cultured blastocyst a rat cell, and culturing the rat cell under conditions sufficient to maintain pluripotency and/or totipotency.

V. Variants, Fragments and Sequence Identity

Active variants and fragments of the disclosed LIF polypeptide, particularly the mouse LIF polypeptide are provided herein. “Variants” refer to substantially similar

sequences. As used herein, a "variant polypeptide" is intended to mean a polypeptide derived from the native protein by deletion (so-called truncation) of one or more amino acids at the N-terminal and/or C-terminal end of the native protein; deletion and/or addition of one or more amino acids at one or more internal sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Variant polypeptides continue to possess the desired biological activity of the native polypeptide, that is, they inhibit the differentiation of rat and/or mouse embryonic stem cells and contribute to stem cell self-renewal. A variant of a polypeptide or disclosed herein (i.e. SEQ ID NOS: 1 or SwissProt Accession No. P09056) will typically have at least about 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity with the reference sequence.

The term "fragment" refers to a portion of an amino acid comprising a specified number of contiguous amino acid. In particular embodiments, a fragment of a polypeptide disclosed herein may retain the biological activity of the full-length polypeptide and hence inhibit the differentiation of rat and/or mouse embryonic stem cells and contribute to stem cell self-renewal. Fragments of a polypeptide sequence disclosed herein (i.e. SEQ ID NOS: 1 or SwissProt Accession No. P09056) may comprise at least 10, 15, 25, 30, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, contiguous amino acids, or up to the total number of amino acids present in a full-length protein.

As used herein, "sequence identity" or "identity" in the context of two polynucleotides or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example,

where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix; or any equivalent program thereof. "Equivalent program" means any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1: Rat ES Cell Derivation.

rESC Characterization. As shown in FIG. 1, rESC grow as compact spherical colonies which routinely detach and float in the dish (close-up, FIG. 4). . B, C, F, G: rESC express pluripotency markers including Oct-4 (FIG. 2A) and Sox2 (FIG. 2B), and express high levels of alkaline phosphatase (FIG. 3, left panel). Karyotype for line DA.2B is 42X,Y(FIG. 4, right panel). rESC often become tetraploid; thus, lines were

pre-screened by counting metaphase chromosome spreads; lines with mostly normal counts were then formally karyotyped.

ACI blastocysts were collected from super-ovulated females obtained commercially; DA blasts were cultured from frozen 8-cell embryos obtained commercially. Zona pellucidae were removed with Acid Tyrodes and blasts were plated onto mitotically inactivated MEFs. Outgrowths were picked and expanded using standard methods. All blasts were plated, cultured and expanded using 2i media (Li et al. (2008) Germline competent embryonic stem cells derived from rat blastocysts, Cell 135:1299-1310; incorporated herein by reference).

Table 3. Rat ES Cell Derivation

	ACI	DA
Embryo source	Blastocysts (Superovulation)	Frozen 8-cell embryos cultured to blastocyst
Blastocysts plated:	107	22
Outgrowths:	32 (30% of blasts)	10 (45% of blasts)
Lines:	16 (50% of outgrowths)	9 (90% of outgrowths)
Karyotyped:	3; all 42X,Y	6: 3 42X,X 3 42X,Y
GLT validated:	1 (ACI.G1)	1 42X,X (DA.2C) 1 42X,Y (DA.2B)

Example 2: Rat Production

Chimeric rats were produced by blastocyst injection and transmission of the rESC genome through the germline. Chimeras produced by blastocyst microinjection using parental ACI.G1 rESC are shown in FIG. 5. F1 agouti pups with albino littermates, sired by the ACI/SD chimera labeled with an asterisk (*) in FIG. 5 are shown in FIG. 6.

Germline transmission of parental rESC. Three euploid rESC lines were evaluated for pluripotency by microinjection into albino SD blastocysts. Chimeras were

identified by agouti coat color indicating rESC contribution. For each line, a majority of chimeras transmitted the rESC genome to F1 offspring (Table 4).

Table 4. Germline Transmission of Parental rESC

Line	Chimeras bred	Germline transmitters	Total pups from GLT chimeras	rESC-derived pups	GLT efficiency (%)
ACI.G1	5	3 (60%)	103	11	11
DA.2B	5	4 (80%)	129	11	9
DA.2C (XX)	3	2 (66%)	45	7	16

Example 3: rESC Targeting: The Rat Rosa 26 Locus.

The rRosa26 locus lies between the Setd5 and Thumpd3 genes as in mouse, with the same spacing. The rRosa 26 locus (FIG. 7, Panel B) differs from the mRosa 26 locus (FIG. 7, Panel A). The mRosa26 transcripts consist of 2 or 3 exons. The rat locus contains a 2nd exon 1 (Ex1b) in addition to the homologous exon to mouse exon1 (Ex1a). No 3rd exon has been identified in rat. Targeting of a rRosa26 allele is depicted in FIG. 7 (bottom), where homology arms of 5kb each were cloned by PCR using genomic DNA from DA rESC. The targeted allele contains a SA-lacZ-hUb-neo cassette replacing a 117bp deletion in the rRosa26 intron.

Targeting efficiency at the rRosa 26 locus was determined (Table 5). Linearized vector was electroporated into DA or ACI rESC, and transfected colonies were cultured in 2i media + G418, using standard techniques. Individual colonies were picked and

screened using a Loss of Allele (LOA) assay (Valenzuela, D. et al. (2003) High-throughput engineering of the mouse genome coupled with high-resolution expression analysis, Nature Biotech. 21:652-660, incorporated herein by reference).

Table 5. rRosa26 Targeting Efficiency			
Cell line	Colonies picked	Reconfirmed positives	Targeting efficiency (%)
DA.2B	192	4	2.1
ACI.G1	96	4	4.2

Chimera production and germline transmission using targeted Rosa26 rESC. Reconfirmed targeted rRosa26 clones were microinjected into SD blastocysts, which were then transferred to pseudopregnant DS recipient females, using standard techniques. Chimeras were identified by coat color; male F0 chimeras were bred to SD females. Germline (agouti) F1 pups were genotyped for the presence of the targeted Rosa26 allele; nine of 22 agouti pups genotyped as heterozygous at the Rosa26 locus (Table 6).

Table 6. Germline Transmission Using Targeted Rosa26 rESC						
Cell line	R26 clones injected	Clones producing Chimeras	Germline Transmitting Clones	Total Pups	rESC-derived Pups	ESC-derived pups (%)
DA.2B	4	3	2	AH7: 64 AE3: 112	AH7: 41 AE3: 6	AH7: 63 AE3: 3
ACI.G1	4	4	1	DE9: 39	DE9: 4	10

Example 3: Derivation of Rat Embryonic Stem Cells.

Superovulation protocol, rats

Day 0: injected with pregnant mare serum: IP, 20 U (0.4 ml).

Day 1: no action

Day 2: (46 hr. later): injected with hCG, IP, 50 U (1 ml).

- set up single female matings.

Day 3: checked plugs. Females were plugged. This is day 0.5.

Day 6 (e3.5): Euthanized females and flushed embryos.

ES Cell derivation protocol (superovulation)

Day 0:

1) Euthanized female rat with CO_2.
2) Swabbed ventral abdomen with 70% ethanol; using scissors, opened the ventral body wall to expose the viscera.
3) Dissected out the oviducts and uterine horns and placed them into a tissue culture dish containing warm N2B27 media. Washed out as much blood as possible and transferred to a new dish with N2B27.
4) Using a 1 ml syringe and a blunt 27g needle, flushed media through the uterine horns and oviducts to eject blastocysts into the media.
5) Collected the blastocysts with a mouth pipet and transfer to embryo culture dish containing KSOM + 2i (1μMPD0325901, 3 μM CHIR99021). KSOM is a culture medium produced by Millipore. Catalog number is MR-106-D.
6) Cultured overnight at 37°; 7.5% CO_2.

ES Cell derivation protocol (frozen embryos)

Day 0:

1) Thawed frozen 8-cell embryos (commercially obtained) into M2 medium. Cultured 10 minutes at room temperature.
2) Transferred to KSOM + 2i and culture overnight.

ES Cell derivation protocol (same for both)

Day 1:

1) Transferred cavitated embryos to 2i medium & culture overnight.
2) Continued culturing un-cavitated embryos in KSOM +2i

Day 2:

1) Transferred all remaining embryos to 2i medium (whether or not they've cavitated).
2) Cultured overnight; continued culturing earlier embryos in 2i medium.

Day 3:

1) Transferred embryos for 30 – 60 seconds with Acid Tyrodes to remove the zona pellucida.
2) Washed embryos 3x in 2i medium to remove Acid Tyrodes.
3) Deposited each embryo into a separate well of a 96-well feeder plate (the well contains a monolayer of mitotically inactivated mouse embryonic fibroblasts (MEFs).
4) Cultured overnight in 2i medium.

Day 4 – 5:

1) Monitored plated embryos for the presence of an outgrowth (an amorphous undifferentiated mass of cells). Outgrowths are ready for transfer when they are approximately twice the size of the plated embryo.
2) Each day: remove spent media with a mircropipet and replace with fresh 2i media.
3) Transferred outgrowths to new feeder wells:
    a. Removed spent media and gently wash well with PBS.
    b. Removed PBS and add 30 μl 0.05% trypsin; incubate for 10 minutes.
    c. Stopped trypsin reaction by adding 30μl 2i + 10% FBS.
    d. Gently dissociated the cells with a micropipettor and transferred entire contents of the well to a new well in a 24-well feeder plate. This was Passage 1 (P1).
    e. Cultured overnight in 2i medium.

Day 5 – 8: (timing depends on how fast each line expands)

1) Changed media each day (2i media) and monitored for the presence of colonies with an ESC morphology.

2) When colonies appear, continued culturing until colonies expand to ~ 50% confluency.
3) Tryspinzed and passaged colonies as before; plated on feeders, 1 well per line, in a 6-well dish. This was Passage 2 (P2).

Ongoing:

1) Continued feeding and monitoring each line until approximately 50% confluent.
2) Trypsinized cells as usual.
3) stopped trypsin with 2i + 10% FBS; pelleted the cells by centrifugation (5', 1200 rpm in Beckman-Coulter tabletop centrifuge).
4) Aspirated the supernatant and gently resuspend the cells in 400 μl Freezing Medium (70% 2i, 20% FBS, 10% DMSO).
5) Distributed the cells into 2 vials and freeze at -80°. This was Passage 3 (P3).
6) For long-term storage, transferred the vials to liquid N_2 storage.

