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(54) **Title**: METHODS FOR SITE-SPECIFIC GENETIC MODIFICATION IN SPERMATOGONIAL STEM CELLS USING ZINC FINGER NUCLEASE (ZFN) FOR THE CREATION OF MODEL ORGANISMS

(57) **Abstract**: Methods for site-specific genetic engineering using zinc finger nuclease (ZFN) of stem cells and gametes, including but not limited to pluripotent cells, totipotent cells, somatic stem cells, spermatogonial stem cells (SSCs), embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, embryos, germ cells, primordial germ cells (PGCs), plant tube cells, pollen cells, and spores are presented herein.

Methods for Site-Specific Genetic Modification in Spermatogonial Stem Cells Using Zinc Finger Nuclease (ZFN) for the Creation of Model Organisms

Government Rights

[0001] This invention was made with government support under Grant Numbers R01-HD061575-02 and R01-HD053889-02 awarded by the National Institutes of Health. The government has certain rights in the invention.

Background of the Invention

[0002] Genetic modification is a process whereby an existing DNA sequence is altered or a new genetic sequence is added in a cell's or organism's genome. Site-specific genetic modification is the intentional alteration of a specific DNA sequence of a cell or organism. Oftentimes, a DNA sequence comprising a gene or gene fragment is chosen. This alteration of the targeted gene may result in a change in the level of RNA and/or protein that is encoded by that gene, or the alteration may result in the targeted gene encoding a different RNA or protein than the untargeted gene. The modified genome may be studied in the context of a cell, or, more preferably, in the context of a genetically modified organism.

[0003] Genetically modified organisms are among the most useful research tools in the biological sciences, as well as having agricultural, pharmaceutical and biotechnology applications. An example of a genetically modified organism is a knockout organism which harbors a genetic modification that results a loss of function to a gene and its encoded protein. Another example of a genetically modified organism is a knockin organism which contains an endogenous gene replaced witha heterologous (i.e., foreign) gene, or gene fragment. Genetically

modified organisms will pass their genetic changes to their progeny if the changes have been incorporated into the organism's germ cells (i.e. sperm or oocytes).

[0004] Genetically modified organisms exhibiting clinically relevant phenotypes are valuable for drug discovery and development and for drug target identification. For example, mutation of somatic or germ cells facilitates the production of genetically modified offspring or cloned organisms having a phenotype of interest. Such organisms have a number of uses, for example as models of physiological disorders (e.g., of human genetic diseases) that are useful for screening the efficacy of candidate therapeutic compounds or compositions for treating or preventing such physiological disorders. Furthermore, identifying the gene(s) responsible for the phenotype provides potential drug targets for modulating the phenotype and, when the phenotype is clinically relevant, for therapeutic intervention. In addition, the manipulation of the genetic makeup of organisms and the identification of new genes have important uses in agriculture, for example in the development of new strains of animals and plants having higher nutritional value or increased resistance to environmental stresses (such as heat, drought, or pests) relative to their wild-type or non-mutant counterparts.

[0005] Methods for producing genetically modified organisms include both random and site-specific mutagenesis and transgenesis. Random methods take advantage of highly active or mutagenic substances such as chemicals, radiation or transposon insertional mutagenesis. Site-specific methods enable precise engineering of genomes in living cells and organisms. Traditionally, site-specific mutagenesis has been carried out by using homologous recombination of an exogenous

sequence which may be a gene, gene fragment or selectable marker at the DNA sequence desired for modification.

[0006] Alternative site-specific genome modification technologies, including nucleases and homing endonucleases have been developed, such as zinc finger nuclease (ZFN). The site-specific technologies, such as ZFNs, can be modified in order to specifically bind to sites within the genome of many organisms. ZFNs may be used to introduce targeted double-stranded or single-stranded breaks in the DNA, which can lead to small deletions at the site of the break during the Non-Homologous End Joining (NHEJ) process, thereby producing gene knockouts in cells and organisms. ZFNs can also generate breaks in the DNA which can increase the frequency of exogenous sequence introduction by homologous recombination, thereby producing gene knock-ins in cells and organisms.

[0007] ZFNs previously have not been used to produce site-specific genetic modifications in spermatogonial stem cells (SSCs) derived from rats, or from SSCs derived from many other agriculturally or biomedically important species. Additionally, ZFNs have not been used to create site-specific mutations in other types of stem cells, such as embryonic stem (ES) cells, induced pluripotent stem cells (iPS), somatic stem cells derived from many other agriculturally or biomedically important species. Stem cells containing site-specific mutations can be used to rapidly and cost-effectively generate genetically modified organisms. While targeted mutations have been described in many somatic cells and cell lines and in embryonic stem cells from a few species, SSCs from rats and most other species have not been successfully targeted using site-specific technologies.

[0008] Thus, there remains a need for compositions and methods for generating site-specific mutations in stem cells that can be used to produce genetic modifications in rats and other agriculturally or biomedically important species.

Brief Summary of the Invention

[0009] In accordance with the purposes of this invention, as embodied and broadly described herein, this invention relates to methods for site-specific genetic engineering using zinc finger nuclease (ZFN) of stem cells and gametes, including but not limited to pluripotent cells, totipotent cells, somatic stem cells, spermatogonial stem cells (SSCs), embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, embryos, germ cells, primordial germ cells (PGCs), plant tube cells, pollen cells, and spores. Methods for site-specific engineering of stem cells include, but are not limited to using site specific DNA binding and cleaving proteins such as ZFNs.

[0010] Site-specific engineering of stem cells results in altered function of gene(s) or gene product(s) and genetically modified organisms, and cell or tissue culture models are produced from these engineered stem cells. Modified stem cells and organisms include knockout and knockin cells and organisms.

[0011] In another aspect, the invention relates to genetically modified organisms created by site-specific engineering using MNs including, but not limited to, mammals, including rats, mice, pigs, rabbits, guinea pigs, dogs, non-human primates, minipigs, as well as plants, including but not limited to maize, soybean, rice, potato, wheat, tobacco, tomato, and Arabidopsis, as well as the descendants and ancestors of such organisms.

[0012] In another embodiment, the invention provides kits that are used to produce site specific-mutations in stem cells, which can be used to generate genetically modified organisms. The kits typically include one or more site-specific genetic engineering technology, such as ZFNs. The kit may also contain one or more sets of stem cells for site-specific modification. The stem cells may include, but is not limited to spermatogonial stem cells (SSCs), as well as media and conditions necessary for growing SSCs. The kits may include exogenous sequences for site-specific genomic introduction, such as but not limited to reporter genes or selectable markers. The kits may include instructions for (i) introducing the ZFNs into the stem cells (ii) identifying stem cells which have been site specifically modified (iii) growing site-specifically modified stem cells in media or conditions necessary and to numbers required for stem cells to produce genetically modified organisms (iv) using the grown stem cells to produce a genetically modified organism (v) identifying which organisms or progeny harbor the site-specific mutation of interest.

[0013] In some embodiments of the invention, a composition or kit comprises one or more stem cells or one or more embryos, wherein the one or more stem cells or one or more embryos comprise one or more of the following mutations: (i) a deletion mutation; (ii) a knockout mutation; and/or (iii) an addition of a heterologous nucleic acid sequence; wherein the one or more mutations of (i), (ii), and/or (iii) are site-specific mutations caused by a zinc finger nuclease (ZFN).

[0014] In some embodiments of the invention, the heterologous nucleic acid sequence is chosen from a selectable marker or an orthologous gene.

[0015] In some embodiments of the invention, the one or more stem cells is chosen from a spermatogonial stem cell (SSC), an embryonic stem cell, or an induced pluripotent stem cell.

[0016] In some embodiments of the invention, the one or more stem cells is derived from the germline lineage of an animal or plant.

[0017] In some embodiments of the invention, the one or more stem cells or the one or more embryos further comprise at least one inverted tandem repeat of a transposon or a variant thereof.

[0018] In some embodiments of the invention, the one or more stem cells is a somatic stem cell.

[0019] In some embodiments of the invention, an organism comprisesone or more stem cells, the one or more stem cells comprise one or more of the following mutations: (i) a deletion mutation; (ii) a knockout mutation; and/or (iii) an addition of a heterologous nucleic acid sequence; the one or more mutations of (i), (ii), and/or (iii) are site-specific mutations caused by a ZFN.

[0020] In some embodiments of the invention, the one or more stem cells comprise an SSC.

[0021] In some embodiments of the invention, the one or more stem cells further comprise at least one inverted tandem repeat of a transposon or variant thereof.

[0022] In some embodiments of the invention, a composition comprises one or more stem cells or one or more embryos and: (a) a ZFN that cleaves a nucleic acid sequence at a pre-determined location within the genome of the one or more stem cells or the one or more embryos; or (b) a nucleic acid sequence that encodes a ZFN that cleaves a nucleic acid of

the stem cell at a pre-determined site within the genome of the stem cell or the embryo; wherein the one or more stem cells is derived from the germline lineage of an animal or plant.

[0023] In some embodiments of the invention, the stem cell is a spermatogonial stem cell derived from a rat or mini pig.

[0024] In some embodiments of the invention, the one or more stem cells or the one or more embryos further comprise at least one inverted tandem repeat of a transposon or a variant thereof.

[0025] In some embodiments of the invention, the one or more stem cells or the one or more embryos further comprise: (a) one or more nucleic acid sequences at least 70% homologous to a nucleic acid sequence chosen from:

[0026] (a)

[0027] CAGTTGAAGTCGGAAGTTTACATACACTTAAGTTGGAGTCAT TAAAACTCGTTTTTCAACTACTCCACAAATTTCTTGTTAACAA ACAATAGTTTTGGCAAGTCAGTTAGGACATCTACTTTGTGCA TGACACAAGTCATTTTTCCAACAATTGTTTACAGACAGATTA TTTCACTTATAATTCACTGTATCACAATTCCAGTGGGTCAGA AGTTTACATACACTAAGT (SEQ ID NO:1);

[0028] (b)

[0029] ATTGAGTGTATGTAAACTTCTGACCCACTGGGAATGTGATGA AAGAAATAAAAGCTGAAATGAATCATTCTCTCTACTATTATT CTGATATTTCACATTCTTAAAATAAAGTGGTGATCCTAACTG ACCTAAGACAGGGAATTTTTACTAGGATTAAATGTCAGGAAT TGTGAAAAAGTGAGTTTAAATGTATTTGGCTAAGGTGTATGT AAACTTCCGACTTCAACTG (SEQ ID NO:2);

[0030] (c)

[0031] CCCTAGAAAGATAGTCTGCGTAAAATTGACGCATGCATTCTT GAAATATTGCTCTCTCTTTCTAAATAGCGCGAATCCGTCGCT GTGCATTTAGGACATCTCAGTCGCCGCTTGGAGCTCCCGTGA GGCGTGCTTGTCAATGCGGTAAGTGTCACTGATTTTGAACTA TAACGACCGCGTGAGTCAAAATGACGCATGATTATCTTTTAC GTGACTTTTAAGATTTAACTCATACGATAATTATATTGTTATT TCATGTTCTACTTACGTGATAACTTATTATATATATATTTTCT TGTTATAGATATC (SEQ ID NO:3); and

[0032] (d)
TAAAAGTTTTGTTACTTTATAGAAGAAATTTTGAGTTTTTGTT TTTTTTTAATAAATAAATAAACATAAATAAATTGTTTGTTGA ATTTATTATTAGTATGTAAGTGTAAATATAATAAAACTTAAT ATCTATTCAAATTAATAAATAAACCTCGATATACAGACCGAT AAAACACATGCGTCAATTTTACGCATGATTATCTTTAACGTA CGTCACAATATGATTATCTTTCTAGGG (SEQ ID NO:4);

[0033] or (b) a fragment of a nucleic acid sequence 70% homologous to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.

[0034] In some embodiments of the invention, a composition comprising one or more progeny of the organism, wherein the one or more progeny comprise any one or more of the one or more mutations (i), (ii), and (iii).

[0035] In some embodiments of the invention, the one or more progeny further comprise at least one inverted tandem repeat of a transposon or variant thereof.

[0036] In some embodiments of the invention, the composition is a colony of mammals.

[0037] In some embodiments of the invention, the organism is a plant or animal.

[0038] In some embodiments of the invention, the organism is a mini pig.

[0039] In some embodiments of the invention, the organism is a rat or mouse.

[0040] In some embodiments of the invention, the organism is chosen from a mouse, pig, rabbit, dog, cat, goat, non-human primate, mini pig, ferret, farm animals, fish, chicken, and bird.

[0041] In some embodiments of the invention, the organism is a plant chosen from: rice, tobacco, wheat, potato, soybean, tomato, Arabidopsis, maize.

[0042] In some embodiments of the invention, the organism is chosen from a salmonoid, carp, tilopia, or tuna.

[0043] In some embodiments of the invention, the organism is an insect.

[0044] In some embodiments of the invention, a mammal comprising one or more stem cells derived from the germline lineage of an animal, wherein the one or more stem cells comprise one or more of the following mutations: (i) a deletion mutation; (ii) a knockout mutation; and/or (iii) an addition of a heterologous nucleic acid sequence; wherein the one or more mutations of (i), (ii), and/or (iii) are site-specific mutations caused by a ZFN.

[0045] In some embodiments of the invention, the one or more stem cells are transplanted from an *in vitro* culture.

[0046] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is at least 70% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4. In some

embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is at least 75% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0047] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is at least 80% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0048] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is at least 85% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0049] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is at least 90% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0050] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is at least 95% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0051] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is at least 96% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4. In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is at least 99% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0052] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is at least 97% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0053] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is at least 98% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0054] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is no more than 99% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0055] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is no more than 98% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0056] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is no more than 97% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0057] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is no more than 96% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0058] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is no more than 95% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0059] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is no more than 90% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0060] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is no more than 85% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0061] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is no more than 80% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0062] In some embodiments of the invention, the one or more stem cells are spermatogonial stem cells.

[0063] In some embodiments of the invention, the mammal is a rat or mini pig.

[0064] In some embodiments of the invention, the mammal further comprises a nucleic acid that comprises a transposon sequence that is at least 70% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0065] In some embodiments of the invention, the one or more stem cells are spermatogonial stem cells.

[0066] In some embodiments of the invention, the mammal is a rat or mini pig.

[0067] In some embodiments of the invention, the mammal is a sterile male rat or sterile male mini pig.

[0068] In some embodiments of the invention, the rat or mini pig is DAZL deficient or DAZL-/-.

[0069] In some embodiments of the invention, a colony of genetically modified organisms comprises:

at least one organism comprising one or more stem cells, wherein the one or more stem cells comprise one or more of the following mutations: (i) a deletion mutation; (ii) a knockout mutation; and/or (iii) an addition of a heterologous nucleic acid sequence; wherein the one or more mutations of (i), (ii), and/or (iii) are site-specific mutations caused by a ZFN. ;and (b)progeny of the organism of subpart (a).

[0070] In some embodiments of the invention, the heterologous nucleic acid is a selectable marker or an orthologous gene.

[0071] In some embodiments of the invention, the at least one organism and the progeny further comprise at least one inverted tandem repeat of a transposon or variant thereof.

[0072] In some embodiments of the invention, the at least one organism and the progeny further comprise a nucleic acid that comprises a transposon sequence that is at least 70% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

[0073] In some embodiments of the invention, the invention relates to a method of generating one or more genetically modified organisms comprising:

(a) contacting at least one stem cell derived from the germline lineage of an animal or plant by the stem cell with: (i) at least one ZFN that mutates a gene of interest; or (ii) at least one expression vector that encodes a ZFN that mutates a gene of interest, thereby creating at least one stem cell comprising at least one mutation at a gene of interest;

(b) expanding an *in vitro* culture of the at least one stem cell comprising at least one mutation at a gene of interest;

(c) implanting one or more stem cells from the culture of step (b) into an organism.

[0074] In some embodiments of the invention, the invention relates to a method of generating one or more genetically modified organisms comprising:

(a) contacting at least a first and second set of stem cells derived from the germline lineage of an animal or plant by the stem cell with: (i) at least one ZFN that mutates a gene of interest; or (ii) at least one expression vector that encodes a ZFN that mutates a gene of interest, thereby creating at least one stem cell comprising at least one mutation at a gene of interest;

(b) expanding an *in vitro* culture of the at least first and second set of stem cells comprising at least one mutation at a gene of interest;

(c) implanting at least the first and second set of stem cells from the culture of step (b) into an organism. In some embodiments, the method further comprises a third, fourth, fifth, sixth, seventh, eighth, ninth, or ten or more sets of stem cells which have been mutated in a site-specific fashion by a ZFN, and , in which case, after expanding each of the third, fourth, fifth, sixth, seventh, eighth, ninth, or ten or more sets of mutated stem cells, each set of transplanted into a single organism. In some embodiments, the single organism that comprises a set of mutated stem cells is a sterile male.

[0075] In some embodiments of the invention, the organism is capable of passing at least one mutation at a gene of interest to progeny by germline transmission.

[0076] In some embodiments of the invention, the genetically modified organism is a mammal.

[0077] In some embodiments of the invention, the genetically modified organism is a rat or mini pig.

[0078] In some embodiments of the invention, the genetically modified organism is a sterile male rat or sterile male mini pig.

[0079] In some embodiments of the invention, the method further comprises: breeding the organism implanted with the one or more stem cells with another animal to generate one or more progeny that comprise the mutated gene of interest.

[0080] In some embodiments of the invention, the progeny are mammals.

[0081] In some embodiments of the invention, a method of breeding a colony of genetically modified organisms comprising:

(a) contacting at least one stem cell derived from the germline lineage of an animal or plant by the stem cell with: (i) at least one ZFN that mutates a gene of interest; or (ii) at least one expression vector that encodes a ZFN that mutates a gene of interest, thereby creating a stem cell comprising at least one mutation at a gene of interest;

(b) expanding an *in vitro* culture of the stem cell comprising at least one mutation at a gene of interest;

(c) implanting the at least one stem cell comprising at least one mutation at a gene of interest from the culture of step (b) into a first organism.

(d) breeding the first organism with a second organism of the same species;

(e) selecting progeny of the first and second organism that comprise the at least one mutation at a gene of interest; and

(f) breeding the progeny to create a colony of organisms that comprise the at least one mutation at a gene of interest.

[0082] In some embodiments of the invention, the first and second organisms are mammals.

[0083] In some embodiments of the invention, the first and second organisms are rats or mini pigs.