The 2i media was prepared as follows in Table 7.

Reagent	Vendor	Concentration
DMEM/F12 basal media	Invitrogen/Life Technologies	1x
Neurobasal media	Invitrogen/Life Technologies	1x
Penicillin/streptomycin	Invitrogen/Life Technologies	1%
L-Glutamine	Invitrogen/Life Technologies	4 mM
2-Mercaptoethanol	Invitrogen/Life Technologies	0.1 mM
N2 supplement	Invitrogen/Life Technologies	1x
B27 supplement	Invitrogen/Life Technologies	1x
LIF	Millipore	100 U/ml
PD0325901 (MEK inhibitor).	Stemgent	1 uM
CHIR99021 (GSK inhibitor).	Stemgent	3 uM

Materials: Pregnant Mare's Serum Gonadotropin (PMSG)
Human Pregnancy Urine Chorionic Gonadotropin (HCG)
Female Rats (5-12 weeks old)
Male rats (12 wks. to 8 mos. old), one per cage
Syringes/needles
Animal room with lights on 6:00-18:00

Procedure:

Day 1: 8:00-10:00 AM
Inject females with 20 IU PMSG (0.4 ml), IP
Discard unused PMSG.
Day 3: 8:00-10:00 AM (48 hours after PMSG injection)
Inject females with 50 IU HCG (1 ml), IP
Place one female per male in mating cage.
Discard unused HCG.
Day 4: 8:00-10:00 AM (24 hrs. after HCG injection)
Check females for plugs.

Hormone suppliers

PMSG: Sigma #G-4877 (1000 IU). Resuspend in PBS to a final [ ] of 50 IU/ml. Store at -20° in 1 ml aliquots.

HCG: Sigma #CG-5 (5000 IU). Resuspend in PBS to a final [ ] of 50 IU/ml. Store at -20° in 1 ml aliquots.

Example 4: Karyotyping of Rat Embryonic Stem Cell Lines

The rat ES cell line generated herein were karyotyped, and the results are summarized in Tables 8-11.

Table 8

ACI.G1 Karyotyping Results	**Number of cells**
Number of cells karyotyped	7
Number of cells analyzed	20
Number of 42, XY cells	18
Number of abnormal cells	2
40, XY, -5, -9	1
41, XY, -14	1
42, XY	18
Other notes: Two analyzed cells were missing different autosomes, which may be a sporadic occurrence due to technical artifact. 90% of analyzed cells had a normal male 42, XY karyotype.	
Figure 9 provides a photograph showing the analysis of the chromosome number of the ACI.G1 rat ES cell line.	

Table 9

DA.2B Karyotyping Results	**Number of cells**
Number of cells karyotyped	6
Number of cells analyzed	20
Number of 42, XY cells	20
Number of abnormal cells	0
42, XY	20
Other notes: All analyzed cells had a normal diploid 42, XY karyotype.	
Figure 10 provides a photograph showing the analysis of the chromosome number of the DA.2B rat ES cell line.	

Table 10

DA.C2 Karyotyping Results	**Number of cells**
Number of cells karyotyped	5
Number of cells analyzed	20
Number of 42, XY cells	20
Number of abnormal cells	0
42, XX	
Other notes: 100% of analyzed cells had normal female XX rat karyotype.	
Figure 11 provides a photograph showing the analysis of the chromosome number of the DA.C2 rat ES cell line.	

5 Table 11

Strain	Blastocysts plated	Lines established	Lines Karyotyped	Karyotypes
BN x SD F1	41	8 (20%)	5	all lines were high % complex polyploid
ACI	27	16 (60%)	3	G1: 90% 42 XY; others were 70-85% euploid
DA	20	9 (45%)	6	2B: 100% 42 XY; 2C: 100% 42 XX; others were 95-100% euploid
F344	4	1 (25%)	0	
Totals	92	34 (37%)		

Example 5: Electroporation of Vector into Rat Embryonic Stem Cell

1. Passaged rat ES cells 24-48 hrs prior to electroporation.
2. Changed media to RVG2i + ROCKi (10μM Y-27632) 24 hr. prior to electroporation
3. Changed media 30' prior to trypsinization.
4. Aliquoted DNA to be electroporated.
5. Allowed DNA to warm at RT for >10 min.
6. Heated DNA for 5' @ 62°C. Place DNA on ice.
7. Trypsinized cells:
    a. Collected floating colonies. Washed plate to collect as many floaters as possible.
    b. Pelleted colonies: 3' @ 750 rpm.
    c. Washed pellet 1x with 5-10ml PBS and re-spin/pellet
    d. Aspirated supernatant; add 500λ trypsin, 0.05% + 1% chicken serum.
        i. Did not pool more than 1 10 cm plate of colonies per tube. If there are too many colonies packed into the bottom of the tube during trypsinization they will clump and most of the cells will be lost.
    e. 4' @ 37°. Pipeted colonies several times to minimize clumping.
    f. Repeated steps 1-2 X: 4' @ 37°.
    g. Stopped trypsin with 500λ RVG2i + 10% FBS.
8. Pelleted cells: 5' @ 1200 rpm.
9. Resuspend cells in 10 ml PBS. Count two 20λ aliquots to determine total cell number.
10. Pelleted cells (5'/1200rpm); calculate total cell number and total resuspension volume to achieve correct cell concentration (target #/75 μl EP buffer).
11. Resuspend in a minimal volume of EP buffer; measure total volume and adjust to target volume with EP buffer. Electroporation buffer is sold by Millipore. The catalog # is ES-003-D. See, Valenzuela *et al.* (2003) *Nature Biotechnology* 21:652-659, which is herein incorporated by reference.
12. Add 75λ cells to 50λ DNA; transfer the 125λ cells/DNA solution to one well of a BTX 48-well cuvette.
    a. Filled the empty wells in the same column with 125λ EP buffer.
13. Pulsed the cuvette once in the BTX electroporator:

a. Settings: 400V; Ω; 100 μF (settings may vary)

14. Placed cuvette on ice for 15' to recover.

15. Removed cells into 5 ml RVG2i + 10μM ROCKi.

16. Added to 15 cm plate with 20 ml RVG2i + 10μM ROCKi. Plate has 2x neoR MEFs (or other MEFs depending on project). The neoR selectable marker is the neomycin phosphotransferase (*neo*) gene of Beck *et al.* (1982) Gene, 19:327-36 or in US Patent No, 7,205,148 or 6,596,541, each of which are herein incorporated by reference.

17. Incubated @ 37°. Begin selection 48hrs later.

ROCK inhibitor used was Y-27632.

Example 6: Selecting Targeted Genetic Modification in a Rat Embryonic Stem Cell.

1. Passaged cells for 24-48 hrs prior to electroporation.
2. Changed media to RVG2i + ROCKi (10μM Y-27632) 24 hr. prior to electroporation
3. Changed media 30' prior to trypsinization.
4. Aliquoted DNA to be electroporated.
5. Allowed DNA warm at RT for >10 min.
6. Heated DNA for 5' @ 62°C. Place DNA on ice.
7. Trypsinized cells:
    h. Collected floating colonies. Washed plate to collect as many floaters as possible.
    i. Pelleted colonies: 3' @ 750 rpm.
    j. Washed pellet 1x with 5-10ml PBS and re-spin/pellet
    k. Aspirated supernatant; add 500λ trypsin, 0.05% + 1% chicken serum.
        i. Did not pool more than 1 10 cm plate of colonies per tube. If there are too many colonies packed into the bottom of the tube during trypsinization they will clump and most of the cells will be lost.
    l. 4' @ 37°. Pipeted colonies several times to minimize clumping
    m. Repeated 1-2 X: 4' @ 37°.
    n. Stopped trypsin with 500λ RVG2i + 10% FBS.
8. Pelleted cells: 5' @ 1200 rpm.

9. Resuspended cells in 10 ml PBS. Count two 20λ aliquots to determine total cell number.

10. Pelleted cells (5'/1200rpm); calculate total cell number and total resuspension volume to achieve correct cell concentration (target #/75 μl EP buffer).

11. Resuspend in a minimal volume of EP buffer; measured total volume and adjusted to target volume with EP buffer.

12. Added 75λ cells to 50λ DNA; transfer the 125λ cells/DNA solution to one well of a BTX 48-well cuvette.

    a. Filled the empty wells in the same column with 125λ EP buffer.

13. Pulsed the cuvette once in the BTX electroporator:

    a. Settings: 400V; 400V; Ω; 100 μF (settings may vary)

14. Placed cuvette on ice for 15' to recover.

15. Removed cells into 5 ml RVG2i + 10μM ROCKi.

16. Added to 15 cm plate with 20 ml RVG2i + 10μM ROCKi. Plate had 2x neoR MEFs (or other MEFs depending on project).

17. Incubated @ 37°. Began selection 48hrs later.

18. G418 selection protocol was as follows:

    a. Day 2 (2^{nd} day after EP): incubated cells in 2i media + G418, 75 μg/ml.
    b. Day 3: incubated cells in 2i media without G418
    c. Day 4: incubated cells in 2i media + G418, 75 μg/ml.
    d. Day 5: incubated cells in 2i media without G418
    e. Day 6: incubated cells in 2i media + G418, 75 μg/ml.
    f. Day 7: incubated cells in 2i media without G418
    g. Day 8: incubated cells in 2i media + G418, 75 μg/ml.
    h. Day 9: incubated cells in 2i media without G418
    i. Day 10: incubated cells in 2i media + G418, 75 μg/ml.
    j. Day 11: incubated cells in 2i media without G418
    k. Day 12: picked colonies to expand for screening. Each colony was dissociated in 0.05% trypsin + 1% chicken serum for 10 minutes and then plated into 1 well of a 96-well feeder plate.

19. Expanded colonies for 3 days in 2i media.

20. Passaged clones 1:1 to new 96-well feeder plates.

21. Expanded clones for 3 days in 2i media.

22. For each clone, dissociated colonies in trypsin. Froze 2/3 of each clone and store at -80°; plated the remaining 1/3 onto laminin plates (96-well plates coated with 10 μg/ml laminin).

23. When the laminin plates were confluent, passed off to the screening lab for genotyping of the clones.

Example 7. Molecular Signature of the Rat Embryonic Stem Cells

The genes listed in Table 13 were expressed at levels 20-fold higher in rat ES cells than the corresponding genes in mouse ES cells. The genes listed in Table 12 were expressed at 20-fold lower in rat ES cells than the corresponding genes in mouse ES cells.

The microarray data in Tables 12 and 13 was generated as follows. Rat ES cells (ACI.G2 and DA.2B) and mouse ES cells (F1H4) were cultured in 2i media for 3 passages until confluent. F1H4 cells were cultured on gelatin-coated plates in the absence of feeders. F1H4 mouse ES cells were derived from 129S6/SvEvTac and C57BL/6NTac heterozygous embryos (see, e.g., US Pat. No. 7,294,754 and Poueymirou, W.T., Auerbach, W., Frendewey, D., Hickey, J.F., Escaravage, J.M., Esau, L., Dore, A.T., Stevens, S., Adams, N.C., Dominguez, M.G., Gale, N.W., Yancopoulos, G.D., DeChiara, T.M., Valenzuela,D.M. (2007), incorporated by reference herein in its entirety).

The following protocol was used for sample prep:

Materials included TRIzol Reagentp; RNA Lysis Buffer (Zymo Kit); and 1.5 mL Eppendorf tubes.

The 1.5mL Eppendorf tubes were labeled with the Sample ID. Cells grown on a plate were rinsed in 37C PBS. PBS was removed and 300 ul of Trizol was added. A scraper was used to break the cells in Trizol. The lysed cells were collected in Trizol in a 1.5mL Epperdorf tube. For cells grown on suspension, the cells were rinsed in 37C PBS. The cells were collected in a 1.5mL tube, the cells were spun down, PBS was removed and 300 ul of Trizol was added. The cells were pipeted up and down to break the cells. Samples were sorted for FACS with 10^1 to 10^5 cells, the volume was concentrated to less than 100uL. 4 volumes of RNA Lysis buffer was added and mix by pipetting. For sample, 320uL RNA Lysis buffer was added to 80uL sample. Samples were stored at –

20°C.

RNA-Seq was used to measure the expression level of mouse and rat genes. Sequencing reads were mapped to mouse and rat reference genome by Tophat, and RPKM (fragments per kilobase of exon per million fragments mapped) were calculated for mouse and rat genes. Homology genes based on gene symbol were selected, and then used t-test to compare gene's expression level between mouse and rat.

miR-632 is in the top 10 highest expressed in rat ESCs but they were not expressed in mouse ES cells. There is therefore no comparative data for these genes. Based on the levels of expression compared to other genes and their known function in the embryonic development, the expression of miR-632 were used as a marker for rat ES cells.

Table 12. The genes listed were expressed at levels 20-fold lower in rat ES cells than the corresponding genes in mouse ES cells.

ID	Notes	Symbol	Entrez Gene Name	Location	Type(s)	Drug(s)
Abcb1b		Abcb1b	ATP-binding cassette, sub-family B (MDR/TAP), member 1B	Plasma Membrane	transporter	
Acta2		ACTA2	actin, alpha 2, smooth muscle, aorta	Cytoplasm	other	
Actg2		ACTG2	actin, gamma 2, smooth muscle, enteric	Cytoplasm	other	
Aebp1		AEBP1	AE binding protein 1	Nucleus	peptidase	
Angptl2		ANGPTL2	angiopoietin-like 2	Extracellular Space	other	
Ankrd1		ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	Cytoplasm	transcription regulator	
Anxa1		ANXA1	annexin A1	Plasma Membrane	other	hydrocortisone
Anxa6		ANXA6	annexin A6	Plasma Membrane	other	
Anxa8		ANXA8L2 (includes others)	annexin A8-like 2	Plasma Membrane	other	
Arhgef25		ARHGEF25	Rho guanine nucleotide exchange factor (GEF) 25	Cytoplasm	other	
Axl		AXL	AXL receptor tyrosine kinase	Plasma Membrane	kinase	cabozantinib
Basp1		BASP1	brain abundant,	Nucleus	transcriptio	

			membrane attached signal protein 1		n regulator	
Bgn		BGN	biglycan	Extracellular Space	other	
Bst2		BST2	bone marrow stromal cell antigen 2	Plasma Membrane	other	
Btf3		BTF3	basic transcription factor 3	Nucleus	transcriptio n regulator	
Btg2		BTG2	BTG family, member 2	Nucleus	transcriptio n regulator	
Capsl		CAPSL	calcyphosine-like	Other	other	
Cav1		CAV1	caveolin 1, caveolae protein, 22kDa	Plasma Membrane	transmemb rane receptor	
Ccdc8 0		CCDC80	coiled-coil domain containing 80	Nucleus	other	
Ccnd 2		CCND2	cyclin D2	Nucleus	other	
Cd24 8		CD248	CD248 molecule, endosialin	Plasma Membrane	other	
Cd44		CD44	CD44 molecule (Indian blood group)	Plasma Membrane	enzyme	
Cd97		CD97	CD97 molecule	Plasma Membrane	G-protein coupled receptor	
Cdc4 2ep5		CDC42E P5	CDC42 effector protein (Rho GTPase binding) 5	Cytoplasm	other	
Cdh1 1		CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)	Plasma Membrane	other	
Cdkn 2a		CDKN2 A	cyclin-dependent kinase inhibitor 2A	Nucleus	transcriptio n regulator	
Cdo1		CDO1	cysteine dioxygenase type 1	Cytoplasm	enzyme	
Clip3		CLIP3	CAP-GLY domain containing linker protein 3	Cytoplasm	other	
Cln5		CLN5	ceroid-lipofuscinosis, neuronal 5	Cytoplasm	other	
Cnn1		CNN1	calponin 1, basic, smooth muscle	Cytoplasm	other	
Col1a 1		COL1A1	collagen, type I, alpha 1	Extracellular Space	other	collagenase clostridium histolyticum
Col1a 2		COL1A2	collagen, type I, alpha 2	Extracellular Space	other	collagenase clostridium histolyticum
Col3a 1		COL3A1	collagen, type III, alpha 1	Extracellular Space	other	collagenase clostridium histolyticum
Col5a 2		COL5A2	collagen, type V, alpha 2	Extracellular Space	other	collagenase clostridium histolyticum
Col6a 2		COL6A2	collagen, type VI, alpha 2	Extracellular Space	other	collagenase clostridium

						histolyticum
Cryab		CRYAB	crystallin, alpha B	Nucleus	other	
Csf1		CSF1	colony stimulating factor 1 (macrophage)	Extracellular Space	cytokine	
Cth		CTH	cystathionase (cystathionine gamma-lyase)	Cytoplasm	enzyme	
Cthrc1		CTHRC1	collagen triple helix repeat containing 1	Extracellular Space	other	
Ctsc		CTSC	cathepsin C	Cytoplasm	peptidase	
Cyr61		CYR61	cysteine-rich, angiogenic inducer, 61	Extracellular Space	other	
Ddx58		DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	Cytoplasm	enzyme	
Dkk3		DKK3	dickkopf WNT signaling pathway inhibitor 3	Extracellular Space	cytokine	
Dmc1		DMC1	DNA meiotic recombinase 1	Nucleus	enzyme	
Dpysl3		DPYSL3	dihydropyrimidinase-like 3	Cytoplasm	enzyme	
Dse		DSE	dermatan sulfate epimerase	Cytoplasm	enzyme	
Dusp1		DUSP1	dual specificity phosphatase 1	Nucleus	phosphatase	
Dusp27		DUSP27	dual specificity phosphatase 27 (putative)	Other	phosphatase	
Dusp9		DUSP9	dual specificity phosphatase 9	Nucleus	phosphatase	
Ece2		ECE2	endothelin converting enzyme 2	Plasma Membrane	peptidase	
Ecm1		ECM1	extracellular matrix protein 1	Extracellular Space	transporter	
Egr1		EGR1	early growth response 1	Nucleus	transcription regulator	
Emp1		EMP1	epithelial membrane protein 1	Plasma Membrane	other	
Emp3		EMP3	epithelial membrane protein 3	Plasma Membrane	other	
Ephx2		EPHX2	epoxide hydrolase 2, cytoplasmic	Cytoplasm	enzyme	
F3		F3	coagulation factor III (thromboplastin, tissue factor)	Plasma Membrane	transmembrane receptor	activated recombinant human factor VII
Fau		FAU	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed	Cytoplasm	other	

Fbn1		FBN1	fibrillin 1	Extracellular Space	other	
Fbxo15		FBXO15	F-box protein 15	Other	transcription regulator	
Fhl2		FHL2	four and a half LIM domains 2	Nucleus	transcription regulator	
Flnc		FLNC	filamin C, gamma	Cytoplasm	other	
Fos		FOS	FBJ murine osteosarcoma viral oncogene homolog	Nucleus	transcription regulator	
Fundc2		FUNDC2	FUN14 domain containing 2	Cytoplasm	other	
Gjb3		GJB3	gap junction protein, beta 3, 31kDa	Plasma Membrane	transporter	
Gpa33		GPA33	glycoprotein A33 (transmembrane)	Plasma Membrane	other	
Gpbp1l1		GPBP1L1	GC-rich promoter binding protein 1-like 1	Other	other	
Gpc3		GPC3	glypican 3	Plasma Membrane	other	
Grb10		GRB10	growth factor receptor-bound protein 10	Cytoplasm	other	
Gstm1		GSTM5	glutathione S-transferase mu 5	Cytoplasm	enzyme	
Hap1		HAP1	huntingtin-associated protein 1	Cytoplasm	other	
Hist1h2bc		HIST2H2BE (includes others)	histone cluster 2, H2be	Nucleus	other	
Hmga2		HMGA2	high mobility group AT-hook 2	Nucleus	enzyme	
Hmgn3		Hmgn3	high mobility group nucleosomal binding domain 3	Nucleus	other	
Hormad1		HORMAD1	HORMA domain containing 1	Nucleus	other	
Hsd17b14		HSD17B14	hydroxysteroid (17-beta) dehydrogenase 14	Cytoplasm	enzyme	
Hspb1		HSPB1	heat shock 27kDa protein 1	Cytoplasm	other	
Hspb8		HSPB8	heat shock 22kDa protein 8	Cytoplasm	kinase	
Htra1		HTRA1	HtrA serine peptidase 1	Extracellular Space	peptidase	
Ifi204		Ifi204 (includes others)	interferon activated gene 204	Nucleus	transcription regulator	
Ifi44		IFI44	interferon-induced protein 44	Cytoplasm	other	
Ifit1		IFIT1B	interferon-induced protein with tetratricopeptide repeats 1B	Cytoplasm	other	