[0084] In some embodiments of the invention, a method of manufacturing a first filial generation of genetically modified organisms comprising two or more distinct subsets of organisms, the method comprising:

(a) contacting a first stem cell with: (i) a ZFN that mutates a first gene of interest; or (ii) an expression vector that encodes a ZFN that mutates a first gene of interest; thereby creating a first stem cell comprising a first mutation;

(b) contacting a second stem cell with a modifying agent, thereby creating a second stem cell comprising a second mutation;

(c) expanding an *in vitro* culture of each of the first and the second stem cells;

(d) implanting a mixed population of stem cells comprising the first and the second stem cells into an organism;

(e) breeding the organism with another organism of the same species.

[0085] In some embodiments of the invention, the first filial generation of genetically modified organisms comprises two or more sets of organisms, each set comprising a distinct mutation of interest derived from a haplotype of distinct stem cells transplanted into a parent of the organism.

[0086] In some embodiments of the invention, at least one stem cell of the mixed population is a spermatogonial stem cell of a mammal.

[0087] In some embodiments of the invention, the organism is a mammal.

[0088] In some embodiments of the invention, a kit comprising:

(a) a ZFN or a nucleic acid sequence that encodes a ZFN that cleaves a nucleic acid sequence at a gene of interest; and

(b) an instruction manual comprising directions; and, optionally

[0089] In some embodiments of the invention, a kit comprising:

(a) In some embodiments of the invention; and, optionally

(b) culture media for the one or more stem cells or one or more embryos.

[0090] In some embodiments of the invention, the kit comprises:

(a) a ZFN or a nucleic acid sequence that encodes a ZFN that cleaves a nucleic acid sequence at a gene of interest; and optionally

(b) culture media for the one or more stem cells or one or more embryos.

[0091] In some embodiments of the invention, the kit comprises:

(a) a ZFN or a nucleic acid sequence that encodes a ZFN that cleaves a nucleic acid sequence at a gene of interest; and

(b) one or more stem cell lines derived from a germline lineage of animal or plant; and, optionally

(c) culture media for the one or more stem cells or one or more embryos; and, optionally

(d) an instruction manual that comprises instructions on how to mutate the one or more stem cells with the ZFN or a nucleic acid sequence that encodes a ZFN that cleaves a nucleic acid sequence at a gene of interest.

[0092] Additional advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended

claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

Brief Description of the Drawing

[0093] This invention, as defined in the claims, can be better understood with reference to the following drawings:

[0094] Figure 1: Schematic of spermatogonial stem cells (SSCs) separated into multiple colonies will be genetically modified with different ZFNs. The genetically modified SSCs will be selected and pooled together to form a pool of SSCs containing different genetic modifications relating to the production of different genetically modified organisms using a single recipient male.

[0095] Figure 2: Schematic of a colony of wild type rat spermatogonial stem cells (SSCs) showed in cell culture.

[0096] Figure 3: Schematic of propagation of SSCs in cell culture

[0097] Figure 4: Western blot of the targeted mutagenesis of the rat transgenic EGFP gene using site-specific zinc finger nuclease (ZFN) in rat spermatogonial stem cells (SSCs).

[0098] Figure 5: Schematic for transfection of SSCs with ZFN and fluorescent marker constructs.

[0099] Figure 6: Zinc finger nuclease (ZFN) recognition site for the transgenic EGFP gene and sequences of genetically modified spermatogonial stem cell (SSC) clones.

[00100] Figure 7: Schematic for transplantation of genetically modified rat SSC transplantation into sterile recipient male rats. The genetically

modified SSCs are used to produce genetically modified rats by mating recipient males with wild type (WT) females.

[0100] Figure 8: Detailed description and sequence of the EGFP ZFN construct as well as ZFN amino acid sequences targeting the transgenic EGFP gene in rat SSCs.

[0101] Figure 9: Detailed description of the minipig Rag1 sequence and proposed ZFN binding and mutation site.

[0102] Table 1: Reagents for the production of SSC medium.

[0103] Table 2: Reagents for passaging and propagation of SSCs.

[0104] Table 3: Transposon inverted terminal repeats (ITRs)

[0105] **Detailed Description of the Invention**

[0106] Described herein are methods for site-specific genetic modification of stem cells which may be used to produce genetically modified organisms. Site-specific genetic modification includes but is not limited to mutations that cause deletions or knockout mutations, as well as mutations that can produce insertions or knockin mutations. In one embodiment, the invention provides site-specific modification of stem cells, especially spermatogonial stem cells (SSCs). The embodiment includes the site-specific technologies, especially zinc finger nuclease (ZFNs). In another embodiment the genetically modified stem cells are somatic stem cells, embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, embryos and other gametes or germ cells and the site-specific technologies include zinc finger nuclease (ZFNs).

[0107] Also described are methods for identifying cells that have acquired site-specific modifications and generating genetically modified organisms from genetifcally modified stem cells. In one embodiment, the invention includes methods for the use of spermatogonia or spermatogonial stem cells (SSCs) containing site-

specific genetic modifications are expanded and grown to adequate numbers and transplanted into azoospermic recipient males that are genetically or chemically sterile. In another embodiment, ES cells, iPS cell-, and embryos or other gametes are used to produce organisms containing site-specific mutations.

[00101] Definitions

[00102] The present invention may be understood more readily by reference to the following detailed description of preferred embodiments of the invention and the Examples included therein and to the Figures and their previous and following description. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All references, publications, patents, patent applications, and commercial materials mentioned herein are incorporated by reference in their entirety for the purpose of describing and disclosing the materials and/or methodologies which are reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

[00103] Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that this invention is not limited to specific synthetic methods, specific recombinant biotechnology methods unless otherwise specified, or to particular reagents unless otherwise specified, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

[00104] Throughout this application, reference is made to various proteins and nucleic acids. It is understood that any names used for proteins or nucleic acids are art-recognized names, such that the reference to the name constitutes a disclosure of the molecule itself.

[00105] As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a pharmaceutical carrier" includes mixtures of two or more such carriers, and the like.

[00106] Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint.

[00107] In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings:

[00108] “Addition of heterologous sequence” is meant to be any introduction of deoxyribonucleotide, nucleotide or DNA sequence within a gene, chromosome or genome of an organism. Also known as a “knock-in” which is meant an alteration in the nucleic acid sequence that replaces the endogenous, normal or wild-type allele with an exogenous allele. The exogenous allele includes but is not limited to a full length gene of

the same or a different species, a section of a gene of the same or different species, a replacement cassette and reporter or selection genes and markers. Knock-in mutations can be produced by homologous recombination, site-specific deletion, repair mechanism provocation via targeting proteins, as well as site specific targeted DNA transposons.

[00109] A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein, or enzyme, i.e., the nucleotide sequence encodes an amino acid sequence for that polypeptide, protein or enzyme. A coding sequence for a protein may include a start codon (usually ATG) and a stop codon.

[00110] "Complementary," as used herein, refers to the subunit sequence complementarity between two nucleic acids, e.g., two DNA molecules. When a nucleotide position in both of the molecules is occupied by nucleotides normally capable of base pairing with each other, then the nucleic acids are considered to be complementary to each other at this position. Thus, two nucleic acids are complementary to each other when a substantial number (at least 50%) of corresponding positions in each of the molecules are occupied by nucleotides which normally base pair with each other (e.g., A:T and G:C nucleotide pairs).

[00111] "DAZL deficient organisms" or "DAZL deficient rats" or "DAZL-/-" or "DAZL knockdown" means male organisms which have a lack of proper function in *Deleted In-Azoospermia (DAZL)* genes. In some embodiments, *DAZL* deficient organisms fail to produce mature haploid gametes. In some embodiments, *DAZL* deficient organisms are infertile.

[00112] A "deletion mutation" means a type of mutation that involves the loss of genetic material,deoxyribonucleotide, nucleotide, DNA, gene or

chromosome which may be from a single base to an entire piece of chromosome. Deletion of one or more nucleotides in the DNA ,ay relate to an altered reading frame or non-reading frame of the gene, chromosome or genome; hence, it could result in a complete absence of the synthesis, synthesis of a nonfunctional, or synthesis followed be degradation of DNA, RNA, peptide, polypeptide or protein.

[00113] The terms "derived from the germline lineage of an animal or plant" mean (as to any animal, cell, tissues or biomaterial--including nucleotides or DNA or genes or chromosomes or genomes or transgenes or mutations that may be passed on to offspring) obtained or originating from the germ cells of a animal or plant. In some embodiments, the one or more progeny of a parent line may contain a mutated gene of interest which originated from the haplotype of a stem cell transplanted into the testes of the parent line. In such an example the mutated gene of interest is derived from the germline of the parent line.

[00114] "Disease state" is a condition of an organism, tissue or tissues, or cells that exhibit unknown or abnormal clinical features, characteristics, or phenotypes. In some embodiments, the condition relates to an organism, tissue or tissues, or cells, that exhibit a phenotype associated with a known disease. The disease state may be characterized by clinical features such as hyperinsulinemia in diabetes or diagnostic features such as biomarker association as well as genetic features such as mutations and polymorphisms.

[00115] "Embryo" is a multicellular diploid eukaryote in early stage of development.

[00116] "Embryonic like cells from umbilical cord blood" or "CBEs" are cells that can be isolated from umbilical cord blood that have embryonic like properties such as the ability to differentiate into multiple germ layers and expression of embryonic markers.

[00117] "Embryonic stem cell" or ES cell is a pluripotent cell derived from the inner mass of the blastocyst or early stage embryo.

[00118] The terms "express" and "expression" mean allowing or causing the information in a gene or DNA sequence to become manifest, for example producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form an "expression product" such as a protein. The expression product itself, e.g. the resulting protein, may also be said to be "expressed". An expression product can be characterized as intracellular, extracellular or secreted. The term "intracellular" means something that is inside a cell. The term "extracellular" means something that is outside a cell. A substance is "secreted" by a cell if it appears in significant measure outside the cell, from somewhere on or inside the cell.

[00119] The term "gene", also called a "structural gene" means a DNA sequence that codes for or corresponds to a particular sequence of amino acids which comprise all or part of one or more proteins or enzymes, and may or may not include introns and regulatory DNA sequences, such as promoter sequences, 5'-untranslated region, or 3'-untranslated region which affect for example the conditions under which the gene is expressed. Some genes, which are not structural genes, may be transcribed from DNA to RNA, but are not translated into an amino acid sequence. Other genes may function as regulators of structural genes or as regulators of DNA transcription.

[00120] "Gene of interest" is meant a nucleotide, nucleotide sequence, DNA, RNA, polypeptide, sequence on a chromosome or within the genome of an organism which is to be genetically modified or altered in some way.

The gene of interest can be mutated or its nucleotide sequence may be altered.

[00121] "Genetic background" or "strain" is meant a genetic composition that is characteristic of an organism. Organisms that have been bred may have a known genetic strain that may be useful for different research reasons. Organisms that evolve in regions of the earth contain different genetic backgrounds which may alter gene function and important physiological functions.

[00122] "Genetic modification associated with the gene of interest" means a mutation or other genetic modification which corresponds to a gene that is being studied or selected for. The genetic modification may involve either endogenous or exogenous genes.

[00123] "Genetically modified" or "genetic modification" means a gene or other DNA sequence that is altered from its native state (e.g. by insertion mutation, deletion mutation, nucleic acid sequence mutation, or other mutation), or that a gene product is altered from its natural state (e.g., by delivery of a transgene that works *in trans* on a gene's encoded mRNA or protein, such as delivery of inhibitory RNA or delivery of a dominant negative transgene). "Mutations" may produce organisms that are genetically modified or a specific genetic modification. "Mutations" may include but are not limited to one or more nucleic acid substitutions, deletions, frameshift mutations, or nonsense mutations.

[00124] A "germ cell" is a cell that gives rise to the gametes of an organism. The germ cell is often a pluripotent cell which can differentiate into gametes as well as other biological cell types. A germ cell includes but is not limited to pluripotent cells, totipotent cells, spermatogonial stem cells (SSCs), embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, embryos, germ cells, primordial germ cells (PGCs), plant tube cells, pollen cells, and spores.

[00125] By "exon" is meant a region of a gene which includes sequences which are used to encode the amino acid sequence of the gene product.

[00126] The term "heterologous" refers to a combination of elements not naturally occurring. For example, heterologous DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell. A heterologous expression regulatory element is such an element operatively associated with a different gene than the one it is operatively associated with in nature.

[00127] As used herein, the term "homology" refers to the subunit sequence identity or similarity between two polymeric molecules e.g., between two nucleic acid molecules, e.g., between two DNA molecules, or two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, e.g., if a position in each of two polypeptide molecules is occupied by phenylalanine, then they are identical at that position. The homology between two sequences, most clearly defined as the % identity, is a direct function of the number of identical positions, e.g., if half (e.g., 5 positions in a polymer 10 subunits in length) of the positions in two polypeptide sequences are identical then the two sequences are 50% identical; if 70% of the positions, e.g., 7 out of 10, are matched or homologous, the two sequences share 70% identity. By way of example, the polypeptide sequences ACDEFG and ACDHIK share 50% identity and the nucleotide sequences CAATCG and CAAGAC share 50% identity.

[00128] “Homologous recombination” is the physical exchange of DNA expedited by the breakage and reunion of two non-sister chromatids. In order to undergo recombination the DNA duplexes must have

complimentarity. The molecular mechanism is as follows: DNA duplexes pair, homologous strands are nicked, and broken strands exchange DNA between duplexes. The region at the site of recombination is called the hybrid DNA or heteroduplex DNA. Second nicks are made in the other strand, and the second strand crosses over between duplexes. After this second crossover event the reciprocal recombinant or splice recombinant is created. The duplex of one DNA parent is covalently linked to the duplex of another DNA parent. Homologous recombination creates a stretch of heteroduplex DNA.

[00129] A “induced pluripotent stem cell” or (iPS) cell is an adult cell that has been reprogrammed back to an embryonic like state. iPS cells can differentiate into many different cell types as well as produce genetically modified organisms.

[00130] “Minipig” or “pig” are breeds of inbred or outbred swine which can be used for research.

[00131] A “modifying agent” or “mutagen” is meant to be a physical or biological or chemical agent that changes genetic material or nucleotides, DNA, genes, chromosomes, genomes or organisms. Modifying agents can include natural and engineered proteins such as ZFNs.

[00132] A "mutation" is a change or in the process of change. A change in the genetic material in the organism, which is transmitted to the organism's progeny. A permanent or heritable change in a nucleotide sequence of a gene or chromosome; the process in which such a change occurs in a gene or chromosome. In some embodiments, a mutation is a change in one or more deoxyribonucleotides, the modification being obtained by, for example, adding, deleting, inverting, or substituting nucleotides.

Exemplary mutations include but are not limited to a deletion mutation, an insertion mutation, a non-sense mutation or a missense mutation. Thus, the terms "mutation" or "mutated" as used herein are intended to denote an alteration in the "normal" or "wild-type" nucleotide sequence of any nucleotide sequence or region of the allele. As used herein, the terms "normal" and "wild-type" are intended to be synonymous, and to denote any nucleotide sequence typically found in nature. The terms "mutated" and "normal" are thus defined relative to one another; where a cell has two chromosomal alleles of a gene that differ in nucleotide sequence, at least one of these alleles is a "mutant" allele as that term is used herein. A mutation may also be a "DNA" or "nucleic acid sequence mutation" or a "frameshift mutation". Any mutation that alters a DNA sequence may cause one or more nucleic acid changes or deletions.

[00133] By "knockout" is meant a mutation or an alteration in the nucleic acid sequence that reduces the biological activity of apeptide, polypeptide, protein, or RNA normally encoded therefrom by at least 80% compared to the unaltered gene. The alteration may be an insertion, deletion, frameshift mutation, or missense mutation. The alteration may be an insertion or deletion, or is a frameshift mutation that creates a stop codon. The knockout mutation may result in complete elimination of the function of a gene or nucleotide sequence. A knockout mutation may also be known as a null mutation.

[00134] "Non-homologous end joining (NHEJ)" is a cellular repair mechanism. The NHEJ pathway is defined by the ligation of blunt ended double stand DNA breaks. The pathway is initiated by double strand breaks in the DNA, and works through the ligation of DNA duplex blunt ends. The first step is recognition of double strand breaks and formation of

scaffold. The trimming, filling in of single stranded overhangs to create blunt ends and joining is executed by the NHEJ pathway. An example of NHEJ is repair of a DNA cleavage site created by a zinc finger nuclease (ZFN). This would normally be expected to create a small deletion mutation.

[00135] "Nucleic Acid sequence mutation" is a mutation to the DNA that involves change of one or multiple nucleotides. A point mutation which affects a single nucleotide can result in a transition (purine to purine or pyrimidine to pyrimidine) or a transversion (purine to pyrimidine or pyrimidine to purine). A point mutation that changes a codon to represent a different amino acid is a missense mutation. Some point mutations can cause a change in amino acid so that there is a premature stop codon; these mutations are called nonsense mutations. A mutation that inserts or deletes a single base will change the entire downstream sequence and are known as frameshift mutations. Some mutations change a base pair but have no effect on amino acid representation; these are called silent mutations. Mutations to the nucleic acid of a gene can have different consequences based on their location (intron, exon, regulatory sequence, and splice joint).

[00136] As used herein, the term "phenotype" means any property of a cell or organism. A phenotype can simply be a change in expression of an mRNA or protein. Examples of phenotypes also include, but are in no way limited to, cellular, biochemical, histological, behavioral, or whole organismal properties that can be detected by the artisan. Phenotypes include, but are not limited to, cellular transformation, cell migration, cell morphology, cell activation, resistance or sensitivity to drugs or chemicals, resistance or sensitivity to pathogenic protein localization within the cell (e.g. translocation of a protein from the cytoplasm to the

nucleus), profile of secreted or cell surface proteins, (e.g., bacterial or viral) infection, post-translational modifications, protein localization within the cell (e.g. translocation of a protein from the cytoplasm to the nucleus), profile of secreted or cell surface proteins, cell proliferation, signal transduction, metabolic defects or enhancements, transcriptional activity, cell or organ transcript profiles (e.g., as detected using gene chips), apoptosis resistance or sensitivity, animal behavior, organ histology, blood chemistry, biochemical activities, gross morphological properties, life span, tumor susceptibility, weight, height/length, immune function, organ function, any disease state, and other properties known in the art. In certain situations and therefore in certain embodiments of the invention, the effects of mutation of one or more genes in a cell or organism can be determined by observing a change in one or more given phenotypes (e.g., in one or more given structural or functional features such as one or more of the phenotypes indicated above) of the mutated cell or organism compared to the same structural or functional feature(s) in a corresponding wild-type or (non-mutated) cell or organism (e.g., a cell or organism that in which the gene(s) have not been mutated).

[00137] By "plasmid" is meant a circular strand of nucleic acid capable of autosomal replication in plasmid-carrying bacteria. The term includes nucleic acid which may be either DNA or RNA and may be single- or double-stranded. The plasmid of the definition may also include the sequences which correspond to a bacterial origin of replication.