Ifitm3		IFITM2	interferon induced transmembrane protein 2	Cytoplasm	other	
Igf2		IGF2	insulin-like growth factor 2 (somatomedin A)	Extracellular Space	growth factor	
Igfbp7		IGFBP7	insulin-like growth factor binding protein 7	Extracellular Space	transporter	
Il1rl1		IL1RL1	interleukin 1 receptor-like 1	Plasma Membrane	transmembrane receptor	
Inhba		INHBA	inhibin, beta A	Extracellular Space	growth factor	
Inhbb		INHBB	inhibin, beta B	Extracellular Space	growth factor	
Irf7		IRF7	interferon regulatory factor 7	Nucleus	transcription regulator	
Isg15		ISG15	ISG15 ubiquitin-like modifier	Extracellular Space	other	
Itga5		ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	Plasma Membrane	transmembrane receptor	
Jun		JUN	jun proto-oncogene	Nucleus	transcription regulator	
Junb		JUNB	jun B proto-oncogene	Nucleus	transcription regulator	
Lgals3bp		LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein	Plasma Membrane	transmembrane receptor	
Lgals9		LGALS9	lectin, galactoside-binding, soluble, 9	Extracellular Space	other	
Lmna		LMNA	lamin A/C	Nucleus	other	
Lox		LOX	lysyl oxidase	Extracellular Space	enzyme	
Loxl2		LOXL2	lysyl oxidase-like 2	Extracellular Space	enzyme	
Loxl3		LOXL3	lysyl oxidase-like 3	Extracellular Space	enzyme	
Lrp1		LRP1	low density lipoprotein receptor-related protein 1	Plasma Membrane	transmembrane receptor	
Mageb16		MAGEB16	melanoma antigen family B, 16	Other	other	
Mcam		MCAM	melanoma cell adhesion molecule	Plasma Membrane	other	
Mgp		MGP	matrix Gla protein	Extracellular Space	other	
Mmp2		MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	Extracellular Space	peptidase	marimastat
Mxra8		MXRA8	matrix-remodelling associated 8	Other	other	

Myl9		MYL9	myosin, light chain 9, regulatory	Cytoplasm	other	
Mylpf		MYLPF	myosin light chain, phosphorylatable, fast skeletal muscle	Cytoplasm	other	
Nab2		NAB2	NGFI-A binding protein 2 (EGR1 binding protein 2)	Nucleus	transcription regulator	
Ndufb4		NDUFB4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 4, 15kDa	Cytoplasm	transporter	
Npm1		NPM1	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	Nucleus	transcription regulator	
Nr0b1		NR0B1	nuclear receptor subfamily 0, group B, member 1	Nucleus	ligand-dependent nuclear receptor	
Nr4a1		NR4A1	nuclear receptor subfamily 4, group A, member 1	Nucleus	ligand-dependent nuclear receptor	
Nrp2		NRP2	neuropilin 2	Plasma Membrane	kinase	
Oas1a		OAS1	2'-5'-oligoadenylate synthetase 1, 40/46kDa	Cytoplasm	enzyme	
Oasl2		Oasl2	2'-5' oligoadenylate synthetase-like 2	Other	enzyme	
P4ha2		P4HA2	prolyl 4-hydroxylase, alpha polypeptide II	Cytoplasm	enzyme	
Parp3		PARP3	poly (ADP-ribose) polymerase family, member 3	Nucleus	enzyme	
Pcolce		PCOLCE	procollagen C-endopeptidase enhancer	Extracellular Space	other	
Pcyt1b		PCYT1B	phosphate cytidylyltransferase 1, choline, beta	Cytoplasm	enzyme	
Pdgfc		PDGFC	platelet derived growth factor C	Extracellular Space	growth factor	
Phlda1		PHLDA1	pleckstrin homology-like domain, family A, member 1	Cytoplasm	other	
Phlda2		PHLDA2	pleckstrin homology-like domain, family A, member 2	Cytoplasm	other	
Pla2g1b		PLA2G1B	phospholipase A2, group IB (pancreas)	Extracellular Space	enzyme	niflumic acid
Pla2g4a		PLA2G4A	phospholipase A2, group IVA	Cytoplasm	enzyme	quinacrine

			(cytosolic, calcium-dependent)			
Porcn		PORCN	porcupine homolog (Drosophila)	Cytoplasm	other	
Postn		POSTN	periostin, osteoblast specific factor	Extracellular Space	other	
Prrx1		PRRX1	paired related homeobox 1	Nucleus	transcription regulator	
Prss23		PRSS23	protease, serine, 23	Extracellular Space	peptidase	
Psmb8		PSMB8	proteasome (prosome, macropain) subunit, beta type, 8	Cytoplasm	peptidase	
Ptgs2		PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	Cytoplasm	enzyme	acetaminophen/pentazocine, acetaminophen/clemastine/pseudoephedrine, aspirin/butalbital/caffeine, acetaminophen/caffeine/dihydrocodeine, aspirin/hydrocodone, aspirin/oxycodone, acetaminophen/aspirin/caffeine, aspirin/pravastatin, acetaminophen/dexbrompheniramine/pseudoephedrine, aspirin/meprobamate, aspirin/caffeine/propoxyphene, aspirin/butalbital/caffeine/codeine, aspirin/caffeine/dihydrocodeine, chlorpheniramine/ibuprofen/pseudoephedrine, licofelone, menatetrenone, icosapent, suprofen, lornoxicam, tiaprofenic acid, lumiracoxib, tenoxicam, naproxen/sumatriptan,

						ibuprofen/phenylephrine, acetaminophen/aspirin/codeine, naproxen/esomeprazole, famotidine/ibuprofen, ibuprofen/phenylephrine/chlorpheniramine, diclofenac/misoprostol, acetaminophen/butalbital/caffeine, hydrocodone/ibuprofen, acetaminophen/hydrocodone, acetaminophen/tramadol, acetaminophen/codeine, acetaminophen/oxycodone, acetaminophen/propoxyphene, niflumic acid, nitroaspirin, ketoprofen, diclofenac, etoricoxib, naproxen, meclofenamic acid, pomalidomide, meloxicam, celecoxib, dipyrone, nimesulide, acetaminophen, mefenamic acid, diflunisal, ibuprofen, GW406381X, phenylbutazone, indomethacin, sulfasalazine, piroxicam, valdecoxib, aspirin, carprofen, zomepirac, rofecoxib, aspirin/methocarbamol, aspirin/caffeine/orphenadrine, aspirin/carisoprodol, aspirin/carisoprod

						ol/codeine, acetaminophen/butalbital, balsalazide, aspirin/dipyridamole, acetaminophen/butalbital/caffeine/codeine, racemic flurbiprofen, phenacetin, sulindac, nabumetone, etodolac, tolmetin, ketorolac, oxaprozin, mesalamine, salsalate, fenoprofen, salicylic acid, acetaminophen/chlorpheniramine/hydrocodone/phenylephrine/caffeine, bromfenac
Ptn		PTN	pleiotrophin	Extracellular Space	growth factor	
Ptrf		PTRF	polymerase I and transcript release factor	Nucleus	transcription regulator	
Rarg		RARG	retinoic acid receptor, gamma	Nucleus	ligand-dependent nuclear receptor	etretinate, adapalene, isotretinoin, tazarotene, acitretin, tretinoin, alitretinoin, fenretinide
Rgs16		RGS16	regulator of G-protein signaling 16	Cytoplasm	other	
Rn45s		Rn45s	45S pre-ribosomal RNA	Other	other	
Rpl10a		RPL10A	ribosomal protein L10a	Other	other	
Rpl31		RPL31	ribosomal protein L31	Other	other	
Rpl37a		RPL37A	ribosomal protein L37a	Cytoplasm	other	
Rps10		RPS10-NUDT3	RPS10-NUDT3 readthrough	Cytoplasm	other	
Rps14		RPS14	ribosomal protein S14	Cytoplasm	translation regulator	
Rps20		Rps20	ribosomal protein S20	Cytoplasm	other	
Rps26		RPS26	ribosomal protein S26	Cytoplasm	other	
Rps9		RPS9	ribosomal protein S9	Cytoplasm	translation regulator	

S100a4		S100A4	S100 calcium binding protein A4	Cytoplasm	other	
S100a6		S100A6	S100 calcium binding protein A6	Cytoplasm	transporter	
Schip1		SCHIP1	schwannomin interacting protein 1	Cytoplasm	other	
Sdc2		SDC2	syndecan 2	Plasma Membrane	other	
Serpine1		SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	Extracellular Space	other	drotrecogin alfa
Serpine2		SERPINE2	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	Extracellular Space	other	
Serpinf1		SERPINF1	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	Extracellular Space	other	
Sh3gl2		SH3GL2	SH3-domain GRB2-like 2	Plasma Membrane	enzyme	
Slc19a2		SLC19A2	solute carrier family 19 (thiamine transporter), member 2	Plasma Membrane	transporter	
Slc25a5		SLC25A5	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	Cytoplasm	transporter	clodronic acid
Slc29a1		SLC29A1	solute carrier family 29 (equilibrative nucleoside transporter), member 1	Plasma Membrane	transporter	
Slc35f2		SLC35F2	solute carrier family 35, member F2	Other	other	
Snrpn		SNRPN	small nuclear ribonucleoprotein polypeptide N	Nucleus	other	
Snx22		SNX22	sorting nexin 22	Other	transporter	
Sparc		SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	Extracellular Space	other	
Spp1		SPP1	secreted	Extracellular	cytokine	

			phosphoprotein 1	Space		
Sult4a1		SULT4A1	sulfotransferase family 4A, member 1	Cytoplasm	enzyme	
Tagln		TAGLN	transgelin	Cytoplasm	other	
Tcea3		TCEA3	transcription elongation factor A (SII), 3	Nucleus	transcription regulator	
Tgfb3		TGFB3	transforming growth factor, beta 3	Extracellular Space	growth factor	
Thbs1		THBS1	thrombospondin 1	Extracellular Space	other	
Thbs2		THBS2	thrombospondin 2	Extracellular Space	other	
Tm4sf1		TM4SF1	transmembrane 4 L six family member 1	Plasma Membrane	other	
Tmbim1		TMBIM1	transmembrane BAX inhibitor motif containing 1	Cytoplasm	other	
Tmem176b		TMEM176B	transmembrane protein 176B	Other	other	
Tnc		TNC	tenascin C	Extracellular Space	other	
Tpd52l1		TPD52L1	tumor protein D52-like 1	Cytoplasm	other	
Tpm2		TPM2	tropomyosin 2 (beta)	Cytoplasm	other	
Usp18		USP18	ubiquitin specific peptidase 18	Cytoplasm	peptidase	
Vim		VIM	vimentin	Cytoplasm	other	
Wfdc2		WFDC2	WAP four-disulfide core domain 2	Extracellular Space	other	
Wisp2		WISP2	WNT1 inducible signaling pathway protein 2	Extracellular Space	growth factor	
Ybx1		YBX1	Y box binding protein 1	Nucleus	transcription regulator	

Table 13. The genes listed were expressed at levels 20-fold higher in rat ES cells than the corresponding genes in mouse ES cells.