[00138] “Pluripotent cells” are stem cells that are capable of differentiating into any of the germ layers and can produce any type of fetal and adult cell.

[00139] A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream

(3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. The promoter may be operatively associated with other expression control sequences, including enhancer and repressor sequences.

[00140] The term "regulatory sequence" is defined herein as including promoters, enhancers and other expression control elements such as polyadenylation sequences, matrix attachment sites, insulator regions for expression of multiple genes on a single construct, ribosome entry/attachment sites, introns that are able to enhance expression, and silencers.

[00141] By "reporter gene" is meant any gene which encodes a product whose expression is detectable. A reporter gene product may have one of the following attributes, without restriction: fluorescence (e.g., green fluorescent protein), enzymatic activity (e.g., lacZ or luciferase), or an ability to be specifically bound by a second molecule (e.g., biotin or an antibody-recognizable epitope).

[00142] By "selectable marker" is meant a gene product which may be selected for or against using chemical compounds, especially drugs. Selectable markers often are enzymes with an ability to metabolize the toxic drugs into non-lethal products. For example, the pac (puromycin acetyl transferase) gene product can metabolize puromycin, the dhfr gene

product can metabolize trimethoprim (tmp) and the bla gene product can metabolize ampicillin (amp). Selectable markers may convert a benign drug into a toxin. For example, the HSV tk gene product can change its substrate, FIAU, into a lethal substance. Another selectable marker is one which may be utilized in both prokaryotic and eukaryotic cells. The neo gene, for example, metabolizes and neutralizes the toxic effects of the prokaryotic drug, kanamycin, as well as the eukaryotic drug, G418.

[00143] By "selectable marker gene" as used herein is meant a gene or other expression cassette which encodes a protein which facilitates identification of cells into which the selectable marker gene is inserted.

[00144] A "site-specific mutation" is used herein to refer to a location in the genome that is predetermined as the position where atargeted mutation will take place. The site-specific mutation may result in a knockout, knock-in or otherwise genetically modified cell. It is also used herein to refer to a specific location in the genome that is modified by any insertion mutation or deletion mutation or nucleic acid sequence mutation or forced repair mutation.

[00145] “Somatic stem cell” or adult stem cell is a potent cell found in organs after embryonic development. Somatic stem cells can be isolated from organs and tissues and have the potential to differentiate into many cell types of that organ and organism.

[00146] “Spermatogonial stem cell” or (SSC) is meant to be a sperm stem cell which maintains spermatogenesis.

[00147] “Sterile” or “sterile animal” or “sterile male” is meant to be a animal which is unable to produce endogenous germ cells or is not capable of producing suitable numbers of endogenous germ cells or not capable of

producing mature germ cells. Sterile animals may not be able to produce sperm or spermatids. Sterile animals may not be able to generate offspring or breed.

[00148] As used herein, the term "targeted genetic recombination" refers to a process wherein recombination occurs within a DNA target locus present in a host cell or host organism. Recombination can involve either homologous or non-homologous DNA.

[00149] "Totipotent cells" are cells that have the ability to divide and differentiate into any cell type including extraembryonic cells.

[00150] The term "transfection" means the introduction of a foreign nucleic acid into a cell. The term "transformation" means the introduction of a "foreign" (i.e. extrinsic or extracellular) gene, DNA or RNA sequence into a cell. In some embodiments, transformation means the introduction of a "foreign" (i.e. extrinsic or extracellular) gene, DNA or RNA sequence into an ES cell or pronucleus, so that the cell will express the introduced gene or sequence to produce a desired substance in an organism or genetically modified organism.

[00151] By "transgenic" is meant any organism which includes a nucleic acid sequence which is inserted by artifice into a cell and becomes a part of the genome of the organism that develops from that cell. Such a transgene may be partly or entirely heterologous to the transgenic organism. Although transgenic mice represent another embodiment of the invention, other transgenic mammals including, without limitation, transgenic rodents (for example, hamsters, guinea pigs, rabbits, and rats), and transgenic pigs, cattle, sheep, and goats are included in the definition.

[00152] A "variant" is a nucleotide, set of nucleotides, DNA, RNA, gene, chromosome, genome, cell or organism which differs. The variant may differ in

nucleotide sequence, gene expression, RNA expression, protein expression and function, genotype, phenotype and characteristics. In some embodiments, the cells or embryos of the invention comprise variant transposon inverted tandem repeats (ITRs) that are at least 70, 75, 80, 85, 90, 95, 96, 97, 98, 99% homologous to known ITRs. in some embodiments, the variant is a variant of a transposon ITR shown in table 3.

[00153] The term "vector" is used interchangeably with the terms "construct", "cloning vector" and "expression vector" and means the vehicle by which a DNA or RNA sequence (e.g. a foreign gene) can be introduced into a host cell, (e.g. ES cell or pronucleus) so as to transform the host and promote expression (e.g. transcription and translation) of the introduced sequence including but not limited to plasmid, phage, transposons, retrotransposons, viral vector, and retroviral vector. By "non-viral vector" is meant any vector that does not comprise a virus or retrovirus.

[00154] A "vector sequence" as used herein, refers to a sequence of DNA comprising at least one origin of DNA replication and at least one selectable marker gene.

[00155] For the purposes of the present invention, the term "zinc finger nuclease" or "ZFN" refers to a chimeric protein molecule comprising at least one zinc finger DNA binding domain effectively linked to at least one nuclease or part of a nuclease capable of cleaving DNA when fully assembled. Ordinarily, cleavage by a ZFN at a target locus results in a double stranded break (DSB) at that locus.

[00156] The present invention provides methods to produce a desired site-specific mutation in a variety of stem cells in order to develop heterozygous or homozygous genetically modified organisms. In one embodiment, the method for producing the site-specific mutation is the use of a zinc finger nuclease (ZFN). Specifically, the invention pertains

to a site-specific mutation generated in a stem cell, which includes but is not limited to somatic stem cells,spermatogonial stem cells (SSCs), embryonic stem (ES) cells, embryos, and induced pluripotent stem (iPS) cells. Stem cells with site-specific mutations are used to produce genetically modified organisms.

[00157] The methods of the present invention can be used to mutate any eukaryotic stem cell, including, but not limited to, haploid, diploid, triploid, tetraploid, or aneuploid. In one embodiment, the cell is diploid. Stem ells in which the methods of the present invention can be advantageously used include, but are not limited to stem cells such as somatic stem cells, SSCs, ES cells, iPS cells, embryos, or any cell capable of developing into organisms.

[00158] In one embodiment, the invention comprises of methods to produce a site-specific knockout, knock-in or otherwise genetically modified stem cell. The site-specific mutation is generated using a ZF-nuclease (ZFN) which cleaves the desired site, followed by NHEJ, resulting in deletion mutations. The site-specific mutation can be produced in spermatogonial stem cells (SSCs) which are used to generate heterozygous or homozygous genetically modified organisms.

[00159] In another embodiment, the invention comprises of methods to produce a site-specific knockout, knock-in or otherwise genetically modified stem cell. The site-specific mutation is generated using a ZFN which cleaves the desired site resulting in deletion mutations. The site specific mutation is produced in embryonic stem (ES) cells, which are used to generate heterozygous or homozygous genetically modified organisms.

[00160] In another embodiment, the invention comprises of methods to produce a site-specific knockout, knock-in or otherwise genetically modified stem cell. The site specific mutation is generated using a ZFN which cleaves the desired site resulting in deletion mutations. The site-specific mutation is produced in induced pluripotent stem (iPS) cells, which are used to generate heterozygous or homozygous genetically modified organisms.

[00161] In another embodiment, the invention comprises of methods to produce a site-specific knockout, knockin or otherwise genetically modified stem cell. The site specific mutation is generated using a ZFN which cleaves the desired site resulting in deletion mutations. The site-specific mutation is produced in embryos which are used to generate heterozygous or homozygous genetically modified organisms.

[00162] In certain embodiments of the invention, cells can be mutated within the organism or within the native environment as in tissue explants (e.g., *in vivo* or *in situ*). Alternatively, tissues or stem cells isolated from the organism using art-known methods and genes can be mutated according to the present methods. The tissues or stem cells are either maintained in culture (e.g., *in vitro*), or re-implanted into a tissue or organism (e.g., *ex vivo*).

[00163] **Zinc Finger Nucleases (ZFN)**

[00164] In another method, a zinc finger nuclease creates site-specific deletions via double stranded DNA breaks that are repaired by non-homologous end joining (NHEJ). Zinc finger nucleases may also be used to create an insertion mutation by combining the ZFN with a cassette that will enter into the genome by homologous recombination to create an insertion mutation. Therefore, this genetic modification method can be

used for both targeted (site-specific) DNA insertions, or knockin mutations, and targeted DNA deletions, or knockout mutations. In one embodiment, the method involves transformation of a cell with a nucleic acid or mRNA construct minimally comprising DNA encoding a chimeric zinc finger nuclease (ZFN) by linking at least one or two zinc finger proteins to the cleavage domain of at least one endonuclease, which can be used to create a DNA deletion. In another embodiment, a second DNA construct can be provided that will serve as a template for repair of the cleavage site by homologous recombination. In this embodiment, a DNA insertion may be created. The DNA insertion may contain a gene trap cassette. In one embodiment, this method can be combined with spermatogonial stem cell (SSC) technology or embryonic stem cell (ESC) technology, induced pluripotent stem cells (iPSCs), embryos and other pluripotent cells. In another embodiment, this method can be combined with mobile DNA technology. In one embodiment ZFNs are used to generate site-specific mutations in SSCs or ESCs or iPSCs or embryos and other pluripotent cells.

[00165] Zinc finger proteins (Cys2-His2) contain sequences of the form (Tyr, Phen)- Xaa-Cys-Xaa2-4, Cys-Xaa3-Phe-Xaa5-Leu-Xaa2-His-Xaa3-5-His in tandem arrays which bind to zinc, thereby forming structural domains that bind the α-helix of the major groove of the DNA double helix. Variations of key amino acids in each DNA binding finger contribute to binding affinity and specificity. The zinc finger domains can be linked together in order to design a protein which binds to a specific DNA sequence. Alternatively, selection of desirable mutants from a library of randomized zinc fingers using phage display can generate DNA-specific binding domains. The DNA cleavage domain of nucleases is fused into the zinc finger domains to produce a hybrid zinc

finger-nuclease or ZFN which binds to a specific site on the DNA and produces mutations.

[00166] Various methods have been described for engineering multi-finger domains. Much research has revealed that a key requirement for constructing high-quality, multi-finger domains is accounting for the context-dependent activities of individual finger domains within the longer array. For example, simple "modular assembly" of pre-selected individual fingers has been shown to have a very low success rate and to yield proteins with low affinities and specificities for their target DNA site.

[00167] The Oligomerized Pool ENgineering (OPEN) method for constructing multi-finger domains addresses the context-dependent activities of individual zinc fingers but is also robust and relatively easier to perform than previously described methods. OPEN is scalable and can be used to generate high quality multi-finger domains for a very large number of different target sites in parallel. To date, OPEN has been used to generate multi-finger domains for over 500 different target sites that function well in a bacterial cell-based assays. Many of these domains have been converted into ZFNs and have been shown to mediate efficient gene modification in organisms including zebrafish, Arabidopsis, tobacco, mouse, and human somatic and pluripotent stem cells.

[00168] Genetic modification of SSCs using ZFNs requires undifferentiated SSCs, transfection of the SSCs with ZFNs and a selection marker, clonal selection of genetically modified SSCs, germline transmission of genetically modified SSCs, and germline transmission of recipient founders.

[00169] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating mutations at higher efficiency due to the unique nature of SSCs, including but not limited to chromatin structure and methylation patterns.

[00170] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in the same SSC or SSC line which relates to generating genetically modified organisms with multiple mutations in fewer experimental steps and in a shorter timeframe than is possible with other systems.

[00171] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in the same SSC or SSC line in multiple and consecutive experiments or transfections which relates to generating genetically modified organisms with multiple mutations in fewer experimental steps and in a shorter timeframe than is possible with other systems.

[00172] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to generating multiple genetically modified organisms containing one or more mutations is fewer experimental steps and in a shorter timeframe than is possible with other systems.(Figure 1). The separate SSCs or SSC lines may be two or more.

[00173] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to

generating multiple genetically modified organisms containing one or more mutations is fewer experimental steps and in a shorter timeframe than is possible with other systems.(Figure 1). The separate SSCs or SSC lines may be three or more.

[00174] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to generating multiple genetically modified organisms containing one or more mutations is fewer experimental steps and in a shorter timeframe than is possible with other systems.(Figure 1). The separate SSCs or SSC lines may be four or more.

[00175] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to generating multiple genetically modified organisms containing one or more mutations is fewer experimental steps and in a shorter timeframe than is possible with other systems.(Figure 1). The separate SSCs or SSC lines may be five or more.

[00176] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to generating multiple genetically modified organisms containing one or more mutations is fewer experimental steps and in a shorter timeframe than is possible with other systems.(Figure 1). The separate SSCs or SSC lines may be six or more.

[00177] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to generating multiple genetically modified organisms containing one or more mutations is fewer experimental steps and in a shorter timeframe than is possible with other systems.(Figure 1). The separate SSCs or SSC lines may be seven or more.

[00178] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to generating multiple genetically modified organisms containing one or more mutations is fewer experimental steps and in a shorter timeframe than is possible with other systems.(Figure 1). The separate SSCs or SSC lines may be eight or more.

[00179] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to generating multiple genetically modified organisms containing one or more mutations is fewer experimental steps and in a shorter timeframe than is possible with other systems.(Figure 1). The separate SSCs or SSC lines may be nine or more.

[00180] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to

generating multiple genetically modified organisms containing one or more mutations is fewer experimental steps and in a shorter timeframe than is possible with other systems.(Figure 1). The separate SSCs or SSC lines may be ten or more.

[00181] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to generating multiple genetically modified organisms containing one or more mutations is fewer experimental steps and in a shorter timeframe than is possible with other systems.(Figure 1). The separate SSCs or SSC lines may be eleven or more.

[00182] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to generating multiple genetically modified organisms containing one or more mutations is fewer experimental steps and in a shorter timeframe than is possible with other systems.(Figure 1). The separate SSCs or SSC lines may be twelve or more.

[00183] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to generating multiple genetically modified organisms containing one or more mutations is fewer experimental steps and in a shorter timeframe than is possible with other systems.(Figure 1). The separate SSCs or SSC lines may be thirteen or more.

[00184] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to generating multiple genetically modified organisms containing one or more mutations is fewer experimental steps and in a shorter timeframe than is possible with other systems.(Figure 1). The separate SSCs or SSC lines may be fourteen or more.

[00185] In some embodiment of the invention, genetic modification of SSCs using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to generating multiple genetically modified organisms containing one or more mutations is fewer experimental steps and in a shorter timeframe than is possible with other systems.(Figure 1). The separate SSCs or SSC lines may be fifteen or more.

[00186] In some embodiment of the invention, increasing the number of distinct or separate pools or lines of genetically modified SSCs, which may be used to generate a genetically modified organism, does not increase the amount of effort, time, and resources used, as well as does not decrease the efficiency of genetically modified organism production. Multiple separate and distinct genetically modified SSCs may be transplanted into a single sterile recipient. The mixed population of distinct genetically modified SSCs, which are derived from separate SSC pools from two or more pools to fifteen or more pools mature within the sterile recipient. The sterile recipient is then bred with multiple wild type females which may be two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more,

ten or more, eleven or more, twelve or more, thirteen or more, fourteen or more, fifteen or more, sixteen or more, seventeen or more, eighteen or more, nineteen or more, twenty or more. These multiple females produce offspring which have incorporated the desired mutation into their germline.

[00187] In some embodiment of the invention, increasing the number distinct or separate pools or lines of genetically modified SSCs, which may be used to generate a genetically modified organism, does not increase the amount of effort, time, and resources used, as well as does not decrease the efficiency of genetically modified organism production. The sterile recipient rat may be a recipient for multiple rounds of separate or distinct genetically modified SSCs. The sterile rat may be a recipient of fifteen or more different genetically modified SSCs and breed with twenty or more wild type females to produce fifteen or more separately genetically modified organisms. Following the first round of breeding, the sterile male may be treated to eliminate the first round of genetically modified SSCs and become a recipient of another round of fifteen or more separately or distinct genetically modified SSCs, breed with twenty or more wild type females to produce fifteen or more separate genetically modified organisms. The sterile male may be a recipient of mixed populations of fifteen or more genetically modified SSCs and breed twenty or more wild type females two times or more, three times or more, four times or more, or five times or more.

[00188] In some embodiment of the invention, increasing the number distinct or separate pools or lines of genetically modified SSCs, which may be used to generate a genetically modified organism, does not increase the amount of effort, time, and resources used, as well as does not decrease the efficiency of genetically modified organism production. Increasing the number of genetically modified SSCs does not

require the effort and resources of other stem cell systems such as embryonic stem (ES) cells or embryos. Increasing the amount of genetically modified ES cells for genetically modified organism production requires an increase in the number of technical steps such as blastocyst injections, as well as the number of oviduct transfer surgeries. In some embodiments of the invention, the method does not comprise blastocyst injection, oviduct transfer, DNA microinjection reimplantation of injected zygotes, ro breeding of chimeric progeny. The SSC system may produce fifteen or more separate genetically modified stem cell populations for genetically modified organism production in a single step, while in order to produce fifteen or more separately genetically modified ES cells, fifteen or more separate steps must be performed on all levels of the procedure, which include but are not limited to blastocyst injection, oviduct transfer, zygote production, preparation of DNA, DNA microinjection, reimplantation of injected zygotes or breeding chimeric progeny.

[00189] In some embodiment of the invention, genetic modification of SSCs using MNs relates to generating genetically modified organisms without requiring the steps required in producing genetically modified organisms from alternative stem cells, including but not limited to embryonic stem cells, embryo's, induced pluripotent stem (iPS) cells and somatic stem cells. Genetic modification in alternative stem cells includes but is not limited to zygote production, preparation of DNA, DNA microinjection, reimplantation of injected zygotes or breeding chimeric progeny.

[00190] In some embodiments, the stem cells of the present invention comprise one or more transposons, one or more inverted tandem repeats (ITRs) of a transposon of variants thereof. In some embodiments, the stem cells of the present invention comprise one or more transposons, one or more inverted tandem repeats (ITRs) of a transposon of variants derived from the sequences of Table 3.

[00191]

[00192] In some embodiments, the present invention comprise one or more transposons, one or more inverted tandem repeats (ITRs) of a transposon wherein the variant sequence inverted tandem repeats are at

least 70 % homologous to known ITRs and known transposon elements (shown in table 3).

[00193] In some embodiments, the present invention comprise one or more transposons, one or more inverted tandem repeats (ITRs) of a transposon wherein the variant sequence inverted tandem repeats are at least 75 % homologous to known ITRs and known transposon elements (shown in table 3).

[00194] In some embodiments, the present invention comprise one or more transposons, one or more inverted tandem repeats (ITRs) of a transposon wherein the variant sequence inverted tandem repeats are at least 80 % homologous to known ITRs and known transposon elements (shown in table 3).

[00195] In some embodiments, the present invention comprise one or more transposons, one or more inverted tandem repeats (ITRs) of a transposon wherein the variant sequence inverted tandem repeats are at least 85 % homologous to known ITRs and known transposon elements (shown in table 3).

[00196] In some embodiments, the present invention comprise one or more transposons, one or more inverted tandem repeats (ITRs) of a transposon wherein the variant sequence inverted tandem repeats are at least 90 % homologous to known ITRs and known transposon elements (shown in table 3).

[00197] In some embodiments, the present invention comprise one or more transposons, one or more inverted tandem repeats (ITRs) of a transposon wherein the variant sequence inverted tandem repeats are at least 95 % homologous to known ITRs and known transposon elements (shown in table 3).

[00198] In some embodiments, the present invention comprise one or more transposons, one or more inverted tandem repeats (ITRs) of a transposon wherein the variant sequence inverted tandem repeats are at least 96 % homologous to known ITRs and known transposon elements (shown in table 3).

[00199] In some embodiments, the present invention comprise one or more transposons, one or more inverted tandem repeats (ITRs) of a transposon wherein the variant sequence inverted tandem repeats are at least 97 % homologous to known ITRs and known transposon elements (shown in table 3).

[00200] In some embodiments, the present invention comprise one or more transposons, one or more inverted tandem repeats (ITRs) of a transposon wherein the variant sequence inverted tandem repeats are at least 98 % homologous to known ITRs and known transposon elements (shown in table 3).

[00201] In some embodiments, the present invention comprise one or more transposons, one or more inverted tandem repeats (ITRs) of a transposon wherein the variant sequence inverted tandem repeats are at least 99 % homologous to known ITRs and known transposon elements (shown in table 3).

[00202] Generating undifferentiated SSCs requires using SSC media and feeder media using DMEM-high glucose + Sodium Bicarbonate Medium contains Dulbecco's Modified Eagle's Medium-high glucose (Sigma, D5648); 1.5g Sodium Bicarbonate (Sigma, S5761), 1L sterile water which are filtered using a 0.2um filter unit and stored at 4C; SSC Feeder Medium contains 225mL DMEM-high glucose + sodium bicarbonate; 25mL Heat Inactivated Fetal Bovine Serum: FBS (Tissue

Culture Biologicals, 104), which are filtered using a 0.2um filter unit and stored at 4C; 0.1% Gelatin is generated by dissolving 1 g gelatin from Porcine Skin- Type A (Sigma, G1890) in 1L ultrapure water. Gelatin is autoclaved on liquid cycle and stored at 4C; Recombinant Rat GDNF (rR-GDNF; R&D Systems, 512-GF-010) is supplied at 10ug, then reconstituted to 100ug/mL (100ng/uL) by adding 100uL 1x PBS/ 0.1% BSA (0.001g BSA-Calbiochem 126609 in 10mL Sigma D8537 1x PBS-sterile filtered). rR-GDNF is pipetted up and down to mix, but not vortexed. Do not freeze thaw rR-GDNF more than 2x and store at -20C, Recombinant Fibroblast Growth Factor- Basic Human (rbH-FGF; Sigma, F0291) is supplied at 25ug, then reconstituted to 25ug/mL (25ng/uL) by adding 1mL- 1x PBS/ 0.1% BSA (0.001g BSA-Calbiochem 126609 in 10mL Sigma D8537 1x PBS-sterile filtered). rbH-FGF is pipetted up and down to mix but not vortexed. Do not freeze thaw rbH-FGF more than 2x and store at -20C, Dilute 2-Mercaptoethanol (Sigma M3148) is prepared by adding 4.7uL stock to 6mL DHF12 (Sigma D8437).

[00203] Spermatogonial Culture Medium (SG Medium) is made by preparing reagents such as in Table 1. in the SG medium the rR-GDNF final concentration is 20 ng/ml, rbH-FGF 20 ng/ml, 2-mercaptoethanol 100 μM, L-glutamine 4mM final concentration – media's overall final concentration glutamine in 6mM, B27 Supplement minus vitamin A, 1x. Sterile filter the medium using 0.2um filter unit, and store at 4C. Media over two weeks old is not to be used.

[00204] Subculturing and preserving rat SSCs for propagation and archiving requires preparing fibroblasts feeder cell lines and cryopreservation of SSCs.

[00205] In order to prepare fibroblasts feeder cell lines coat dish with 0.1% gelatin and incubate at 37C -1 hour and wash 1x with 1x PBS. Thaw IRR mouse embryonic fibroblasts (Global Stem) by placing frozen vial at 37C immediately after removing from liquid nitrogen until ice crystals disappear. Transfer contents into 9mL of 37C DR4 Feeder medium. Spin at 1000 rpm for 5 minutes, discard supernatant, and resuspend in SSC Feeder medium. Plate on gelatin coated surface in SSC Feeder medium for 16-48 hr. Using 6-well plate (Costar) – 0.43 x 10^6 cells/well and 10cm dish – 2.6 x 10^6 cells/dish rinse with 1x PBS (sigma D8537) and then pre-incubate in SG medium for an additional 16-48 hr. The SG medium used for pre-incubation is then discarded and spermatogonia are passaged onto the MEFs in fresh SG medium (Table 1).

[00206] In order to sub-culture SSC lines The initial passage of spermatogonial cultures after thawing onto MEF feeder layers requires a 1:1 to 1:2 split into the same size wells at 10-12 days after their initial seeding onto the MEFs. Additionally, if required, fresh MEFs (2x104/cm2) can also "spiked" into the on-going spermatogonial cultures on day 11-12 so to by-pass the need to immediately passage the spermatogonia before expanding to larger numbers. Using 6-well plates (Costar) – 0.19 x 10^6 cells/well and 10cm dish – 1.16 x 10^6 cells/dish once established after the first couple passages on MEFs post-thaw, cultures of spermatogonia are passaged at ~1:3 dilutions onto a fresh monolayer of MEFs every 10-14 days at ~3x104 cells/cm2 for over 5 months (i.e. ~12 passages).

[00207] Requirements for passaging and propagation are shown in Table 2. For passaging, cultures are first harvested by gently pipetting them free from the MEFs. After harvesting, the "clusters" of spermatogonia are

dissociated by gentle trituration with 20-30 strokes through a P1000 pipet tip in SG Medium. The dissociated cells are pelleted at 400 x g for 4 minutes and the number of cells recovered during each passage is determined by counting on a Hemocytometer (Note: spermatogonial clusters are not disrupted for counting until the second passage on MEFs). Spermatogonia are easily distinguished during counting as the predominant population of smaller, round cells with smooth surfaces, as compared to occasionally observed, larger and often irregular shaped irradiated MEFs. Typically, 2-4 x 106spermatogonia can be harvested from a single, 10 cm dish (Figure 3).

[00208] Cryopreservation of SSCs for archiving is achieved by preparing Spermatogonial Freezing Medium (SG Freezing Medium) by adding DMSO (Sigma, D2650) at a concentration of 10% (v/v) in SG Medium. Filter-sterilize and cool the prepared freezing medium on ice prior to use. Prepare a 5100 Cryo 1°C Freezing Container "Mr. Frosty" (Thermo Fisher Scientific Nalgene, Inc.,15-350-50) by adding 200 ml fresh isopropanol to the outer chamber. Chill the container by equilibrating it to ~4C in a refrigerator prior to use. Suspend the harvested spermatogonial pellet in ice-cold, SG Freezing Medium at 2x105 to 2x106 cells/ml and then aliquot stocks into cryovials (Thermo Fisher Scientific Nalgene, 03-337-7D) at 1ml/vial. Work quickly and place filled cryovials on ice while finishing aliquots. Place cryovials of spermatogonial stocks into the pre-chilled "Mr Frosty" and close container firmly. Store the freezing container of spermatogonial stocks at -80C for 24 hours, then transfer vials into a liquid nitrogen cryostorage unit.

[00209] Transfection of Rat Spermatogonia with zinc finger nuclease (ZFNs) construct and plasmid DNA such as selection fluorescent markers and

homologous recombination vectors using Lipofectamine 2000 requires a number of reagents and methods.

[00210] ZFN constructs and plasmids used for transfection into SSCs may contain a CAG or CMV promoter, followed by the ZFN encoding gene, as well as a FLAG tag (Figure 8). Some vectors will also express more than one ZFN by joining the coding sequences together in-frame with cleaving sequences.

[00211] Reagents include undifferentiated spermatogonia, SG Medium (pre-warmed), Opti-MEM (cat. no. 31985-062; Invitrogen, Inc.), Lipofectamine 2000 (cat. no. 11668-019; Invitrogen), highly purified ZFN construct and plasmid DNA containing selection markers or homologous recombination vectors in TE buffer at 1-2 μg/μl, Gelatin-coated plates, and plates with fresh MEF feeder layers.

[00212] In some embodiment, the site-specific technology using ZFN was employed. ZFN DNA binding domains can be engineered to bind to a sequence of choice. A rat spermatogonial stem cell line derived from transgenic rats expressing EGFP was transfected (2 million cells/transfection) with 6.7 ug of plasmid pCK181 + 3.3 ug pUBC-dsRED2-1 using Amaxa Solution L and program A020 in an AmaxaNucleofectorAppratus. Transfected spermatogonia were plated into a 10 cm dish containing DR4 mouse embryonic fibroblasts (DR4 MEFs; Applied Stem Cell, Inc.) and maintained for 7 days in Spermatogonial Culture Medium (SG Medium). Cells were then harvested and red fluorescent, dsRed2-1 positive cells were sorted (FACS) into a single well of a 6-well dish (9.6cm2) and maintained for 11 days in SG Medium on DR4 MEFs. Spermatogonia were then harvested and expanded into 4 wells of a 6-well dish at ~30 thousand cells/well and maintained for 13 days in SG Medium on

DR MEFS. 96 individual spermatogonial colonies were picked using a p200 pipette and passaged into individual wells of 2 x 48 plates on DR4 MEFs in SG Medium. Colonies were maintained for 14 days and then split into replicate plates on DR4 MEFs in SG Medium. After expansion for an additional 7 days genomic DNA was isolated form 81 of 96 picked colonies and processed for analysis by PCR cloning and DNA sequencing of regions amplified with PCR primers flanking Zinc Finger Recognition Site 304 (ZFN 304; EG304). 1 of 81 picked colonies (i.e. Colony H9) showed Zinc Finger Endonuclease cleavage and DNA repair at the predicted Recognition Site (Figure 4 and 6).

[00213] Prepare a Transfection Mixture containing Lipofectamine 2000 (Invitrogen) ZFN construct and plasmid DNA in Opti-MEM, as follows: In a 1.5 ml microfuge tube, dilute 1 μg DNA/100 μlOpti-MEM. In a separate 1.5 ml microfuge tube, dilute 2 μlLipofectamine 2000/100 μlOpti-MEM. Incubate tubes separately for 5-10 min. Combine contents of each tube together and incubate at room temperature for at least 20 minutes (but no longer than 6 hr) to obtain the Transfection Mixture. During this incubation step, proceed to harvesting cells for transfection. Harvest cultures of proliferating spermatogonia grown on MEFs. If using proliferating cultures of spermatogonia maintained on MEF feeder layers, first plate the cells onto a fresh gelatin-coated plate and incubate for 30-45 min (37°C, 5% CO2) to deplete the number of residual MEFs present in the cell suspension. Suspend spermatogonia to ~106 cells/ml in SG Medium, or DHF12-FBS + 30 μM 2ME. Add Transfection Mixture to the cell suspension at a ratio of 20% volume Transfection Mixture: 80% volume spermatogonial suspension, and incubate at 37°C, 5% CO2 for 40- 120 min (routinely 80 min) in a vented tube. As a typical example,

40 µl volume of the Transfection Mixture is used to transfect ~2x105 spermatogonia in a total transfection volume of 200 µl. During transfections lasting longer than 1 h, mix the transfection by gently pipetting cells up and down two times midway through the incubation period. After the transfection incubation period, wash spermatogonia by first suspending the transfection suspension to 20 times its volume using fresh culture medium (i.e. 4 ml medium/200 µl transfection reaction), and then pellet the cells for 5 min at 400 x g. Discard the supernatant fluid, and wash the pellet(s) two additional times using fresh culture medium at an equivalent of the 20x volume/wash. After the third wash, suspend the cell pellet in fresh medium and then plate transfected cells onto fresh MEF feeder layers for selection of genetically modified spermatogonial lines.

[00214] Clonal selection for genetically modified SSCs is done by using the following reagents: established, proliferating line of rat spermatogonial stem cells, geneticin selective antibiotic: G418 (cat no 11811-031, Invitrogen Inc.), DNA Constructs expressing a resistance gene that selects for survival in G418 containing medium (i.e. neomycin phosphotransferase gene), fibroblast feeder cell line expressing a resistance gene that selects for survival in G418 containing medium.

[00215] After transfecting spermatogonia from an established proliferating line with zinc finger construct plasmid DNA, or a selectable marker, the treated spermatogonia are plated directly into SG Medium at an equivalent of ~3x105 spermatogonia/well (9.5 cm2) in a 6-well plate containing freshly prepared MEFs. The transfected (or virally transduced) spermatogonia are then allowed to proliferate in cell number for ~18 days after transfection with plasmid DNA. The culture medium is replenished every two days; and, fresh MEFs are spiked

onto cultures of the transfected spermatogonia after ~10 days. At ~18 days following gene-transfer with ZFN construct and plasmid DNA or selectable marker (or, after ~8 days following lentiviral transduction), cultures are harvested and then passaged onto freshly prepared MEFs in SG medium and maintained for an additional 2-3 days before initiating clonal selection in SG medium containing ~75 μg/ml G418 (Invitrogen, Inc.). After initiating selection, cultures are fed fresh SG medium containing G418 every two days during an 8-10 day selection period. Thereafter, cells are fed every two days using SG medium alone to expand clonally enriched lines of rat spermatogonia that can be used to produce transgenic rats, as described in the following sections.

[00216] In some embodiment of the invention, genetic modification of SSCs produced using ZFNs relates to generating multiple mutations in the same SSC or SSC line, which relates to generating genetically modified organisms with multiple mutations. The embodiment relates to transfection with multiple ZFN constructs targeting multiple DNA sequences and locations within the same SSC or SSC line in a single transfection. The embodiment of the invention relates to clonal selection and screening for multiple mutations in single SSCs or SSC lines.

[00217] In some embodiment of the invention, genetic modification of SSCs produced using ZFNs relates to generating multiple mutations in the same SSC or SSC line in multiple and consecutive experiments or transfections. The embodiment of the invention relates to clonal selection and screening for multiple mutations in single SSCs or SSC lines. The embodiment of the invention relates to generating genetically modified organisms or a colony of genetically modified organisms with multiple mutations in single SSCs or SSC lines.

[00218] In some embodiment of the invention, genetic modification of SSCs produced using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to generating multiple genetically modified organisms containing one or more mutations. In the embodiment of the invention SSCs or SSC lines are separated by including, but not limited to, different media, colonies or transfection dishes. The separated SSCs or SSC lines undergo one or more experiments or transfections. The separate SSCs or SSC lines are then brought together for production of multiple genetically modified organisms in a single injection into a recipient male followed by a single breeding step.

[00219] In some embodiment of the invention, genetic modification of SSCs produced using ZFNs relates to generating multiple mutations in the same SSC or SSC line, which relates to generating genetically modified organisms with multiple mutations. The embodiment relates to transfection with multiple ZFN constructs targeting multiple DNA sequences and locations within the same SSC or SSC line in a single transfection. The embodiment of the invention relates to clonal selection and screening for multiple mutations in single SSCs or SSC lines.

[00220] In some embodiment of the invention, genetic modification of SSCs produced using ZFNs relates to generating multiple mutations in the same SSC or SSC line in multiple and consecutive experiments or transfections. The embodiment of the invention relates to clonal selection and screening for multiple mutations in single SSCs or SSC lines. The embodiment of the invention relates to generating genetically

modified organisms or a colony of genetically modified organisms with multiple mutations in single SSCs or SSC lines.

[00221] In some embodiment of the invention, genetic modification of SSCs produced using ZFNs relates to generating multiple mutations in separate SSCs or SSC lines followed by pooling or combining separate SSCs or SSC lines and injecting into a single recipient male, which relates to generating multiple genetically modified organisms containing one or more mutations. In the embodiment of the invention SSCs or SSC lines are separated by including, but not limited to, different media, colonies or transfection dishes. The separated SSCs or SSC lines undergo one or more experiments or transfections. The separate SSCs or SSC lines are then brought together for production of multiple genetically modified organisms in a single injection into a recipient male followed by a single breeding step.

[00222] In some embodiment of the invention, the SSCs are derived from an organism. The SSCs may be collected by spermatocyte harvest, the SSCs may be selected and purified using laminin selection, and propagated, cryopreserved and validated by cell surface marker identification.

[00223] In some embodiment of the invention, the SSCs are derived from a tissue sample. The SSCs may be collected by spermatocyte harvest, the SSCs may be selected and purified using laminin selection, and propagated, cryopreserved and validated by cell surface marker identification.

[00224] In some embodiment of the invention, the SSCs are derived from cells. The SSCs may be collected by spermatocyte harvest, the SSCs may be

selected and purified using laminin selection, and propagated, cryopreserved and validated by cell surface marker identification.

[00225] In some embodiment, the SSCs used for production of organisms are derived from an organism or tissue with a well-characterized disease state. The SSCs are used for the production of organisms, which may be further genetically modified.

[00226] In some embodiment, the SSCs used for production of organisms are derived from a well-characterized disease state wherein the disease state is a metabolic disorder, which is not limited to diabetes. The SSCs are used for the production of organisms which may be further genetically modified.

[00227] In some embodiment, the SSCs used for production of organisms are derived from a well-characterized disease state wherein the disease state is an oncology disorder, which is not limited to prostate cancer. The SSCs are used for the production of organisms which may be further genetically modified.

[00228] In some embodiment, the SSCs used for production of organisms are derived from a well-characterized disease state wherein the disease state is an autoimmune disorder, which is not limited to arthritis. The SSCs are used for the production of organisms which may be further genetically modified.

[00229] In some embodiment, the SSCs used for production of organisms are derived from a well-characterized disease state wherein the disease state is a cardiovascular disorder, which is not limited to atherosclerosis. The SSCs are used for the production of organisms which may be further genetically modified.