ID	Notes	Symbol	Entrez Gene Name	Location	Type(s)	Drug(s)
Ajap1		Ajap1	adherens junction associated protein 1	Other	other	
Amd1		AMD1	adenosylmethionine decarboxylase 1	Cytoplasm	enzyme	
Ankrd2		ANKRD2	ankyrin repeat domain 2 (stretch responsive muscle)	Nucleus	transcription regulator	
Arhgef9		ARHGEF9	Cdc42 guanine nucleotide exchange factor (GEF) 9	Cytoplasm	other	

Atp5h		Atp5h	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	Cytoplasm	enzyme	
Btg3		BTG3	BTG family, member 3	Nucleus	other	
Car6		CA6	carbonic anhydrase VI	Extracellular Space	enzyme	methazolamide, hydrochlorothiazide, acetazolamide, trichloromethiazide, chlorothiazide, chlorthalidone, benzthiazide, sulfacetamide, topiramate
Camk4		CAMK4	calcium/calmodulin-dependent protein kinase IV	Nucleus	kinase	
Capn12		CAPN12	calpain 12	Other	peptidase	
Cct6b		CCT6B	chaperonin containing TCP1, subunit 6B (zeta 2)	Cytoplasm	transporter	
Cdx2		CDX2	caudal type homeobox 2	Nucleus	transcription regulator	
Cldn5		CLDN5	claudin 5	Plasma Membrane	other	
Clec3a		CLEC3A	C-type lectin domain family 3, member A	Other	other	
Clic6		CLIC6	chloride intracellular channel 6	Plasma Membrane	ion channel	
Dhrsx		DHRSX	dehydrogenase/reductase (SDR family) X-linked	Other	enzyme	
Dpysl2		DPYSL2	dihydropyrimidinase-like 2	Cytoplasm	enzyme	
Dusp26		DUSP26	dual specificity phosphatase 26 (putative)	Cytoplasm	enzyme	
Eci3		Eci3	enoyl-Coenzyme A delta isomerase 3	Other	enzyme	
Eef2k		EEF2K	eukaryotic elongation factor-2 kinase	Cytoplasm	kinase	
Efna1		EFNA1	ephrin-A1	Plasma Membrane	other	
Epha4		EPHA4	EPH receptor A4	Plasma Membrane	kinase	
Fank1		FANK1	fibronectin type III and ankyrin repeat domains 1	Nucleus	transcription regulator	
Fhit		FHIT	fragile histidine triad	Cytoplasm	enzyme	
Filip1		FILIP1	filamin A interacting protein 1	Cytoplasm	other	
Fmod		FMOD	fibromodulin	Extracellular Space	other	
Foxe1		FOXE1	forkhead box E1 (thyroid	Nucleus	transcription regulator	

			transcription factor 2)			
Fry		FRY	furry homolog (Drosophila)	Extracellular Space	other	
Gjb5		GJB5	gap junction protein, beta 5, 31.1kDa	Plasma Membrane	transporter	
Gpx2		GPX2	glutathione peroxidase 2 (gastrointestinal)	Cytoplasm	enzyme	
Grxcr2		GRXCR2	glutaredoxin, cysteine rich 2	Other	other	
Hecw2		HECW2	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2	Extracellular Space	enzyme	
Hey2		HEY2	hairy/enhancer-of-split related with YRPW motif 2	Nucleus	transcription regulator	
Icos		Icos	inducible T-cell co-stimulator	Plasma Membrane	other	
Ifitm1		IFITM1	interferon induced transmembrane protein 1	Plasma Membrane	transmembrane receptor	
Il1f8		Interleukin 36 beta				
Il28ra		Interleukin 28 receptor, alpha				
Igfbpl1		IGFBPL1	insulin-like growth factor binding protein-like 1	Other	other	
Ipcef1		IPCEF1	interaction protein for cytohesin exchange factors 1	Cytoplasm	enzyme	
Lctl		Lctl	lactase-like	Cytoplasm	other	
Ldhd		LDHD	lactate dehydrogenase D	Cytoplasm	enzyme	
Lef1		LEF1	lymphoid enhancer-binding factor 1	Nucleus	transcription regulator	
Lefty1		LEFTY1	left-right determination factor 1	Extracellular Space	growth factor	
Lifr		LIFR	leukemia inhibitory factor receptor alpha	Plasma Membrane	transmembrane receptor	
Lpar2		LPAR2	lysophosphatidic acid receptor 2	Plasma Membrane	G-protein coupled receptor	
Mog		MOG	myelin oligodendrocyte glycoprotein	Extracellular Space	other	
Morn5		MORN5	MORN repeat containing 5	Other	other	
Pigz		NCBP2	nuclear cap binding protein subunit 2, 20kDa	Nucleus	other	

Nptxr		NPTXR	neuronal pentraxin receptor	Plasma Membrane	transmembrane receptor	
Ntm		NTM	neurotrimin	Plasma Membrane	other	
Nutf2		NUTF2	nuclear transport factor 2	Nucleus	transporter	
Ocln		OCLN	occludin	Plasma Membrane	enzyme	
Olr1		OLR1	oxidized low density lipoprotein (lectin-like) receptor 1	Plasma Membrane	transmembrane receptor	
Pabpc4		PABPC4	poly(A) binding protein, cytoplasmic 4 (inducible form)	Cytoplasm	translation regulator	
Pde11a		PDE11A	phosphodiesterase 11A	Cytoplasm	enzyme	dyphylline, nitroglycerin, aminophylline, dipyridamole, tolbutamide, tadalafil, theophylline, pentoxifylline
Pdyn		PDYN	prodynorphin	Extracellular Space	transporter	
Per3		PER3	period circadian clock 3	Nucleus	other	
Pllp		PLLP	plasmolipin	Plasma Membrane	transporter	
Ppp1r14c		PPP1R14C	protein phosphatase 1, regulatory (inhibitor) subunit 14C	Cytoplasm	other	
Pramel6		Pramel6	preferentially expressed antigen in melanoma like 6	Other	other	
Ptpn18		PTPN18	protein tyrosine phosphatase, non-receptor type 18 (brain-derived)	Nucleus	phosphatase	
Pycr1		PYCR1	pyrroline-5-carboxylate reductase 1	Cytoplasm	enzyme	
Rab26		RAB26	RAB26, member RAS oncogene family	Plasma Membrane	enzyme	
Ramp2		RAMP2	receptor (G protein-coupled) activity modifying protein 2	Plasma Membrane	transporter	pramlintide
Rbm24		RBM24	RNA binding motif protein 24	Other	other	
Rhag		RHAG	Rh-associated glycoprotein	Plasma Membrane	peptidase	
Rpl3		RPL3	ribosomal protein L3	Cytoplasm	other	homoharringtonine
Sall3		SALL3	sal-like 3 (Drosophila)	Nucleus	other	
Satb1		SATB1	SATB homeobox 1	Nucleus	transcription regulator	
Scg2		SCG2	secretogranin II	Extracellul	cytokine	

				ar Space		
Slc15a1		SLC15A1	solute carrier family 15 (oligopeptide transporter), member 1	Plasma Membrane	transporter	
Slc1a1		SLC1A1	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1	Plasma Membrane	transporter	riluzole
Slc24a5		Slc24a5	solute carrier family 24 (sodium/potassium/calcium exchanger), member 5	Other	other	
Slc37a2		SLC37A2	solute carrier family 37 (glucose-6-phosphate transporter), member 2	Other	transporter	
40424		SNTB1	syntrophin, beta 1 (dystrophin-associated protein A1, 59kDa, basic component 1)	Plasma Membrane	other	
St6galnac3		ST6GALNAC3	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3	Cytoplasm	enzyme	
Tex12		TEX12	testis expressed 12	Nucleus	other	
Tex15		TEX15	testis expressed 15	Extracellular Space	other	
Tfap2a		TFAP2A	transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	Nucleus	transcription regulator	
Tmc1		TMC1	transmembrane channel-like 1	Plasma Membrane	other	
Tmem130		TMEM130	transmembrane protein 130	Other	other	
Tmem30b		TMEM30B	transmembrane protein 30B	Other	other	
Tomm20		TOMM20	translocase of outer mitochondrial membrane 20 homolog (yeast)	Cytoplasm	transporter	
Tox3		TOX3	TOX high mobility group box family member 3	Other	other	
Ttc25		TTC25	tetratricopeptide repeat domain 25	Cytoplasm	other	
Tymp		TYMP	thymidine phosphorylase	Extracellular Space	growth factor	

Ubb		Ubb	ubiquitin B	Cytoplasm	other	
Vamp7		VAMP7	vesicle-associated membrane protein 7	Cytoplasm	transporter	
Wfdc12		Wfdc12	WAP four-disulfide core domain 12	Extracellular Space	other	
Wfdc15a		Wfdc15a	WAP four-disulfide core domain 15A	Other	other	
Wfdc6a		Wfdc6a	WAP four-disulfide core domain 6A	Other	other	

Table 14. A subset of genes from Table 13 which are expressed at levels 20-fold higher in rat ES cells than the corresponding genes in mouse ES cells.

ID	Entrez Gene Name
Ajap1	Adheres Junctions Associate Protein
Cldn5	Claudin 5
Arhgef9	Cdc42 guanine nucleotide exchange facter 9
Camk4	Calcium/calmodulin-dependent protein kinase IV
Efna1	ephrin-A1
Epha4	EPH receptor A4
Gjb5	gap junction protein beta 5
Igfbpl1	Insulin-like growth factor binding protein-like 1
Il1f8	Interleukin 36 beta
Il28ra	Interleukin 28 receptor, alpha
Lefty1	left-right determination factor 1
Lifr	Leukemia inhibitory factor receptor alpha
Lpar2	Lysophosphatidic acid receptor 2
Ntm	Neuronal pentraxin receptor
Ptpn18	Protein tyrosine phosphatase non-receptor type 18
Cdx2	Caudal type homeobox 2
Fank1	Fibronectin type III and ankyrin repeat domains 1
Foxe1	Forkhead box E1 (thyroid transcription factor 2)
Hey2	Hairy/enhancer-of-split related with YRPW motif 2
Lef1	Lymphoid enhancer-binding factor 1

Sall3	Sal-like 3 (Drosophila)
Satb1	SATB homeobox 1

An additional molecular signature employing the pluripotency markers/genes for the rat ES cells has also been developed. Table 15 provides a gene list and their expression ranks from the RNA profiling data. mRNA was isolated from rat ES cells and the expression level of various pluripotency markers were compared relative to each other. The "pluripotency genes" which are listed are genes that other groups have used (mostly in mouse, but also in rat) as markers of ES cells. Mesoderm endoderm and neural are similarly defined. By "rank" is refers to the expression in our experiment: the higher the rank (1 is highest) the higher the expression. For example, Oct4's rank of 13 means that, of all the genes assayed, it was expressed higher than all but 12 genes. Background in this experiment was any expression value below 30; 6107 genes had expression values of 30 or higher.

Table 15. Rat ES cell molecular signature employing the pluripotency, mesodermal, endodermal, neural and trohectoderm markers/genes.

Pluripotency	Pluripotency Rank	Mesodermal	Meso-dermal Rank	Endodermal	Endodermal Rank	Neural	Neural Rank	Troph-ectoderm	Troph-ectoderm Rank
c-Myc	8248	Brachyury	7542	Gata6	11195	Nestin	7761	Cdx2	739
Dnmt3L	127	Flk1	?	Sox17	11418	Pax6	13570		
Dppa2	Not tested	Nodal	3050	Hhex1	4571	Sox2	681		
Dppa5	Not tested	Bmp4	3072	Nodal	3050				
Ecat1	9714	Bmpr2	6382	Ext1	6091				
Eras	2541			Sox7	10284				
Err-beta	1368								
Fbxo15	1369								
Fgf4	3440								
Fthl17	Not tested								
Gdf3	2771		**Rank > 6107 = bkg expression**						
Klf4	836								
Lef1	1313								
LIF receptor	724								
Lin28	828								
Nanog	774								
Oct4	13								
Rexo1	6119								
Sox15	4524								

Sox2	681								
SSEA1	Not tested								
SSEA4	Not tested								
Stella	Not tested								
Tcl1	Not tested								
Utf1	1501								

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. Unless otherwise apparent from the context of any embodiment, aspect, step or feature of the invention can be used in combination with any other. Reference to a range includes any integers within the range, any subrange within the range. Reference to multiple ranges includes composites of such ranges.

THAT WHICH IS CLAIMED:

1. An isolated rat ES cell of a strain selected from an ACI or a Dark Agouti (DA) rat, wherein the isolated rat ES cell is capable of transmitting its genome through the germline.

2. The isolated rat ES cell of claim 1, wherein the rat ES cell is derived from an ACI rat.

3. The isolated rat ES cell of claim 1, wherein the rat ES cell is derived from a DA rat.

4. The isolated rat ES cell of claim 1, 2, or 3, wherein the rat ES cell is euploid and capable of transmitting a targeted genetic modification through the germline.

5. The isolated rat ES cell of claim 4, wherein the rat ES cell comprises a germline transmission efficiency of the targeted genetic modification of at least 3%.

6. The isolated rat ES cell of claim 4, wherein the rat ES cell has a germline transmission efficiency of the targeted genetic modification of at least 60%.

7. The isolated rat ES cell of any one of claims 1-6, wherein the rat ES cell exhibits a targeting efficiency of homologous recombination of at least 2%.

8. The isolated rat ES cell of any one of claims 1-7, wherein the rat ES cell is capable of transmitting a targeted genetic modification into progeny following a successive round of electroporation.

9. The isolated rat ES cells of any one of claims 1-8, wherein the rat ES cell comprises one or more, two or more, or three or more targeted genetic modifications .

10. The isolated rat ES cell of claim 9, wherein the targeted genetic modification comprises an insertion, a deletion, a knockout, a knockin, a point mutation, or a combination thereof.

11. The isolated rat ES cell of claim 9, wherein the targeted genetic modification comprises at least one insertion of a heterologous polynucleotide into a genome of the cell.

12. The isolated rat ES cell of claim 11, wherein the heterologous polynucleotide comprises a selection marker.

13. The isolated rat ES cell of claim 12, wherein (a) the selection marker comprises a non-attenuated selection marker gene operably linked to a promoter; or (b) the rat ES cell comprises at least 2 copies of the polynucleotide encoding the selection marker.

14. The isolated rat ES cell of claim 12, wherein the selection marker has an increased activity compared to a wild type selection marker.

15. The isolated rat ES cell of any one of claims 1-14, wherein the rat ES cell forms a sphere-like colony when plated on a feeder cell layer in culture comprising a leukemia inhibitory factor (LIF) polypeptide, a GSK3 inhibitor, and a MEK inhibitor.

16. The isolated rat ES cell of any one of claims 1-15, wherein the rat ES cell, when cultured *in vitro*, loosely adheres to a feeder cell layer.

17. The isolated rat ES cell of any one of claims 1-16, wherein the rat ES cell does not require paracrine LIF signaling for maintenance of pluripotency.

18. The isolated rat ES cell of any one of claims 1-17, wherein the rat ES cell is a male (XY) rat ES cell.

19. The isolated rat ES cell of any one of claims 1-17, wherein the rat ES cell is a female (XX) rat ES cell.

20. The isolated rat ES cell of any one of claims 1-19, wherein the rat ES cell can be passaged up to at least 11 times in a medium comprising a GSK3 inhibitor and a MEK inhibitor without decreasing its targeting efficiency or germline transmission efficiency of a targeted genetic modification.

21. The isolated rat ES cell of any one of claims 1-20, wherein the rat ES cell expresses at least one pluripotency marker selected from Dnmt3L, Eras, Err-beta, Fbxo15, Fgf4, Gdf3, Klf4, Lef1, LIF receptor, Lin28, Nanog, Oct4, Sox15, Sox2, Utf1, and a combination thereof.

22. The isolated rat ES cell of any one of claims 1-21, wherein the rat ES cell does not express one or more pluripotency markers selected from c-Myc, Ecat1, Rexo1, and a combination thereof.

23. The isolated rat ES cell of any one of claims 1-22, wherein the rat ES cell does not express one or more mesodermal markers selected from Brachyury, Bmpr2, and a combination thereof.

24. The isolated rat ES cell of any one of claims 1-23, wherein the rat ES cell does not express one or more endodermal markers selected from Gata6, Sox17, Sox7, and a combination thereof.

25. The isolated rat ES cell of any one of claims 1-24, wherein the rat ES cell does not express one or more neural markers selected from Nestin, Pax6, and a combination thereof.

26. The isolated rat ES cell of any one of claims 1-25, wherein the rat ES cell expresses a pluripotency marker comprising Oct-4, Sox2, alkaline phosphatase, and a combination thereof.

27. The isolated rat ES cell of any one of claims 1-26, wherein the rat ES cell is characterized by the expression of one or more of a rat ES cell -specific gene selected from one or more of Adherens Junctions Associated Protein (Ajap1), Claudin 5 (Cldn5), Cdc42 guanine nucleotide exchange factor 9 (Arhgef9), Calcium/calmodulin-dependent protein kinase IV (Camk4), ephrin-A1 (Efna1), EPH receptor A4 (Epha4), gap junction protein beta 5 (Gjb5), Insulin-like growth factor binding protein-like 1 (Igfbpl1), Interleukin 36 beta (Il1f8), Interleukin 28 receptor, alpha (Il28ra), left-right determination factor 1 (Lefty1), Leukemia inhibitory factor receptor alpha (Lifr), Lysophosphatidic acid receptor 2 (Lpar2), Neuronal pentraxin receptor (Ntm), Protein tyrosine phosphatase non-receptor type 18 (Ptpn18), Caudal type homeobox 2 (Cdx2), Fibronectin type III and ankyrin repeat domains 1 (Fank1), Forkhead box E1 (thyroid

transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Lymphoid enhancer-binding factor 1 (Lef1), Sal-like 3 (Drosophila) (Sall3), SATB homeobox 1 (Satb1), miR-632, and a combination thereof.

28. An isolated population of rat ES cells, wherein at least 70% of the rat ES cells are euploid and form sphere-like colonies when plated on a feeder cell layer *in vitro*.

29. The isolated population of rat ES cells of claim 28, wherein the rat ES cells are derived from an ACI rat.

30. The isolated population of rat ES cells of claim 28, wherein the rat ES cells are derived from a Dark Agouti (DA) rat.

31. The isolated population of rat ES cells of any one of claims 28-30, wherein the rat ES cells are capable of transmitting their genome through the germline.

32. The isolated population of rat ES cells of any one of claims 28-31, wherein the rat ES cells have a germline transmission efficiency of a targeted genetic modification of at least 3%.

33. The isolated population of rat ES cells of any one of claims 28-31, wherein the rat ES cells have a germline transmission efficiency of a targeted genetic modification of at least 60%.

34. The isolated population of rat ES cells of any one of claims 28-33, wherein the rat ES cells exhibit a targeting efficiency of homologous recombination of at least 2%.

35. The isolated population of rat ES cells of any one of claims 28-34, wherein the rat ES cells are capable of transmitting a targeted genetic modification into progeny following a successive round of electroporation.

36. The isolated population of any one claims 28-35, wherein the rat ES cells comprise one or more, two or more, or three or more targeted genetic modification and can transmit the targeted genetic modification through the germline.