[00230] In some embodiment, the SSCs used for production of organisms are derived from a well-characterized disease state wherein the disease state is a neurodegenerative disorder, which is not limited to Alzheimer's disease. The SSCs are used for the production of organisms which may be further genetically modified.

[00231] In some embodiment, the SSCs used for production of organisms are derived from a well-characterized disease state wherein the disease state is a behavioral disorder, which is not limited to Schizophrenia. The SSCs are used for the production of organisms which may be further genetically modified.

[00232] In some embodiment, the SSCs used for production of organisms are derived from induced pluripotent stem (iPS) cells from a well, characterized disease state wherein the disease state is a metabolic disorder, which is not limited to diabetes. The SSCs are used for the production of organisms which may be further genetically modified.

[00233] In some embodiment, the SSCs used for production of organisms are derived from induced pluripotent stem (iPS) cells from a well-characterized disease state wherein the disease state is an oncology disorder, which is not limited to prostate cancer. The SSCs are used for the production of organisms which may be further genetically modified.

[00234] In some embodiment, the SSCs used for production of organisms are derived from induced pluripotent stem (iPS) cells from a well-characterized disease state wherein the disease state is an autoimmune disorder, which is not limited to arthritis. The SSCs are used for the production of organisms which may be further genetically modified.

[00235] In some embodiment, the SSCs used for production of organisms are derived from induced pluripotent stem (iPS) cells from a well-

characterized disease state wherein the disease state is a cardiovascular disorder, which is not limited to atherosclerosis. The SSCs are used for the production of organisms which may be further genetically modified.

[00236] In some embodiment, the SSCs used for production of organisms are derived from induced pluripotent stem (iPS) cells from a well-characterized disease state wherein the disease state is a neurodegenerative disorder, which is not limited to Alzheimer's disease. The SSCs are used for the production of organisms which may be further genetically modified.

[00237] In some embodiment, the SSCs used for production of organisms are derived from induced pluripotent stem (iPS) cells from a well-characterized disease state wherein the disease state is a behavioral disorder, which is not limited to Schizophrenia. The SSCs are used for the production of organisms which may be further genetically modified.

[00238] In some embodiment, the SSCs used for production of organisms are derived from induced pluripotent stem (iPS) cells from a well-characterized genetic background.

[00239] In some embodiment, the SSCs used for production of organisms are derived from induced pluripotent stem (iPS) cells from a well-characterized genetic background wherein the genetic background is associated with different established strains of organism.

[00240] In some embodiment, the SSCs used for production of organisms are derived from induced pluripotent stem (iPS) cells from a well-characterized genetic background wherein the genetic background is associated with known ethnic or regional genetic make-ups.

[00241] In one embodiment, SSCs containing site-specific mutations are generated to produce genetically modified organisms.

[00242] In another embodiment, SSCs containing site specific mutations are generated to produce genetically modified mammals.

[00243] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified rodents.

[00244] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified rats.

[00245] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified mice.

[00246] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified pigs

[00247] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified rabbits

[00248] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified guinea pigs.

[00249] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified dogs.

[00250] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified cats.

[00251] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified goats.

[00252] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified chickens.

[00253] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified non-human primates.

[00254] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified ferrets.

[00255] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified birds.

[00256] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified farm animals.

[00257] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified fish.

[00258] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified slamonoids.

[00259] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified carp.

[00260] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified tilapia.

[00261] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified tuna.

[00262] In another embodiment, the invention provides kits that are used to produce site specific-mutations in stem cells, which can be used to generate genetically modified organisms. The kits typically include one or more site-specific genetic engineering technology, such as ZFNs. The kit may also contain one or more sets of stem cells for site-specific modification. The stem cells may include, but is not limited to spermatogonial stem cells (SSCs), as well as media and conditions necessary for growing SSCs. The kits may include exogenous sequences for site-specific genomic introduction, such as but not limited to reporter genes or selectable markers. The kits may include

instructions for (i) introducing the ZFNs into the stem cells (ii) identifying stem cells which have been site specifically modified (iii) growing site-specifically modified stem cells in media or conditions necessary and to numbers required for stem cells to produce genetically modified organisms (iv) using the grown stem cells to produce a genetically modified organism (v) identifying which organisms or progeny harbor the site-specific mutation of interest.

[00263] In some embodiment, the invention provides a kit which includes a mixed population of different or distinct genetically modified SSCs which may be custom made. The mixed population of genetically modified SSCs may be provided in suitable quantities for direct injection into a sterile male recipient for the production of multiple genetically modified organisms in a single step. The mixed population of separate or distinct genetically modified SSCs may consist of at least two genetically modified SSCs, at least two genetically modified SSCs, at least three genetically modified SSCs, at least four genetically modified SSCs, at least five genetically modified SSCs, at least six genetically modified SSCs, at least seven genetically modified SSCs, at least eight genetically modified SSCs, at least nine genetically modified SSCs, at least ten genetically modified SSCs, at least twenty genetically modified SSCs, at least thirty genetically modified SSCs, at least forty genetically modified SSCs, at least fifty genetically modified SSCs, at least one hundred genetically modified SSCs, at least one thousand genetically modified SSCs, at least ten thousand genetically modified SSCs, at least thirty thousand genetically modified SSCs or genetically modified SSCs which harbor genetic modification within every gene in the organisms genome.

[00264] In some embodiment, the invention provides a kit which includes one or more sets of SSCs for site-specific modification. The sets of SSCs may be derived from well-characterized organisms with different disease states. The SSCs may contain multiple mutations, which may be derived from genetic modification or naturally or by any method. The kit may include the media and conditions to grow disease state SSCs, as well as the sterile recipient male for the production of genetically modified organisms.

[00265] In some embodiment, the invention provides a kit which includes the necessary tools for the derivation of SSC lines from an organism or tissue sample, as well as the necessary tools to genetically modify the derived SSC and produce a genetically modified organism from the derived SSCs. The kit may include cell collection tools such as spermatocytes for harvest, and SSC selection tools such as laminin selection, and SSC propagation and cryopreservation tools as well as SSC validation tools which may include cell surface marker staining. The kit may also include media and conditions for growing the SSCs, tools for genetic modification of the SSCs as well as sterile recipient males for production of genetically modified organisms from the SSCs.

[00266] In some embodiment, the invention provides a kit which includes SSCs which have been generated from induced pluripotent stem (iPS) cells. The iPS cells may be derived from well characterized different genetic backgrounds including disease states as well as regional, strain, ethnic genetic backgrounds. The kit may also include media and conditions for growing the SSCs, tools for genetic modification of the SSCs as well as sterile recipient males for production of genetically modified organisms from the SSCs.

[00267] Germline transmission from genetically modified SSCs can be carried out by using the following reagents: Disposable Pasteur Pipettes (cat. no. 13-678-20C, Thermo Fisher Scientific Inc.), 30G Precision Glide Needles (cat. no. 305106, BD, Inc.), 1 ml Syringes (cat. no. 309602, BD Inc.), Busulfan (cat. no. 154906, MP Biomedicals), Dimethyl Sulfoxide (DMSO) (cat. no. 317275, Calbiochem), Trypan Blue (cat. no. T6146-25G, Sigma Inc.), Triadine Prep Solution, (10% povidone iodine solution, cat. no. 10-8208, Triad Disposables), Ethanol 200 Proof (cat. no. 111000200, Pharmco-AAPER), PBS: Dulbecco's phosphate-buffered saline (PBS; cat. no.D8537, Sigma Inc.) 200 mg/L KCl (w/v), 200 mg/L KH2PO4 (w/v), 8 g/L NaCl (w/v), 1.15 g/L Na2HPO4 (w/v)., Kimwipes (cat. no. 34155, Kimberly-Clark), Bead Sterilizer; Germinator 500 (Cellpoint Scientific Inc), Flaming/Brown Micropipette Puller; Model P-97 (Sutter Instruments Co.),Glass Capillaries for needles; 100 µl micropipette (cat. no 1-000-1000, Drummond Scientific Co.), Heat Therapy Pump (cat. no HTP-1500, Kent Scientific Corporation or other suitable model), Reusable Warming Pad (cat. no. TPZ-1215EA, Kent Scientific Corporation), 10 ml Syringes (cat. no. 309604, BD, Inc.), Acepromazine (cat. no. 038ZJ03, Vedco), Rompun (cat. no. LA33806A, Lloyd Laboratories) Ketaset (cat. no. 440761, Fort Dodge Animal Health), Buprenex Injectable (cat. no. 12496-0757-1, Reckitt Benckiser), Shaving Razors – Stainless Steel Surgical Prep Blades (cat. no. 74-0001, Personna), Suture Thread; Spool Suture (cat. no. SUT-15-2, Roboz Surgical Inc.), Suture Needles; Eye 3/8 circle (cat. no. RS-7981-4, Roboz Surgical Inc.), Michel Wound Clips (cat. no. RS-9272, Roboz Surgical Inc.), Michel Wound Clip Forceps (cat. no. RS-9294, Roboz Surgical Inc.), Ear Puncher – 2 mm diameter (cat. no. RS-9902, Roboz Surgical Inc.), Hemostat (cat. no. RS-7110, Roboz Surgical Inc.), Straight Sharp

Microdissecting Scissors (cat. no. RS-5882, Roboz Surgical Inc.) Curved, Sharp Microdissecting Scissors (cat. no. RS-5883, Roboz Surgical Inc.), Full-Serve Microdissecting Forceps (cat. no. RS-5137, Roboz Surgical Inc.), Straight Tip, Dumostar Tweezers (cat. no. RS-4978, Roboz Surgical Inc.), 5/45 INOX Tweezers (cat. no. RS-5005, Roboz Surgical Inc.) Polyethylene capillary tubing (cat. No. 19-0040-01, GE Heatlthcare, Inc.), 24 day old, busulfan-treated, male, Sprague Dawley rats.

[00268] Generation of recipient-founders by testicular transplantation is carried out by using busulfan-treated wildtype Sprague Dawley rats (Harlan, Inc), or male-sterile *DAZL*- deficient, Sprague Dawley rats at 24 days of age can be used as recipients for spermatogonial lines. To prepare recipients for transplanting spermatogonia, rats arrive from the supplier at 8-10 days of age, together with mother, 14 –16 days prior to the transplantation procedure which is performed at 24 days of age. At 12 days of age (i.e. 12 days prior to the transplantation procedure), each rat is administered a single dose of busulfan (12.5 mg/kg, i.p. for wildtype Sprague Dawley rats; 12.0 mg/kg for *DAZL*-deficient Sprague Dawley rats), and then housed in a quiet, clean and well ventilated location within an approved animal facility. *Under guidelines of an approved safety plan**, a 4 mg/ml working stock of busulfan in 50% DMSO is prepared by first dissolving busulfan in 100% DMSO at 8 mg/ml, and then adding and equal volume of filter-sterilized, deionized water.

[00269] On the day of transplantation, genetically modified rat spermatogonia are harvested from culture and suspended in ice cold, culture medium (i.e. either SG medium or DHF12-FBS-2ME) at concentrations ranging from 4-6x105spermatogonia/100 µl. The cellular suspension is transferred to a sterile microfuge tube and maintained on ice until the

time of transplantation. Just prior to transplantation, the cell suspension is supplemented with a 20% volume of a filter-sterilized, 0.04% trypan blue solution made fresh in PBS the same day. Once spermatogonia are harvested, the first busulfan-treated recipient rat is anesthetized by intraperitoneal (i.p.) injection of a cocktail containing 100 mg/ml ketaset, 20 mg/ml rompun, and 10 mg/ml acepromazine at 0.1 ml/100g body weight to achieve a surgical plane of anesthesia (as demonstrated by the lack of a pedal reflex in the toe pinch test). The recipient is layed on its back. The abdominal skin is then opened just rostral to the pelvis, and the testis is exposed. The efferent ductules leading into the rete testis are then accessed by blunt dissection using micro-dissection forceps. The ductules are further dissected up to the base of their respective testis to yield visible access to the rete, which will be the site of injection. Once the rete is exposed, the harvested spermatogonial suspension is mixed gently by pipetting up and down ~5 times with a p200 tip and then ~70-80 µl of the suspension is loaded into a 100 µl glass capillary injection needle (~50 µm opening) using a flame pulled, transfer pipette (i.e. made from Pasteur pipettes) and rubber squeeze bulb. The injection needle containing spermatogonia is manually inserted into the rete of the testis, and the cells are transferred into the testis by injection using a stationary 10 ml syringe (i.e. simply taped to the work bench), which is connected to the glass capillary injection needle by flexible plastic tubing. The injected testis is then carefully placed back into the abdominal cavity and the same procedure can be performed on the contra-lateral testis to achieve more optimal breeding. Once injected and placed back in the abdominal cavity, the abdominal wall (sutured) and skin (wound clips) are surgically closed. The procedure can then be repeated on subsequent recipients using the same spermatogonial suspension. Spermatogonial suspensions can be

maintained on ice in SG medium for up to 5 hours during the transplantation of multiple recipients.

[00270] After surgically closing the abdominal cavity and skin, all animals are maintained on a warming pad set to 34oC and receive post-operative care to assure their safe recovery from anesthesia and to alleviate pain and distress. For recovery from anesthesia, each animal is observed with respect to its breathing rate, muscle control and external stimuli until ambulatory, prior to being housed in a quiet, well ventilated location within the animal facility.

[00271] As a post-operative analgesic to alleviate pain, each rat is administered a single dose of buprenorphine hydrochloride (25 μg/kg) (Buprenex Injectable, Reckitt Benckiser) as it starts to regain consciousness. An additional dose is given every 6-12 hr for the next 48 hr upon signs of discomfort or pain. Wound clips are removed at 12-14 days post-surgery. The recipients are then housed together for ~60 days prior to initiating breeding studies. In some embodiments, the male recipient are housed for between about 45 and 75 days. In some embodiments, the recipient males transplanted with spermatogonial lines are housed for no more than 45 days. In some embodiments, the recipient males transplanted with spermatogonial lines are housed for no more 50 days. In some embodiments, the recipient males transplanted with spermatogonial lines are housed for no more than 55 days. In some embodiments, the recipient males transplanted with spermatogonial lines are housed for no more than 60 days. In some embodiments, the recipient males transplanted with spermatogonial lines are housed for no more than 65 days. In some embodiments, the recipient males transplanted with spermatogonial lines are housed for no more than 70 days.

[00272] Recipient males transplanted with spermatogonial lines are paired with wild-type female Sprague Dawley rats of similar age at 60-70 days post-transplantation. Typically, the first F1 progeny are born between 100 and 150 days post-transplantation and recipients can continue to sire litters for greater than 300 days

post-transplantation due to the long-term spermatogenesis colony forming potential of laminin-binding rat spermatogonia. Transgenic rat progeny from recipient-founders and wild-type females are identified by genomic PCR and/or Southern Blot analysis using probes specific to the mutation of interest.

Stem Cell Technologies

[00273] **Spermatogonial stem cells (SSCs)**

[00274] Site-specific genetic modification to sperm cell progenitors prior to differentiation and development can be carried out by modifying spermatogonial stem cells (SSCs) which develop into spermatozoa through the process known as spermatogenesis. Site-specific genetic modification of enriched SSCs is possible *in vitro* through use of various site specific genetic modification technologies. Transplantation of SSCs containing site-specific mutations into the seminiferous tubules of bulsulfan treated and/or genetically sterile male rats lacking the germ-line specific gene product DAZL results in maturation of SSCs into genetically modified spermatids. The genetically modified germ line recipient males are then bred with wild type females to produce offspring that harbor the site specific mutation (Production and Use of Rat Spermatogonial Stem Cell Lines (PCT/US2009/066275, WO/2010/065550, which are incorporated by reference in their entirety).

[00275] **Embryonic stem (ES) cells**

[00276] Embryonic stem cells are a pluripotent cell derived from the inner mass of the blastocyst or early stage embryo. Genetically modified ESCs from a donor are microinjected into a recipient blastocyst. Recipient blastocysts containing genetically modified ES cells are implanted into pseudopregnant surrogate females. The progeny, some of which have

a genetic modification to the germline can then be established, and lines homozygous for the genetic modification can be produced by interbreeding.

[00277] Induced pluripotent stem (iPS) cells

[00278] Induced pluripotent stem cells are artificially derived pluripotent cells from a less or non pluripotent cell, typically a somatic cell. There are multiple methods for which iPS cells can be "reprogrammed" to a pluripotent state from non pluripotent cells, including the expression of reprogramming factors. Genetically modified iPS cells from a donor are microinjected into a recipient blastocyst. Recipient blastocysts containing genetically modified ES cells are implanted into pseudopregnant surrogate females. The progeny, some ofwhich have a genetic modification to the germline can then be established, and lines homozygous for the genetic modification can be produced by interbreeding.

[00279] Somatic Stem Cells

[00280] Somatic stem cells or adult stem cells are potent cells found in organs after embryonic development. Somatic stem cells can be isolated from organs and tissues and have the potential to differentiate into many cell types of that organ and organism. For example, cord blood stem cells can be isolated from umbilical cord blood (CBEs) (McGuckin et al. (2008) *Nature Protocols.* 3, 6, 1046-1055. These cells are then expanded and used in the production of genetically modified organisms. CBEs are known as "embryonic-like" due to the expression of similar markers as embryonic stem cells. CBEs are a very small fraction of the cells present in umbilical cord blood. The CBE fraction is depleted of hematopoietic stem cells which stimulate hematopoietic

commitment. CBEs are plated at high concentrations (10 million cells per 1 ml) in TPOFLK medium which is supplemented with extracellular matrix (ECM) proteins. The ECM proteins are essential for cell survival and aggregate formation similar to embryoid bodies which promotes cell-cell interactions and secretion of growth factors. Dynamic cell culture conditions are maintained based on cell phenotype: formation of floating aggregates, size and number of cell aggregates, cell adhesion and differentiation.

[00281] Genetically modified somatic stem cells from a donor are microinjected into a recipient blastocyst. For example, fresh or frozen cleavage stage embryos, produced from *in vitro* fertilization (IVF) can be cultured to blastocyst stage. The inner cell masses are isolated to produce ES cell lines that are capable of undifferentiated proliferation *in vitro*. Recipient blastocysts containing genetically modified somatic stem cells are implanted into pseudopregnant surrogate females. The progeny, some of which have a genetic modification to the germline can then be established, and lines homozygous for the genetic modification can be produced by interbreeding. Alternatively, genetically modified somatic stem cells can be reprogrammed into iPS cells in order to produce genetically modified organisms.