37. The isolated population of rat ES cells of claim 36, wherein the targeted genetic modification is at the rat Rosa26 locus.

38. The isolated population of rat ES cells of claim 36, wherein the targeted genetic modification comprises an insertion, a deletion, a knockout, a knockin, a point mutation, or a combination thereof.

39. The isolated population of rat ES cells of claim 36, wherein the targeted genetic modification comprises at least one insertion of a heterologous polynucleotide into a genome of the cell.

40. The isolated population of rat ES cells of claim 39, wherein the heterologous polynucleotide comprises a selection marker.

41. The isolated population of rat ES cells of claim 40, wherein

(a) the selection marker comprises a non-attenuated selection marker gene operably linked to a promoter; or

(b) the rat ES cell comprises at least 2 copies of the polynucleotide encoding the selection marker.

42. The isolated population of rat ES cells of claim 40, wherein the selection marker has an increased activity compared to a wild type selection marker.

43. The isolated population of rat ES cells of any one of claims 28-42, wherein the cells form a sphere-like colony when plated on a feeder cell layer in culture comprising a LIF polypeptide, a GSK3 inhibitor, and a MEK inhibitor.

44. The isolated population of rat ES cells of any one of claims 28-43, wherein the cells, when cultured *in vitro*, loosely adhere to a feeder cell layer.

45. The isolated population of rat ES cells of any one of claims 28-44, wherein the cells do not require paracrine LIF signaling for maintenance of pluripotency.

46. The isolated population of rat ES cells of any one of claims 28-45, wherein the rat ES cells are male (XY) rat ES cells.

47. The isolated population of rat ES cells of any one of claims 28-45, wherein the rat ES cells are female (XX) rat ES cells.

48. The isolated population of rat ES cells of any one of claims 28-47, wherein the rat ES cells can be passaged up to at least 11 times in a medium comprising a GSK3 inhibitor and a MEK inhibitor without decreasing its targeting efficiency or germline transmission efficiency of a targeted genetic modification.

49. The isolated population of rat ES cells of any one of claims 28-48, wherein the rat ES cells express at least one pluripotency marker selected from Dnmt3L, Eras, Err-beta, Fbxo15, Fgf4, Gdf3, Klf4, Lef1, LIF receptor, Lin28, Nanog, Oct4, Sox15, Sox2, Utf1, and a combination thereof.

50. The isolated population of rat ES cells of any one of claims 28-49, wherein the rat ES cells do not express one or more pluripotency markers selected from c-Myc, Ecat1, Rexo1, and a combination thereof.

51. The isolated population of rat ES cells of any one of claims 28-50, wherein the rat ES cells do not express one or more mesodermal markers selected from Brachyury, Bmpr2, and a combination thereof.

52. The isolated population of rat ES cells of any one of claims 28-51, wherein the rat ES cells do not express one or more endodermal markers selected from Gata6, Sox17, Sox7, and a combination thereof.

53. The isolated population of rat ES cells of any one of claims 28-52, wherein the rat ES cells do not express one or more neural markers selected from Nestin, Pax6, and a combination thereof.

54. The isolated population of rat ES cells of any one of claim 28-53, wherein the rat ES cells express a pluripotency marker comprising Oct-4, Sox2, alkaline phosphatase, or a combination thereof.

55. The isolated population of any one of claims 28-54, wherein the rat ES cells are characterized by the expression of one or more rat ES cell-specific genes selected from Adherens Junctions Associated Protein (Ajap1), Claudin 5 (Cldn5), Cdc42 guanine nucleotide exchange factor 9 (Arhgef9), Calcium/calmodulin-dependent protein kinase IV (Camk4), ephrin-A1 (Efna1), EPH receptor A4 (Epha4), gap junction protein beta 5 (Gjb5), Insulin-like growth factor binding protein-like 1 (Igfbpl1), Interleukin 36 beta (Il1f8), Interleukin 28 receptor, alpha (Il28ra), left-right determination factor 1 (Lefty1), Leukemia inhibitory factor receptor alpha (Lifr), Lysophosphatidic acid receptor 2 (Lpar2), Neuronal pentraxin receptor (Ntm), Protein tyrosine phosphatase non-receptor type 18 (Ptpn18), Caudal type homeobox 2 (Cdx2), Fibronectin type III and ankyrin repeat domains 1 (Fank1), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Lymphoid enhancer-binding factor 1 (Lef1), Sal-like 3 (Drosophila) (Sall3), SATB homeobox 1(Satb1), miR-632, and a combination thereof.

56. The isolated population of rat ES cells of any one of claims 28-55, wherein the population comprises at least 10^4 cells.

57. The isolated population of rat ES cells of any one of claims 28-56, wherein the rat ES cells have one or more characteristic comprising:

a. at least 90% of the rat ES cells are euploid;

b. at least 70% of the rat ES cells express at least one pluripotency marker; wherein the at least one pluripotency marker comprises Oct-4, Sox2, alkaline phosphatase, or a combination thereof;

c. a cell from the rat ES cell population, when combined with a rat host embryo transmits the genome of the rat ES cell line into an offspring;

d. the rat ES cells form sphere-like colonies when plated on a feeder cell layer *in vitro*; and/or,

e. the rat ES cells maintain pluripotency when cultured *in vitro* in a medium comprising a GSK3 inhibitor, a MEK inhibitor, and LIF, and on a feeder cell layer that is not genetically modified to express LIF.

58. The isolated population of any one of claims 28-57, wherein (a) the rat ES cells are derived from a rat blastocyst; (b) the rat ES cells are derived from a rat morula stage embryo; and/or, (c) the rat ES cells are derived from a superovulated rat.

59. An *in vitro* culture comprising a feeder cell layer, a population of rat embryonic stem (ES) cells, and a medium comprising a Leukemia Inhibitory Factor (LIF), a GSK3 inhibitor, and a MEK inhibitor, wherein at least 70% of the rat ES cells are euploid and the rat ES cells form a sphere-like colony.

60. The *in vitro* culture of claim 59, wherein the rat ES cells, loosely adhere to the feeder cell layer.

61. The *in vitro* culture of claim 59 or 60, wherein the rat ES cells are capable of transmitting their genome through the germline.

62. The *in vitro* culture of any one of claims 59- 61, wherein the rat ES cells are derived from an ACI rat.

63. The *in vitro* culture of any one of claims 59-61, wherein the rat ES cells are derived from a Dark Agouti (DA) rat.

64. The *in vitro* culture of any one of claims 59-63, wherein the rat ES cells are capable of transmitting a targeted genetic modification through the germline.

65. The *in vitro* culture of claim 64, wherein the rat ES cells comprise a germline transmission efficiency of the targeted genetic modification of at least 3%.

66. The *in vitro* culture of claim 64, wherein the rat ES cells have a germline transmission efficiency of the targeted genetic modification of at least 60%.

67. The *in vitro* culture of any one of claims 59-66, wherein the rat ES cells exhibit a targeting efficiency of homologous recombination of at least 2%.

68. The *in vitro* culture of any one of claims 59-67, wherein the rat ES cell is capable of transmitting a targeted genetic modification into progeny following a successive round of electroporation.

69. The *in vitro* culture of any one of claims 59-68, wherein the rat ES cells comprise one or more, two or more, or three or more-targeted genetic modification.

70. The *in vitro* culture of claim 69, wherein the targeted genetic modification comprises an insertion, a deletion, a knockout, a knockin, a point mutation, or a combination thereof.

71. The *in vitro* culture of claim 69, wherein the targeted genetic modification comprises at least one insertion of a heterologous polynucleotide into a genome of the rat ES cells.

72. The *in vitro* culture of claim 71, wherein the heterologous polynucleotide comprises a selection marker.

73. The *in vitro* culture of claim 72, wherein (a) the selection marker comprises a non-attenuated selection marker gene operably linked to a promoter; or (b) the rat ES cell comprises at least 2 copies of the polynucleotide encoding the selection marker.

74. The *in vitro* culture of claim 72, wherein the selection marker has an increased activity compared to a wild type selection marker.

75. The *in vitro* culture of any one of claims 59-74, wherein the cell does not require paracrine LIF signaling for maintenance of pluripotency.

76. The *in vitro* culture of any one of claims 59-75, wherein the rat ES cells are male (XY) rat ES cells.

77. The *in vitro* culture of any one of claims 59-75, wherein the rat ES cells are female (XX) rat ES cells.

78. The *in vitro* culture of any one of claims 59-77, wherein the rat ES cells can be passaged up to at least 11 times in a medium comprising a GSK3 inhibitor

and a MEK inhibitor without decreasing its targeting efficiency or germline transmission efficiency of a targeted genetic modification.

79. The *in vitro* culture of any one of claims 59-78, wherein the rat ES cells express at least one pluripotency marker selected from Dnmt3L, Eras, Err-beta, Fbxo15, Fgf4, Gdf3, Klf4, Lef1, LIF receptor, Lin28, Nanog, Oct4, Sox15, Sox2, Utf1, and a combination thereof.

80. The *in vitro* culture of any one of claims 59-79, wherein the rat ES cells do not express one or more pluripotency markers selected from c-Myc, Ecat1, Rexo1, and a combination thereof.

81. The *in vitro* culture of any one of claims 59-80, wherein the rat ES cells do not express one or more mesodermal markers selected from Brachyury, Bmpr2, and a combination thereof.

82. The *in vitro* culture of any one of claims 59-81, wherein the rat ES cells do not express one or more endodermal markers selected from Gata6, Sox17, Sox7, and a combination thereof.

83. The *in vitro* culture of any one of claims 59-82, wherein the rat ES cells do not express one or more neural markers selected from Nestin, Pax6, and a combination thereof.

84. The *in vitro* culture of any one of claims 59-83, wherein the cell expresses a pluripotency marker comprising Oct-4, Sox2, alkaline phosphatase, or a combination thereof.

85. The *in vitro* culture of any one of claims 59-84, wherein the rat ES cells are characterized by the expression of one or more of a rat ESC-specific gene selected from Adherens Junctions AssociatedProtein (Ajap1), Claudin 5 (Cldn5), Cdc42 guanine nucleotide exchange factor 9 (Arhgef9), Calcium/calmodulin-dependent protein kinase IV (Camk4), ephrin-A1 (Efna1), EPH receptor A4 (Epha4), gap junction protein beta 5 (Gjb5), Insulin-like growth factor binding protein-like 1 (Igfbpl1), Interleukin 36 beta (Il1f8), Interleukin 28 receptor, alpha (Il28ra), left-right determination factor 1 (Lefty1), Leukemia inhibitory factor receptor alpha (Lifr), Lysophosphatidic acid

receptor 2 (Lpar2), Neuronal pentraxin receptor (Ntm), Protein tyrosine phosphatase non-receptor type 18 (Ptpn18), Caudal type homeobox 2 (Cdx2), Fibronectin type III and ankyrin repeat domains 1 (Fank1), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Forkhead box E1 (thyroid transcription factor 2) (Foxe1), Hairy/enhancer-of-split related with YRPW motif 2 (Hey2), Lymphoid enhancer-binding factor 1 (Lef1), Sal-like 3 (Drosophila) (Sall3), SATB homeobox 1 (Satb1), miR-632, and a combination thereof.

86. The *in vitro* culture of any one of claims 59-85, wherein the concentration of LIF is about 50 U/ml to about 150 U/ml.

87. The *in vitro* culture of any one of claims 59-85, wherein the concentration of LIF is about 100 U/ml.

88. The *in vitro* culture of any one of claims 59-87, wherein the LIF is from mouse or comprises at least 91% sequence identity to SEQ ID NO: 1.

89. The *in vitro* culture of any one of claims 59-88, wherein the feeder cell layer is not genetically modified to express LIF.

90. The *in vitro* culture of any one of claims 59-89, wherein the feeder cell layer comprises a monolayer of mitotically inactivated mouse embryonic fibroblasts (MEFs).

91. The *in vitro* culture of any one of claims 59-90, wherein the MEK inhibitor comprises PD0325901.

92. The *in vitro* culture of any one of claim 59-91, wherein the GSK-3 inhibitor comprises CHIR99021.

93. The *in vitro* culture of any one of claims 59-92, wherein the rat ES cells are derived from a rat blastocyst-stage embryo or a rat morula-stage embryo.

94. The *in vitro* culture of claim 93, wherein the blastocyst-stage or the morula-stage rat embryo further comprises an outgrowth of an amorphous undifferentiated mass of rat ES cells.

95. The *in vitro* culture of claim 93, wherein the population of rat ES cells comprises an isolated outgrowth of an amorphous undifferentiated mass of rat ES cells.

96. A method for generating a rat embryonic stem (ES) cell line comprising:

(a) culturing *in vitro* a first feeder cell layer and a morula or a blastocyst-stage rat embryo, wherein the zona pellucida of the morula or blastocyst-stage rat embryo has been removed, and wherein the culture conditions maintain pluripotency of a rat ES cell and comprise a medium having mouse leukemia inhibitory factor (LIF) or a sequence having at least 91% sequence identity to SEQ ID NO:1 and having LIF activity, and a GSK3 inhibitor, and a MEK inhibitor; and,

(b) transferring an outgrowth of an amorphous undifferentiated mass of rat ES cells to an *in vitro* culture well comprising a second feeder cell layer; and,

(c) culturing the outgrowth under conditions comprising the medium having the mouse LIF or an active variant of the mouse LIF, and thereby maintaining pluripotency of the rat ES cells; and, establishing a rat ES cell line therefrom.

97. The method of claim 96, wherein the rat ES cell line is passaged at least 5 times.

98. The method of claim 96 or 97, wherein the rat ES cell line is passaged at least 10 times.

99. The method of claim 96, 97, or 98, wherein the medium comprises about 50 U/ml to about 150 U/ml of mouse LIF.

100. The method of any one of claims 96-99, wherein the medium comprises about 100 U/ml of mouse LIF.

101. The method of any one of claims 96-100, wherein the first and second feeder cell layers are not genetically modified to express LIF.

102. The method of any one of claims 96-101, wherein the first and second feeder cell layers comprise a monolayer of mitotically inactivated mouse embryonic fibroblasts (MEFs).

103. The method of any one of claims 96-102, wherein the MEK inhibitor comprises PD0325901.

104. The method of any one of claims 96-103, wherein the GSK-3 inhibitor comprises CHIR99021.

105. The method of any one of claim 96-104, wherein (a) the rat ES cell line is derived from an ACI rat or derived from a Dark Agouti (DA) rat; and/or, (b) the rat ES cell line is derived from a morula-stage or a blastocyst-stage embryo from a superovulated rat.

106. The method of any one of claims 96-105, wherein the medium further comprises at least one of an FGF receptor inhibitor, a ROCK inhibitor, or an ALK inhibitor.

107. The method of claim 106, wherein the FGF receptor inhibitor comprises PD184352, the ROCK inhibitor comprises Y-27632, or the ALK inhibitor comprises A-83-01.

108. The method of any one of claims 96-107, wherein the rat ES cell line has a germline transmission efficiency of a targeted genetic modification of at least 3%.

109. The method of any one of claims 96-108, wherein the germline transmission efficiency of a targeted genetic modification is at least 60%.

110. A method of selecting a rat embryonic stem (ES) cell having stably incorporated into its genome a heterologous polynucleotide comprising: (a) providing an *in vitro* population of rat ES cells; (b) introducing into at least one rat ES cell a heterologous polynucleotide comprising a selection marker operably linked to a promoter active in the rat ES cell; and, (c) culturing *in vitro* the rat ES cell population in an alternating first and second culture media, wherein the first culture medium comprises an effective amount of a selection agent for a first time period and the second culture

medium does not comprise the selection agent, wherein the *in vitro* culture conditions are sufficient to maintain pluripotency; thereby selecting the rat ES cell having stably integrated into its genome the heterologous polynucleotide.

111. The method of claim 110, wherein the first and second culture media are alternated every 24 hours.

112. The method of claim 110 or 111, wherein the selection marker imparts resistance to an antibiotic.

113. The method of any one of claims 110-112, wherein the antibiotic comprises G418.

114. The method of any one of claims 110-113, wherein the selection marker comprises neomycin phosphotransferase (neo^r), hygromycin B phosphotransferase (hyg^r), puromycin-N-acetyltransferase ($puro^r$) , blasticidin S deaminase (bsr^r), xanthine/guanine phosphoribosyl transferase (gpt), and herpes simplex virus thymidine kinase (HSV-tk), or a combination thereof.

115. The method of any one of claims 110-114, wherein (a) the selection marker has an increased activity compared to the wild type selection marker; and/or (b) multiple copies of the selection marker are stably incorporated into the genome of the rat ES cell.

116. The method of claim 115, wherein the selection marker is a non-attenuated selection marker.

117. A method for genetically modifying an isolated rat embryonic stem (ES) cell comprising introducing into the genome of an isolated rat ES cell of any one of claims 1-27 a heterologous polynucleotide to form a genetically modified rat ES cell.

118. A method of making a genetically modified rat comprising:

(a) introducing into the genome of the isolated rat embryonic stem (ES) cell of any one of claims 1-27, a heterologous polynucleotide to form a rat ES cell having a targeted genetic modification;

(b) introducing at least one of the rat ES cells comprising the targeted genetic modification into a rat host embryo to produce an F0 embryo;

(c) implanting the F0 embryo into a surrogate mother; and,

(d) gestating the F0 embryo in the surrogate mother to term; and, (e) identifying an F0 rat having the targeted genetic modification.

119. The method of claim 118, further comprising breeding a male F0 rat with a wild type female rat to produce an F1 progeny that is heterozygous for the targeted genetic modification.

120. The method of claim 119, further comprising breeding a male rat of the F1 progeny with a female rat of the F1 progeny to obtain an F2 progeny that is homozygous for the genetic modification.

121. The method of any one of claims 118-120, wherein at least 3% of the F0 rats having the genetic modification transmit the genetic modification to the F1 progeny.

122. The method of any one of claims 118-121, wherein at least 10% of the F0 rats having the genetic modification transmit the genetic modification to the F1 progeny.

123. The method of any one of claims 118-122, wherein at least 60% of the F0 rats having the genetic modification transmit the genetic modification to the F1 progeny.

124. The method of any one of claims 118-123, wherein the genetically modified rat ES cell is from the same rat strain as the rat host embryo.

125. The method of any one of claims 118-123, wherein the genetically modified rat ES cell is from a different rat strain as the rat host embryo.

126. The isolated population of rat ES cells of any of the preceding claims, the in vitro culture of any of the preceding claims, or the method of any of the preceding claims, wherein the rat ES cells in the population comprise:

(a) at least 90% of the rat ES cells are euploid;

(b) at least 70% of the rat ES cells express at least one pluripotency marker; wherein the at least one pluripotency marker comprises Oct-4, Sox2, alkaline phosphatase, or a combination thereof;

(c) a cell from the rat ES cell population, when combined with a rat host embryo transmits the genome of the rat ES cell into an offspring;

(d) the rat ES cells when cultured *in vitro* loosely adhere to a feeder cell layer;

(e) the rat ES cells form sphere-like colonies when plated on a feeder cell layer *in vitro*;

(f) the rat ES cells maintain pluripotency when cultured *in vitro* in a medium comprising a GSK3 inhibitor, a MEK inhibitor, and LIF, and on a feeder cell layer that is not genetically modified to express LIF;

(g) the rat ES cells exhibit a targeting efficiency of homologous recombination of at least 2%;

(h) the rat ES cells maintain pluripotency *in vitro* without requiring paracrine LIF signaling;

(i) at least 70% of the rat ES cells are euploid and form sphere-like colonies when plated on a feeder cell layer *in vitro*;

(j) the rat ES cells express at least one pluripotency marker selected from Dnmt3L, Eras, Err-beta, Fbxo15, Fgf4, Gdf3, Klf4, Lef1, LIF receptor, Lin28, Nanog, Oct4, Sox15, Sox2, Utf1, or a combination thereof;

(k) the rat ES cells do not express one or more differentiation markers selected from- c-Myc, Ecat1, or Rexo1.

(l) the rat ES cells do not express one or more mesodermal markers selected from Brachyury, Bmpr2, or a combination thereof;

(m) the rat ES cells do not express one or more endomermal markers selected from Gata6, Sox17, Sox7, or combination thereof; and/or

(n) the rat ES cells do not express one or more neural markers selected from Nestin, Pax6, or combination thereof.

5