[00282] **Embryos**

[00283] An embryo is a multicellular diploid eukaryote in early stage of development. Embryos can be genetically modified *in vitro* or *in vivo*. Embryos containing site-specific mutations may be implanted into pseudopregnant surrogate females. The progeny which have a genetic modification to the germline can then be established, and lines homozygous for the genetic modification can be produced by interbreeding.

Methods

[00284] The methods used in the present invention are comprised of a combination of genetic introduction methods, site-specific genetic modification or mutagenesis mechanisms of stem cells, and generation of site-specific genetically modified organisms from the stem cells. For all genetic modification or mutagenesis mechanisms one or more introduction and delivery method may be employed. The invention may include but is not limited to the methods described below.

[00285] Genetic Introduction Methods

[00286] In one introduction method, the site-specific mutationis produced in a stem cell. These stem cells can proliferate as cultured cells and be genetically modified without affecting their ability to differentiate into other cell types, including germline cells. In the case of embryonic stem cells, genetically modified stem cells from a donor are microinjected into a recipient blastocyst, or in the case of spermatogonial stem cells,genetically modified cells can be injected into the rete testis of a recipient animal. Recipient genetically modified blastocysts are implanted into pseudopregnant surrogate females. The progeny, some of which have a genetic modification to the germ line can then be established, and lines homozygous for the genetic modification can be produced by interbreeding.

[00287] In one embodiment, spermatogonial stem cells (SSCs) with site specific mutations are used to generate genetically modified organisms. Preparing SSCs for site-specific genetic modification involves preparing feeder cell lines, and sub-culturing SSC lines. Preparing feeder cells may be carried out by thawing embryonic fibroblasts (EF), and placing on gelatin coated surface in SSC feeder medium. Sub-

culturing SSCs may be carried out by seeding SSCs on EF medium. A 1:1 to 1:2 split passage is required before expanding into larger SSC numbers. Once established after the first several passages on EFs, cultures of spermatogonia are passaged at ~1:3 dilutions onto a fresh monolayer of EFs. For passaging, cultures are first harvested by gently pipetting them free from the EFs. After harvesting, the "clusters" of spermatogonia are dissociated by gentle trituration. Spermatogonia are easily distinguished during counting as the predominant population of smaller, round cells with smooth surfaces, as compared to occasionally observed, larger and often irregular shaped irradiated EFs.

[00288] Site-Specific Genetic Modification or Mutagenesis Methods

[00289] The invention pertains to a site-specific mutation generated in a stem cell, which includes but is not limited to somatic stem cells,spermatogonial stem cells (SSCs), embryonic stem (ES) cells, embryos, and induced pluripotent stem (iPS) cells. Stem cells containing site-specific mutations are used to produce a genetically modified organism.

[00290] Generating site-specific mutations in stem cells, which can then be used to produce a genetically modified organisms first involves the design and development of a protein such as a ZFN whose DNA binding domain is engineered for a specific target site within the genome. A protein consisting of both a DNA binding domain and a cleavage or insertional mutagenesis domain is developed.

[00291] In one embodiment of the invention, a site-specific mutagenesis technology is expressed in SSCs generating site-specific mutations. The binding domains of the site-specific mutagenesis technologies are modified to bind a particular location in the genome. The site-specific

mutagenesis technology may be introduced into SSCs via transfection using lipofetamine. A transfection mixture may be prepared by mixing transfectamine with the site specific mutagenesis technology ZFN. After harvesting undifferentiated SSCs, one may then add transfection mixture to the cell suspension, incubate, wash and plate the SSCs onto fresh EF feeder layers.

[00292] In some embodiments, the stem cells of the present invention comprise one or more transposons, one or more inverted tandem repeats (ITRs) (shown in table 3) of a transposon of variants thereof.

[00293] In some embodiments, the present invention comprise one or more transposons, one or more inverted tandem repeats (ITRs) of a transposon wherein the variant sequence inverted tandem repeats are at least 70, 75, 80, 85, 90, 95, 96, 97, 98, 99% homologous to known ITRs and known transposon elements including but not necessarily limited to those ITRs shown in table 3.

[00294] In one embodiment of the invention, clonal selection of SSCs containing site-specific muations may be carried out by first plating treated spermatogonia. The genetically modified SSCs are allowed to proliferate in cell number by replenishing the medium with fresh EFs. Selection for genetically modified SSCs may be carried out in several methods. Selection using a reporter gene or selectable marker and, followed by culturing with an antibiotic or cell sorting. Specific mutations may be identified by selecting clones, isolating DNA and DNA sequencing.

[00295] Screening for ZFN mediated site specific modification such as knockout mutations via NHEJ or knockin mutations using homologous recombination (HR) is done by selection with co-transfected vectors. SSCs are co-transfected with a ZFN and a selection marker vector such as a fluorescent marker or antibody resistance within a lipid-based

transfection reagent. 1ug total DNA is transfected with a ratio of 500ng ZFN to 500ng selection vector. Clones are isolated and propagated to sufficient numbers to isolate DNA for screening and sequencing.

Generation of Genetically Modified Organisms from Stem Cells Containing Site-Specific Mutations

[00296] The invention pertains to a site-specific mutation generated in a stem cell, which includes but is not limited to somatic stem cells, spermatogonial stem cells (SSCs), embryonic stem (ES) cells , induced pluripotent stem (iPS) cells and embryos. Stem cells containing site-specific mutations are used to produce a genetically modified organism.

[00297] In one embodiment, SSCs containing site-specific mutations are generated to produce genetically modified organisms.

[00298] In another embodiment, SSCs containing site specific mutations are generated to produce genetically modified mammals.

[00299] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified rodents.

[00300] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified rats.

[00301] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified mice.

[00302] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified pigs

[00303] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified rabbits

[00304] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified guinea pigs.

[00305] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified dogs.

[00306] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified cats.

[00307] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified goats.

[00308] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified chickens.

[00309] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified non-human primates.

[00310] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified ferrets.

[00311] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified birds.

[00312] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified farm animals.

[00313] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified fish.

[00314] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified slamonoids.

[00315] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified carp.

[00316] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified tilapia.

[00317] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified tuna.

[00318] In another embodiment, SSCs containing site-specific mutations are generated to produce genetically modified rats. The method for producing such organisms involves germline transmission of the genetically modified SSCs. Wild type and genetically sterile *DAZL* deficient organisms are prepared for transplantation of SSCs containing site-specific mutations into seminiferous tubules of the testes. A cellular suspension of SSCs containing site-specific mutations is transferred to a sterile microfuge. Genetically sterile recipients are placed in the supine position. The abdominal skin is then opened just rostral to the pelvis, and the testis exposed. The efferent ductules leading into the rete testis are then accessed by blunt dissection using micro-dissection forceps. The ductules are further dissected up to the base of their respective testis to yield visible access to the rete, which will be the site of injection. The injection needle holding SSCscontaining site-specific mutations is manually inserted into the rete of the testis, and the cells are transferred into the testis by injection. The injected testis is then carefully placed back into the abdominal cavity and the same procedure can be performed on the contra-lateral testis to achieve more optimal breeding. Once injected and placed back in the abdominal cavity, the abdominal wall (sutured) and skin (wound clips) are surgically closed. Recipient males transplanted with SSCscontaining site-specific mutations are mated with wild-type females to produce genetically modified organisms.

[00319] Mating between *DAZL* deficient males carrying genetically modified sperm and wild type females will produce progeny with approximately 50% of the offspring being heterozygous for the site specific genetic modification. Breeding and maintaining the colony involves PCR genotyping to identify which animals harbor the mutation. Once the animals are identified proper genetic crosses can be set up to produce numbers of homozygous, heterozygous and wilt type littermates.

In some embodiments, the invention relates to a composition comprising: (i) one or more stem cells or one or more embryos; and (ii) a ZFN; and, optionally, a culture media for the one or more stem cells or the one or more embryos.

Examples

Generation of Knockout Rats: Gene Disruption Technique Targeting the EGFP Gene

[00320] Examples include using the site-specific ZFN technology, which can specifically bind and cleave designated DNA sequences for mutation of the targeted sequence. Site-specific ZFN can be used to genetically modify rat spermatogonial stem cells (SSCs) Wild type SSCs in colony are shown in Figure 2.

[00321] In one example, the site-specific technology using ZFN was employed. ZFN DNA binding domains can be engineered to bind to a sequence of choice. A ZFN was designed to bind and mutate the EGFP gene (Figure 8) and co-transfected into rat SSCs with a fluorescent marker (schematic figure 5). A rat spermatogonial stem cell line derived from transgenic rats expressing EGFP was transfected (2 million cells/transfection) with 6.7 ug of plasmid pCK181 + 3.3 ug pUBC-dsRED2-1 using Amaxa Solution L and program A020 in an AmaxaNucleofectorAppratus. Transfected spermatogonia were plated

into a 10 cm dish containing DR4 mouse embryonic fibroblasts (DR4 MEFs; Applied Stem Cell, Inc.) and maintained for 7 days in Spermatogonial Culture Medium (SG Medium). Cells were then harvested and red fluorescent, dsRed2-1 positive cells were sorted (FACS) into a single well of a 6-well dish (9.6cm2) and maintained for 11 days in SG Medium on DR4 MEFs. Spermatogonia were then harvested and expanded into 4 wells of a 6-well dish at ~30 thousand cells/well and maintained for 13 days in SG Medium on DR MEFS. 96 individual spermatogonial colonies were picked using a p200 pipette and passaged into individual wells of 2 x 48 plates on DR4 MEFs in SG Medium. Colonies were maintained for 14 days and then split into replicate plates on DR4 MEFs in SG Medium. After expansion for an additional 7 days genomic DNA was isolated form 81 of 96 picked colonies and processed for analysis by PCR cloning and DNA sequencing of regions amplified with PCR primers flanking Zinc Finger Recognition Site 304 (ZFN 304; EG304). 1 of 81 picked colonies (i.e. Colony H9) showed Zinc Finger Endonuclease cleavage and DNA repair at the predicted Recognition Site (Figure 4 and 6).

Genetically modified SSCs will be expanded and germline transmission will be carried out in the method described above to produce genetically modified Rag1 knockout rats (schematic figure 7).

Generation of Knockout Minipigs: Gene Disruption Technique Targeting the Rag1 Gene (Prophetic)

[00322] Site-specific zinc finger nuclease (ZFN) technology, which can specifically bind and cleave designated DNA sequences for mutation of the targeted sequence. Site-specific ZFN will be used to genetically modify minipig spermatogonial stem cells (SSCs).

[00323] In one example, the site-specific technology using zinc finger nuclease (ZFN) will be employed. ZFN DNA binding domains can be engineered to bind to a sequence of choice. The ZFN binding domain will be engineered to bind to the minipig Rag1 gene at prophetic binding sequence shown in figure 9. The minipig Rag1-specific ZFN will be expressed in minpig spermatogonial stem cells (SSCs) along with a selection marker (e.g. fluorescent marker or homologous recombination vector) which indicates that the ZFN construct was successfully transfected into the cell. The ZFN and selectable marker co-transfection will result in site-specific double-stranded DNA breaks followed by NHEJ repair. Minipig SSC clones will be sorted and mutation screening identified knockout clones. Propagation of ZFN-mediated genetically modified minpig SSCs is required in order to generate ample numbers for recipient injection and germline transmission. Genetically modified SSCs will be expanded and germline transmission will be carried out in the method described above to produce genetically modified Rag1 knockout minipigs.

CLAIMS

1. A composition comprising one or more spermatogonial stem cells, wherein the one or more spermatogonial stem cells comprise one or more of the following mutations: (i) a deletion mutation; (ii) a knockout mutation; and/or (iii) an addition of a heterologous nucleic acid sequence; wherein the one or more mutations of (i), (ii), and/or (iii) are site-specific mutations caused by a zinc finger nuclease (ZFN).

2. The composition of claim 1, wherein the heterologous nucleic acid sequence is chosen from a selectable marker or an orthologous gene.

3. The composition of claim 1, wherein the one or more spermatogonial stem cells is derived from the germline lineage of an animal.

4. The composition of claim 1, wherein the one or more spermatogonial stem cells further comprise at least one inverted tandem repeat of a transposon or a variant thereof.

5. An organism comprising one or more spermatogonial stem cells, wherein the one or more spermatogonial stem cells comprise one or more of the following mutations: (i) a deletion mutation; (ii) a knockout mutation; and/or (iii) an addition of a heterologous nucleic acid sequence; wherein the one or more mutations of (i), (ii), and/or (iii) are site-specific mutations caused by a ZFN.

6. The organism of claim 5, wherein the one or more stem cells further comprise at least one inverted tandem repeat of a transposon or variant thereof.

7. A composition comprising one or more spermatogonial stem cells and: (a) a ZFN that cleaves a nucleic acid sequence at a pre-determined location within the genome of the one or more spermatogonial stem cells; or (b) a nucleic acid sequence that encodes a ZFN that cleaves a nucleic acid of the stem cell at a pre-determined site

within the genome of the spermatogonial stem cell; wherein the one or more spermatogonial stem cells is derived from the germline lineage of an animal.

8. The composition of claim 7, wherein the stem cell is a spermatogonial stem cell derived from a rat or mini pig.

9. The composition of claim 7, wherein the one or more spermatogonial stem cells further comprise at least one inverted tandem repeat of a transposon or a variant thereof.

10. The composition of claim 7, wherein the one or more spermatogonial stem cells further comprise: (a) one or more nucleic acid sequences at least 70% homologous to a nucleic acid sequence chosen from:

(i)

CAGTTGAAGTCGGAAGTTTACATACACTTAAGTTGGAGTCATTAAAACTCG
TTTTTCAACTACTCCACAAATTTCTTGTTAACAAACAATAGTTTTGGCAAGT
CAGTTAGGACATCTACTTTGTGCATGACACAAGTCATTTTTCCAACAATTG
TTTACAGACAGATTATTTCACTTATAATTCACTGTATCACAATTCCAGTGG
GTCAGAAGTTTACATACACTAAGT (SEQ ID NO:1);

(ii)

ATTGAGTGTATGTAAACTTCTGACCCACTGGGAATGTGATGAAAGAAATA
AAAGCTGAAATGAATCATTCTCTCTACTATTATTCTGATATTTCACATTCTT
AAAATAAAGTGGTGATCCTAACTGACCTAAGACAGGGAATTTTTACTAGG
ATTAAATGTCAGGAATTGTGAAAAAGTGAGTTTAAATGTATTTGGCTAAGG
TGTATGTAAACTTCCGACTTCAACTG (SEQ ID NO:2);

(iii)

CCCTAGAAAGATAGTCTGCGTAAAATTGACGCATGCATTCTTGAAATATTG
CTCTCTCTTTCTAAATAGCGCGAATCCGTCGCTGTGCATTTAGGACATCTCA
GTCGCCGCTTGGAGCTCCCGTGAGGCGTGCTTGTCAATGCGGTAAGTGTCA
CTGATTTTGAACTATAACGACCGCGTGAGTCAAAATGACGCATGATTATCT
TTTACGTGACTTTTAAGATTTAACTCATACGATAATTATATTGTTATTTCAT
GTTCTACTTACGTGATAACTTATTATATATATATTTTCTTGTTATAGATATC
(SEQ ID NO:3); and

(iv)
TAAAAGTTTTGTTACTTTATAGAAGAAATTTTGAGTTTTTGTTTTTTTTTAA
TAAATAAATAAACATAAATAAATTGTTTGTTGAATTTATTATTAGTATGTA
AGTGTAAATATAATAAAACTTAATATCTATTCAAATTAATAAATAAACCTC
GATATACAGACCGATAAAACACATGCGTCAATTTTACGCATGATTATCTTT
AACGTACGTCACAATATGATTATCTTTCTAGGG (SEQ ID NO:4);

or (b) a fragment of a nucleic acid sequence 70% homologous to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.

11. A composition comprising one or more progeny of the organism of claim 5, wherein the one or more progeny comprise any one or more of the one or more mutations (i), (ii), and (iii).

12. The composition of claim 11, wherein the one or more progeny further comprise at least one inverted tandem repeat of a transposon or variant thereof.

13. The organism of claim 5 or the composition of claim 11, wherein the composition is a colony of mammals.

14. The organism of claim 5 or the composition of claim 11, wherein the organism is an animal.

15. The organism of claim 5 or the composition of claim 11, wherein the organism is a mini pig.

16. The organism of claim 5 or the composition of claim 11, wherein the organism is a rat.

17. The organism of claim 5 or the composition of claim 11, wherein the organism is chosen from a mouse, pig, rabbit, dog, cat, goat, non-human primate, mini pig, ferret, farm animals, fish, chicken, and bird.

18. The organism of claim 5 or the composition of claim 11, wherein the organism is chosen from a salmonoid, carp, tilapia, or tuna.

19. The organism of claim 5 or the composition of claim 11, wherein the organism is an insect.

20. A mammal comprising one or more spermatogonial stem cells derived from the germline lineage of an animal, wherein the one or more spermatogonial stem cells comprise one or more of the following mutations: (i) a deletion mutation; (ii) a knockout mutation; and/or (iii) an addition of a heterologous nucleic acid sequence; wherein the one or more mutations of (i), (ii), and/or (iii) are site-specific mutations caused by a ZFN.

21. The mammal of claim 20, wherein the one or more spermatogonial stem cells are transplanted from an *in vitro* culture.

22. The mammal of claim 20, wherein the mammal further comprises a nucleic acid that comprises a transposon sequence that is at least 70% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

23. The mammal of claim 20, wherein the mammal is a rat or mini pig.

24. The mammal of claim 20, wherein the mammal is a sterile male rat or sterile male mini pig.

25. The rat of claim 24, wherein the rat or mini pig is DAZL deficient or DAZL-/-.

26. A colony of genetically modified organisms comprising:

(a) at least one organism comprising one or more spermatogonial stem cells, wherein the one or more spermatogonial stem cells comprise one or more of the following mutations: (i) a deletion mutation; (ii) a knockout mutation; and/or (iii) an addition of a heterologous nucleic acid sequence; wherein the one or more mutations of (i), (ii), and/or (iii) are site-specific mutations caused by a ZFN. ; and

(b) progeny of the organism of subpart (a).

27. The colony of claim 26, wherein the heterologous nucleic acid is a selectable marker or an orthologous gene.

28. The colony of claim 26, wherein the at least one organism and the progeny further comprise at least one inverted tandem repeat of a transposon or variant thereof.

29. The colony of claim 26, wherein the at least one organism and the progeny further comprise a nucleic acid that comprises a transposon sequence that is at least 70% homologous to: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4.

30. A method of generating one or more genetically modified organisms comprising:

(a) contacting at least one spermatogonial stem cell derived from the germline lineage of an animal with: (i) at least one ZFN that mutates a gene of interest; or (ii) at least one expression vector that encodes a ZFN that mutates a gene of interest,

thereby creating at least one spermatogonial stem cell comprising at least one mutation at a gene of interest;

(b) expanding an *in vitro* culture of the at least one spermatogonial stem cell comprising at least one mutation at a gene of interest;

(c) implanting one or more spermatogonial stem cells from the culture of step (b) into an organism.

31. The method of claim 30, wherein the organism is capable of passing at least one mutation at a gene of interest to progeny by germline transmission.

32. The method of claim 30, wherein the genetically modified organism is a mammal.

33. The method of claim 30, wherein the genetically modified organism is a rat or mini pig.

34. The method of claim 30, wherein the genetically modified organism is a sterile male rat or sterile male mini pig.

35. The method of claim 30, wherein the method further comprises: breeding the organism implanted with the one or more spermatogonial stem cells with another animal to generate one or more progeny that comprise the mutated gene of interest.

36. The method of claim 35, wherein the progeny are mammals.

37. A method of manufaturing a colony of genetically modified organisms comprising:

(a) contacting at least one spermatogonial stem cell derived from the germline lineage of an animal with: (i) at least one ZFN that mutates a gene of interest;

or (ii) at least one expression vector that encodes a ZFN that mutates a gene of interest, thereby creating a stem cell comprising at least one mutation at a gene of interest;

(b) expanding an *in vitro* culture of the spermatogonial stem cell comprising at least one mutation at a gene of interest;

(c) implanting the at least one spermatogonial stem cell comprising at least one mutation at a gene of interest from the culture of step (b) into a first organism.

(d) breeding the first organism with a second organism of the same species;

(e) selecting progeny of the first and second organism that comprise the at least one mutation at a gene of interest; and

(f) breeding the progeny to create a colony of organisms that comprise the at least one mutation at a gene of interest.

38. The method of claim 37, wherein the first and second organisms are mammals.

39. The method of claim 37, wherein the first and second organisms are rats or mini pigs.

40. A method of manufacturing a first filial generation of genetically modified organisms comprising two or more distinct subsets of organisms, the method comprising:

(a) contacting a first spermatogonial stem cell with: (i) a ZFN that mutates a first gene of interest; or (ii) an expression vector that encodes a ZFN that mutates a first gene of interest; thereby creating a first stem cell comprising a first mutation;

(b) contacting a second spermatogonial stem cell with a modifying agent, thereby creating a second spermatogonial stem cell comprising a second mutation;

(c) expanding an *in vitro* culture of each of the first and the second spermatogonial stem cells;

(d) implanting a mixed population of spermatogonial stem cells comprising the first and the second spermatogonial stem cells into an organism;

(e) breeding the organism with another organism of the same species.

41. The method of claim 40, wherein the first filial generation of genetically modified organisms comprises two or more sets of organisms, each set comprising a distinct mutation of interest derived from a haplotype of distinct spermatogonial stem cells transplanted into a parent of the organism.

42. The method of claim 40, wherein the organism is a mammal.

43. A kit comprising:

(a) a ZFN or a nucleic acid sequence that encodes a ZFN that cleaves a nucleic acid sequence at a gene of interest; and

(b) an instruction manual comprising directions; and, optionally

(c) one or more spermatogonial stem cells; and, optionally

(d) culture media for the one or more spermatogonial stem cells.

44. A kit comprising:

(a) the composition of claim 1; and, optionally

(b) culture media for the one or more spermatogonial stem cells.

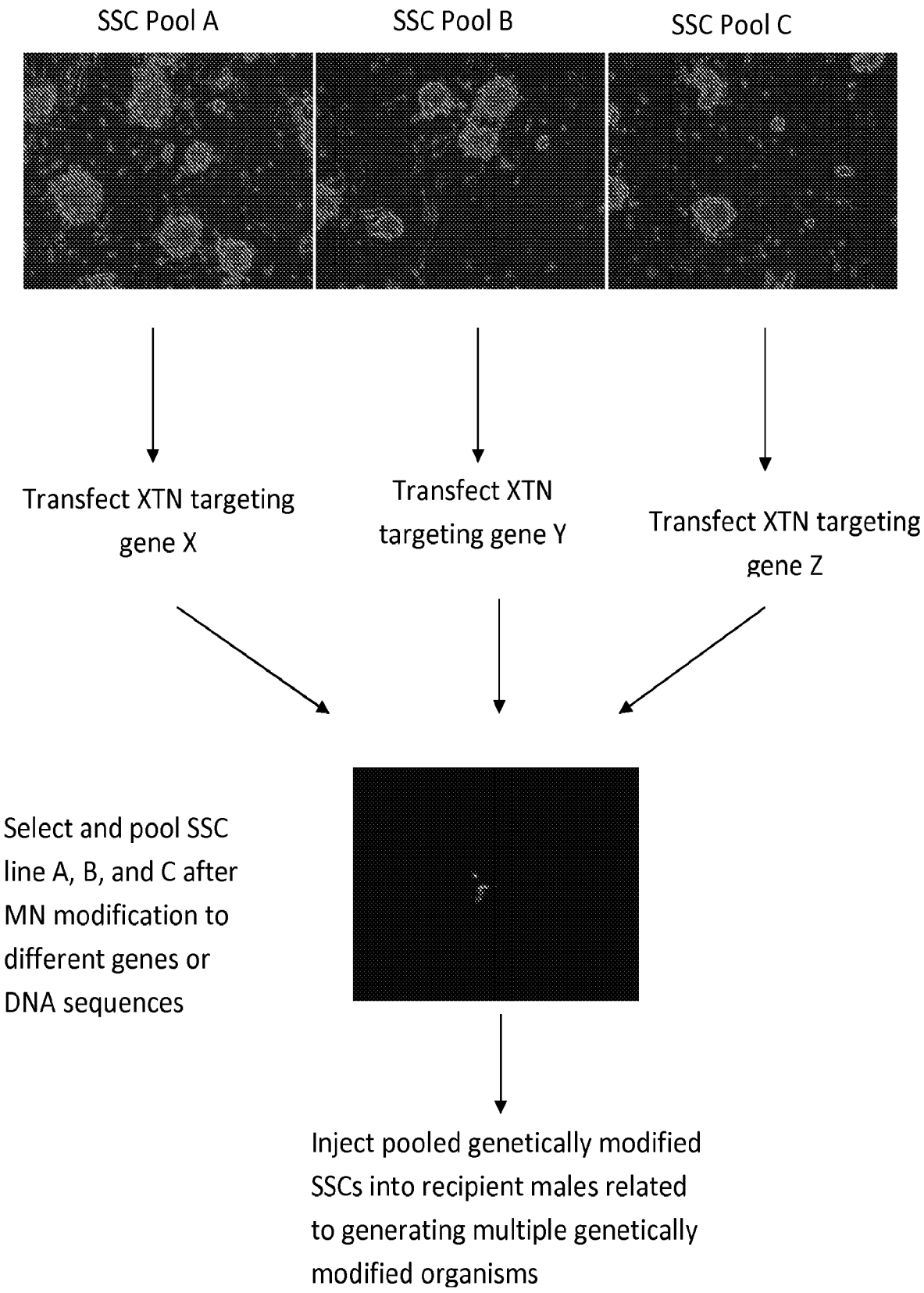

SSC Pool A
SSC Pool B
SSC Pool C
Transfect XTN targeting gene X
Transfect XTN targeting gene Y
Transfect XTN targeting gene Z
Select and pool SSC line A, B, and C after MN modification to different genes or DNA sequences
Inject pooled genetically modified SSCs into recipient males related to generating multiple genetically modified organisms

Figure 1

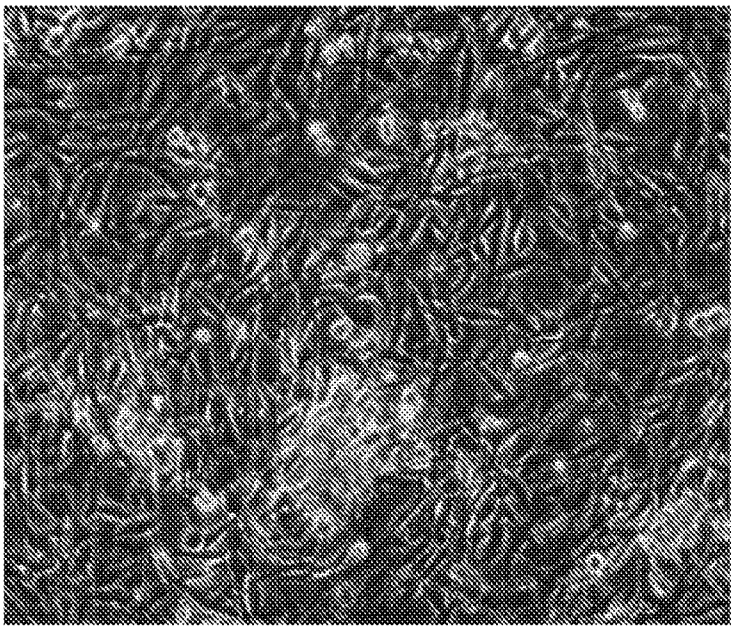


Figure 2

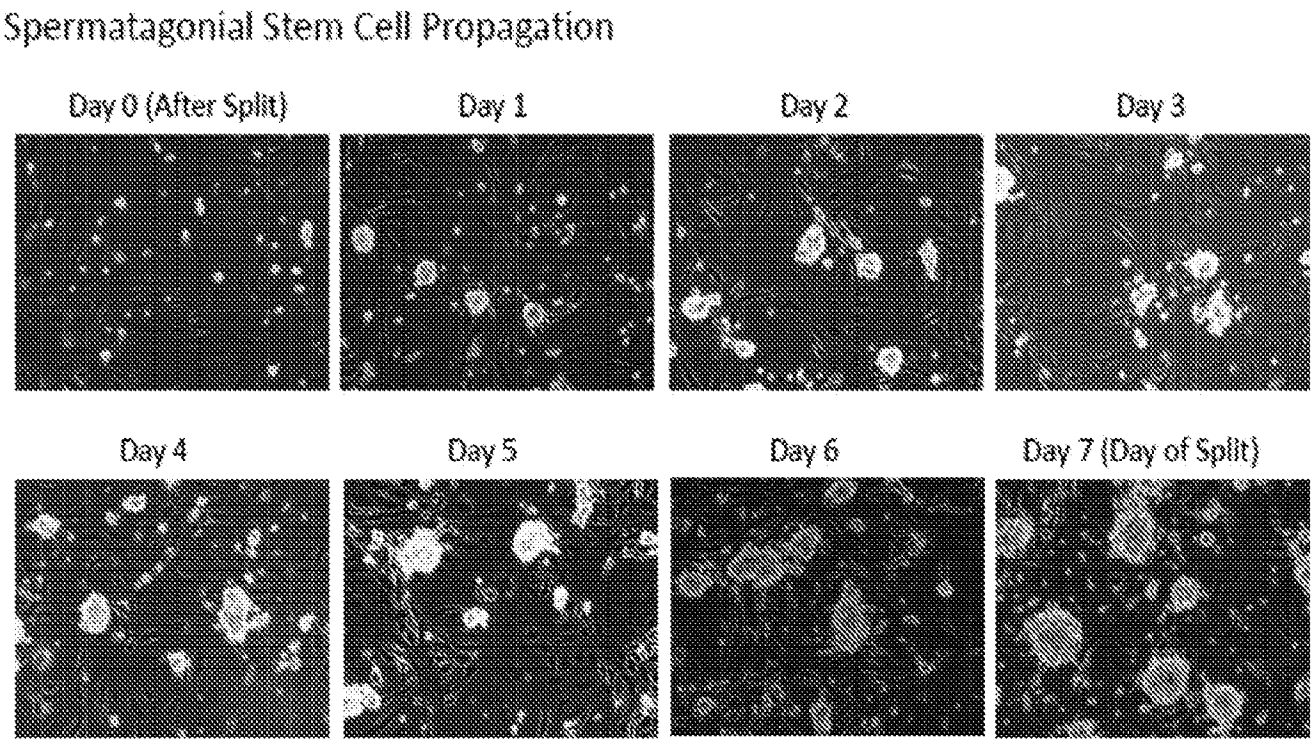
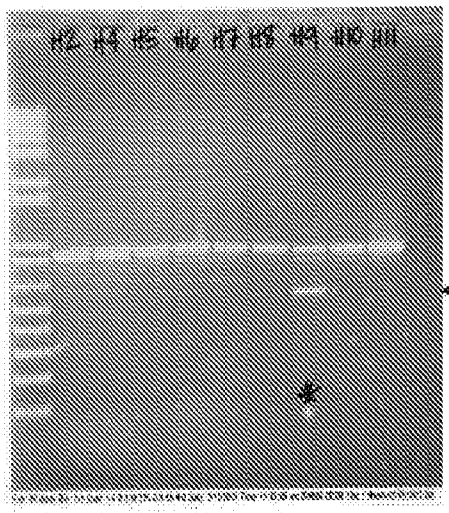

Spermatagonial Stem Cell Propagation
Day 0 (After Split)
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7 (Day of Split)

Figure 3

Figure 4



Targeted Zinc Finger Nuclease Mediated Mutatgenesis in Rat Spermatogonial Stem Cells. Shown is Taq Polymerase PCR reaction using genomic DNA from individually picked and expanded transgenic *EGFP* spermatogonial colonies as template and Primers flanking the Zinc Finger Recognition Site "ZFN 304 (EG304), at 52 days after transfection with pCK181.

PCR Product Sequenced from Colony H9

Figure 5

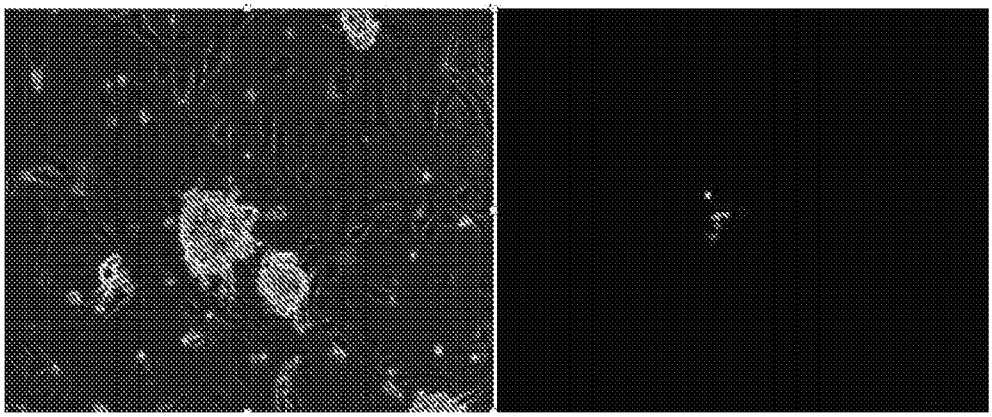
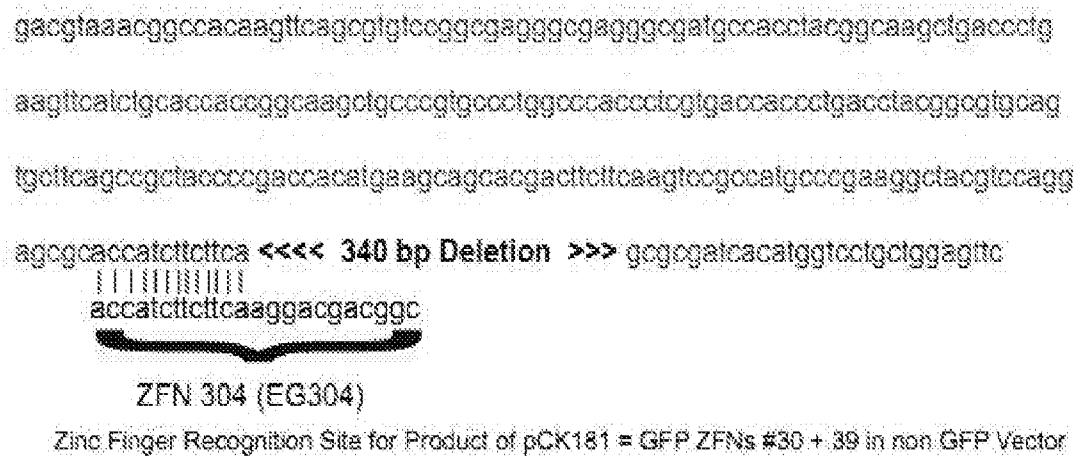


Figure 6



Target site (5' to 3'; 9 bp ZF binding and mutation half sites in CAPS)

cACCATCTTCttcaagGACGACGGCa

gATCCGCCACaacatcGAGGACGGCa

Figure 7

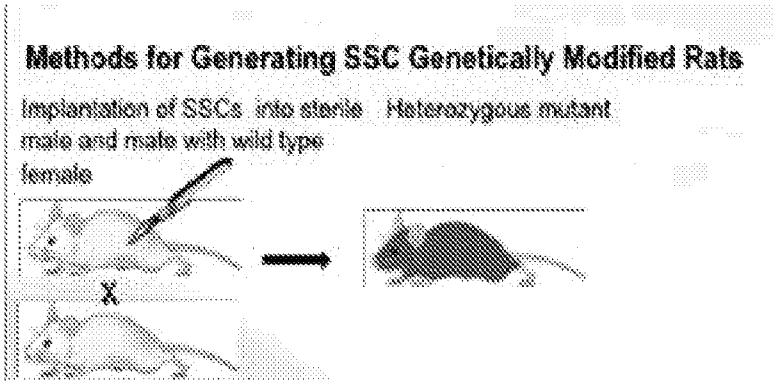


Figure 8

CMV promoter = 235-822
Zinc Finger start codon at 902-904
NLS = 910-930
FLAG Tag = 940-963
Zinc finger in between unique XbaI and BamHI sites

ZFN 1 Amino Acid Sequence

MGPKKKRKVAAADYKDDDDKSRPGERPFQCRICMRNFSTNQKLEVHTRTHT
GEKPFQCRICMRNFSVRHNLQRHLRTHTGEKPFQCRICMRNFSQHPNLTRHLK
THLRGSQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEF
FMKVYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQAD
EMQRYVKENQTRNKHINPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTR
LNHKTNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINF

ZFN 2 Amino Acid Sequence

MGPKKKRKVAAADYKDDDDKSRPGERPFQCRICMRNFSAPSKLDRHTRTHT
GEKPFQCRICMRNFSLGENLRRHLRTHTGEKPFQCRICMRNFSDGGNLGRHLK
THLRGSQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEF
FMKVYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQAD
EMERYVEENQTRNKHLNPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTR
LNHITNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINF.

ZFN 3 Amino Acid Sequence

MGPKKKRKVAAADYKDDDDKSRPGERPFQCRICMRNFSTRQNLDTHTRTHT
GEKPFQCRICMRNFSRRDTLERHLRTHTGEKPFQCRICMRNFSRPDALPRHLK
THLRGSQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEF
FMKVYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQAD
EMQRYVKENQTRNKHINPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTR
LNHKTNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINF.

ZFN 4 Amino Acid Sequence

MGPKKKRKVAAADYKDDDDKSRPGERPFQCRICMRNFSAPSKLDRHTRTHT
GEKPFQCRICMRNFSDESNLRRHLRTHTGEKPFQCRICMRNFSRVDNLPRHLK
THLRGSQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEF
FMKVYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQAD
EMERYVEENQTRNKHLNPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTR
LNHITNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINF.

Figure 9:

Minipig Rag1 ZFN Binding and Mutation Site: GTCGGGGACTCAAGAGGAAGAG

Minipig Rag1 sequence:

GGGGGCAGACACCAGAAGTAAGAGAGGCTACAGCTCTATTATCCGTAAAAAGGTCACCACGCCAAAAAC
CTATAAAAATGAAAAGACAGAACTATAATTCAGATGAGGGAGAAAGGAAAAACCCCAGAAAATCAGCTA
ATCGGTGAGGAGGTTCTCAGCCTCCAGGAAAAAGACTTTAGACCTGATGCTGAAGATGATGCAGGACAT
TGGAAATAAACTGGAGGCAAAGATGGATAACTTACAGAAAACACTGAGCAAAGAGATACAAGATATAAA
ACTTAAACAAGAAGGGGTATGAAATACCTTGTTTAAAAAAAAAAAAGTTACCTTTTGTGGAAGAAACAA
CATGAAATTTGGAGACAAAAGGCCTGAGCTCAAATTCCAGATCAGGTATTTACTATCCTTGTAACTTGG
GCAAATCATTGAACCCTCCACCAAATGGGAATCATAATAATGGCTACCCCACAGGGTGGTAATGAGGGA
AAAACAAGGAAAAATGAATGTGGAAATATTCTAAAAATGGAAGGAAAAAAAAAAAAAAACCTCTGCAAAT
GTTAACTTCCACTGTAAGCAGTTTCCATCTAAACTTAGCAGGTGAGAAAAGTACAAAGATATCCTGCTT
TCAAAAATAACTGAATACACTGAAAGTAAACCGATCATAATTTAAAAAATAAATAACATAATGCAGAAA
GCAAGCCTTCATGAAAACCAGATTTAGCAAAACTGACCCAGGATTCATATGGAAAAAACCTGGCAGAAA
TTTTCAGCAAGACATTAATTCTGCTTTTATACAATGAGAAAACCCAGATTTTAAAAGTCTCTGTGGCTT
TGAGATTGTAGGGGGAAAACATTGGAGAATCTAAATTAATTAAAAGTAAAAATAAAAATCATCCTTGGC
TACTGATAGCTAAATAAACACACACTATTGCAAGATTTAATAACTAACTGCAGCTAACAGTTTTCTTCT
TCCAACGTTATTATTGAAAAACCTAGGGAGTTCCCATTGTGGCGAAGTGGAAAACGAATCTGACTAGGA
ACCATGAGGTTTTGGGTTCGATCCCTGGCCTCGCTCAGTGGGTTAAGGATCTAGTGTTGCCATGAGCTG
TGGTATAGGTCGCAGACACGGCTCGGATCTGGCATTGCTGTGGCTCTGGTGTAGGCCGGCAGCAATAGC
TCCAAATGCATCCCTAGCCTGGGAACCTCCATATGCTGCGGGTGCGGACCTAAAAAGACAAAAAACCAA
AAACAACAAAAAAAGAAAAACCTATGGTAGCAGATGATCTTCTAAACTCAAGGTTACAATTCTGACATC
GTTTAAACTCAGAAAATGACTTATTTTCCAAAAATATTCTACATAAGTGTACCAAATGGGAGCATTTTG
AAAGGTCATTGTCGCATAGTGTAAGATATTATTAGTAGAGAAATCTGAGAAGTCTTTTTCTTTTTCTTT
TGTGGCCACTCCATGGCCCATGGCGTTCCCGGACCAGGGATCAGATTCGAGCCACAGTTGTGACCTTAG

Figure 9 (Cont'd)

CTACAGCTGCAGCAACGCTGGATCCTTAACCCACCATGCCAGGCTGGGGCTGGAACCTGTGTCCCAGCA
CTCCCAAGATGCCACATTGTGCCACAGCGGGAATTCTTGAAAACCTTTTTTAAATCTTGTAAAAAAATA
AGCCAAAATATGTGAAATTTATAAAATAAAAAATTCTGCAAAAGTGATATGAAAAACACCTCTCAGTAT
AAACTAGAATAACTTGGGCAGGGGGAATGGAAACCACAAGTCAAATTCCTTTTCTGGCTTCACTGGCTG
TCCTTTGACAACCTTGGCTTTGACTTTGGCATCACTCAATCCTCTCCACATACATCTTTGTTCACCTCT
GGTCCCCTGCAGTTACCCCTGTTCTCAGCTATGGAGCTTTCAAATAACTGGTTTCTCCTGTTGCTTTTC
TGAACTCCCTAAACAGCTCAAGCAATCGGCTTACCAGTTACCTATTCCTGACATGGGTTCACCATCTCT
GGCCTCATCAACCTACCACCATTTGGGTGGTGAAGCCCCACACCCAGGAGTTAAGGGTGCCAGGAGAAG
CTCTCCATGCAACCCAGAGAAAACCCATTTGCAAAACCAATGCCTAGGCAGCTTCCCTGCTTAAAATTC
CATCAAATCTTGAGATTCTGGAGAGTTCTCTAGGTCTAATGTGTCCCCTGAACTTCTCTGCAAGTTTAT
GGTAAACCCTGAGTTTTTAAATGCTTTATGAGCATTCATAAACTTCTGCAGGTATTTGGAGGTGTACAA
CCAGTGATGTTTCAAAACATCTTCCATTTCATAGTACTTGGACTGCCTGGCATTCATTTTTCGGAAGCG
CCTGAACAGCTTGTTCCCAGACTCATTTCCCTCGCTAGCCCATGCCCCAATGGAGCCGTCCCTCTCGAT
AATTTCCGGGACGTGGGCCAGTGTCTTGTGAAAATAATTGGTGATTTTGCCCTCATATCTGTACTTGAA
CTTGGTGGAGAGGAGCTCAGCAAAACGCTGCGAATTGAAACTATACTGGCAGAGGGATTCCGGGCACTC
TTTAGCAGGGCATGACGATCGCCAGACGGGTTTCATCTTCAGGTAAAGGTCCATCAGTTCCCTCAGAGC
TTCATGCCTCTCCTCGGAGGGAATTAACTCACAGACTGCTTCCACAGTCTCTTTGGTCATGAGCTTCCT
GGCAAAGTTGCCATTCATCCTCATGATGGGCTTCAGATTCATCTTCTTGCGGAGGTGCTTGTCCAAGGT
CGCCTGCCATCTCTTCCTTTCCTCCTTGGAGGCATGGGGGTTCTTATACGCCTCCCCTATCTCGAGCTG
GAAAATCTTGTAGAACTCGGCTGCATTGCCAATGTCACAGTGGAGGGCATCTATGGAAGGCACCGTCTC
AATGAAGGGTTTGGCCGAGACCCCTTTCACCCGGTCCCGAAGTTCATCCACCGTCTCATGGTATGGGTT
GGAACGCCAGACCTCATAGCGCTCCAAATTCTCCGCGTGGCTTCTGGTTATGGAGTGGAAGACCAGATT
TTGAGAGGCTTCCAGGCGGGTGGCATCACAGAGAGTACAGATGTAGACAGAGCCAGAAGCCTCAAGGCC
TTCCACTTCCCGGACCAGTTTCTCATCATATCCGGTGCCCCTGAAGATGAACTTGAAAGTCCGGAGGAT
GCCTCCCATCTCCAGCATTAGCTGGCTGCTCTTCATGGCCTCCCTCTCGGCAATGAGAGGGCTCAGGAT
GGCCGTCAGGGTCTCATGGTCGGATTCGTCGGCCAGCATGAGGCACAAGGGCTTGCAGCATAGTTCAGA
GTTAGGCTTGGCTTCCTCAAACACCTTCACGTTCTGTGACCCGTGTGCGATGGTGATTTTCATGACTGT
GAAGGAAAACCGAACGGCCTTTTCCGGCACGACCGGCCCACTGCCGTGCTTCTCACTCACGTCTCCCAT
CCCATCACAAGACTCCTTCACCACCACAGTGAAGGGGCCATTCAGGTAGTCGTCAAGGTCTTGGGCTCT
CATACCCTCCAGGATGTCTTCTCACATGTCCATGAGAGCGGACACCAGAGCCGAGTCATAGCGGAAGCG
CTGGCAATNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGTCTTCTTGTGGGAGGCCTTGACTTGCAGC
TTGAGCTCACTCGACGGTGTTTCTGAGCCCTCCGCGTCAGGGACAGGAGATGCTGGCGGGGCCGGCCCC
NCTTTATTAATATGCACAAATGTCTCCTTCGACTCCTTGTGGCTTGAGATATGGTGATTATATTTTTCC
AAGCTGATCTCCTCGTTGCACTCCTTTGCTGGGCATTTCACCATCAGGGTATTCAAGATGCTCAGAAAA
GACTTCACTGGACTCTCCAGGTCAGTAGGGAAACAGGGATAGTGGCAAGAGGGACAACTGCTGCCCATG
ACTTTGAGGCACCTGAGAATGCAGATCCTGCAAAACACGTGCTTGCAGCTGGTCTCCACCGGGTCGGCC
AGGATGTGTTCACAAATCTGGCAGGAGATAGATTTCACAAAGTGCGCCGGGAAGTCCACTGCCAGGAGC
TTGGGGCTAAGATGTATCTGACCGCAGTTGGCGATCTTCTTCATCAGTTCCTTGCTGCTGATCCTGGCC
TGAGCTCTCCTCTTGCGCTGACGGGCTTGTCTCGCTCGGTCAATCACAGTTTTGAGTTTTTTGCTGAGC

Figure 9 (Cont'd)

TGCATGTTTGGCTGCTGACTCTTCCTCTTGAGTCCCCGACGTGCAATGTGGCAGATGTCACAGTTTAGG
GTGTGGGGGTGCCACTCCATGGTTGCATTCCTTGGGGAGTAAACCTCACATGGGGTGCTGCTAAACTTC
CTGTGCATGAAGCTCCAGCAGTTATGGCAGAACTCAGTGGGGTGGATCGAGTCAACATCTGCCTTCACA
TCGATCCGGAAAACTTTGGCAATGAGGTCTGGCCAGGACGTGGCCCTCTTTTCCTTCTTCCGTAAAAGG
ACTTGGGTTTTACCATCCACAGGCCCGTGGACTGGATACCTTCTCTTGTGCCCAGTGGTGTTGAAAGAA
TTCCCACAGATGCGGCAGAGACGTCTCAGGTTGGCTTGGTGGATGGCTTTGTCTCTTGCTTTCCCATCT
TCGTGGGATTCCTTTAAAAACTTTGGATGGGGCTTGAATGCTGGTTGAGGCAGGGCTGACTTCTGACCA
CCAGGCTTGTCCAGGACTGCTGGAGATTGCTCCAGCGAGGGTTTCCCCTCGGAGGAATCCTGCTTTTCC
GTTTGAGCCTTTTCAGGTGCCTTTTCAAAGGATCTCACCCTGAATAGCTTAAACTTCCATTCTGAAAAT
TTAATGTGGGGGTGCTGGATTTCATCTGGGGCGGAACTGAGTCCCAGAGTGGGTGGCAAAGAGACAGCC
ATGCTGGCTGAGGTACCTGGGAGCAATAAGGACAAGTCAGGTTAGGAAAAAAAATGTAAATAACCCACT
GATGCAACTTGGAAGGAATACCAATTAGTACATTCAATTATTCATTTATTTTTTATTTTATTTTATTAT
TTATTTTATTTTTTTGTCTTTTTTGCCTTTTTTCCAGAACCAGCACACAGAGGTTCCCAGGCTAGGGGAG
CTGCAGCCGCCGGCCTACGCCAGAGCCACAACCGCTTGGGACCTGAGCCGCATCTGCGACCTACACCAC
AGATCATGGCAATACCGGATCCTTAACCCACTGAGTAAAGGCAGGGATCGAACCCACAACCTCATGGTT
CCTAGTTGGATTCATTAACCACTGCGCCACAATGGGAACGCCTATTCATTTATTAAAGCATTCAGAAAG
TTAATTGTTTGGCTTCCTATGTTAATATACTATGTTAGGGCTGATCATATACAGAAAACAGAGTCATTT
CCTGTTCTTTCAGTGGCATTGTCCATTTACATGTTTCTGCGTTCACGCATCATCACCATGGTCTATTTT
ACATATATGTGTCCAATCATTTCTTTTATAAAAATTATTCTTTTTTATTGAGATATAGTTGATGTACAA
TATTATCTATGTACGATATTATTTCCTTTCCACCAAAACCTGGACATTTTTTAGGACGGAATCTTTGAG
TGATTTATCTCTGTCTCCCCCAAGCTTTCTAGCACACATTAGATGCTCAATAGTTATCTGCTGAATAAA
TGCAAGGAAAAACTGGTTACTAAAACCAAAGAAAAGTCATATTTTCCGCGCTGTATCAGTCTCATTGCA
CTAGCTGGACAATGTATTTCCAGCTTTTTAAAGCAGTCTGCCAGGAGGTTGGTTGTTTATTTCTCTGAA
ACGGGACATAAAGGGATGATAAAGCTACAAATCCAAAGAAACTAATTACTCGCTACTTGAAAGGATGAA
GTGAATTTTGAGCTTTATGAATGCACACTAATAACAAGTATCATGGATAGTAATTATGCTTACAATTTT
AATTAGTGAACGCTGCTGTAAATTATAAGGCTGTGTCTCCATTAGGAACAAAACCCACAGAACTCTCTA
AACCTGCAACAAACCACAGTGTGCATGTACAGAGACTCCAAAGGACTTTTAAGATGAAAAATTCAAGTA
GTTTCAGGTCGAATTCACACAGGCCATCCCAAAAGTCATTACTGGGCACTGGTCATTAGCTCTATCGTT
TGTAATAAGAAGAGGTGGGAGTAATAATTTTCTTCTTCAAAAAAAACATTGGCCATTATATATTCCAGG
AAACCCTAGCAACCTGGGTTGTGGTGTAGGCTTCAACAGAGCTGTAGAAAAAAGTTTAAAAATGCCATT
TGAGTGTCTCTAGACCCAATTAGCACAATATTCTCAAAATTCCTTAGTATCAGCATGACTGGAAAAACA
GCAATTGTATTTTCAAACTACAATCTAAATTTTCCATATTTTCCTGGGTTAGTGCTCTTGACCCGCCAA
TTATATCAAACAATGCTTCCCCTTCAATTAACTAACTAACCAAGTTGATGATATTGTTATTGTCCTGTG
GTATGGAGACTAATCCTCTGATGAACACACTTTGCCCAGGGGTGGGATTTGGGGGAGGGGAAAGGAAAG
CCACAAGGGTGATCCTGACTGCTGCTAACAAAGCTTCCTGTTAGGTCATCCCTTTCAACCACAGAAGTT
TGTTCCATCTTGCATGCATGATGCTTCAGAGATTCATGTTTTTCATAAAGACGAAAAGCCAATTTCCAA
GTCTTGGTTGAAAAGGAAATCCTGTTACACTGTTCTATATGCAGGACTGTGTGAGAACTAAACAGTTCC
AAACCATTTCAAAGGCCTCATTAACCTCAAAAGTATTTCAACTGCAGTCAGACCAACGCCTCAAAGTTG
CTTATAGTTCAGTTATGTTCCTACATTCAGGATCAGCGGCAAGGATGTCACTTCTGTCCCACTGGTTTG

Figure 9 (Cont'd)

AACTTATTTCCATGTGTTTTGTTTCTTTAGCCCTTCCTTTCCCTGTGTCTGGGCCAAGGATTGAACCGA
CATCACAGCAGTGACCTGAGCCACAACAGAGACAATGCTGGGTCCTTAACCCATTGAGCTA

Table 1: Reagents for SSC culture medium (SG Medium)

SG Medium

	500mL	**250mL**	**200mL**	**100mL**	**50mL**
DHF12 - Sigma D8437	469.5mL	234.75mL	189.8mL	93.9mL	46.95mL
100ug/mL rR-GDNF	100uL	50uL	40uL	20uL	10uL
25ug/mL rbH-FGF	400uL	200uL	160uL	80uL	40uL
Diluted 2-mercaptoethanol –Sigma M3148	5mL	2.5mL	2mL	1mL	500uL
l-glutamine (100x) – Invitrogen 25030-149	10mL	5mL	4mL	2mL	1mL
B27 Supplement Minus Vitamin A (50x) – Invitrogen 12587-010	10mL	5mL	4mL	2mL	1mL
Antibotic/mytotic (100x) - Invitrogen 15240062	5mL	2.5mL	2mL	1mL	500uL

Table 2 Passaging SSCs

Size of Culture Vessel	Growth Area (cm^2)	Splitting SSC density	Nominal Fill Volume
96 well	0.32	9.6 x 10^3	200 ul
8 well chambered coverslip	0.8	2.4 x 10^4	400 ul
24 well	1.88	5.6 x 10^4	500 ul
12 well	3.83	1.2 x 10^5	1.0 ml
6 well	9.4	2.8 x 10^5	2 ml
35 mm	8	2.4 x 10^5	2 ml
60 mm	21	6.3 x 10^5	5 ml
10 cm	55	1.7 x 10^6	10 ml
Flasks:	25	7.5 x 10^5	5 ml
	75	2.3 x 10^6	10 ml

Table 3: Transposon ITRs

Transposon

Sleeping Beauty

5' Inverted Tandem Repeat:
CAGTTGAAGTCGGAAGTTTACATACACTTAAGTTGGAGTCATTAAAACTCG
TTTTTCAACTACTCCACAAATTTCTTGTTAACAAACAATAGTTTTGGCAAGT
CAGTTAGGACATCTACTTTGTGCATGACACAAGTCATTTTTCCAACAATTGT
TTACAGACAGATTATTTCACTTATAATTCACTGTATCACAATTCCAGTGGGT
CAGAAGTTTACATACACTAAGT

3' Inverted Tandem Repeat:
ATTGAGTGTATGTAAACTTCTGACCCACTGGGAATGTGATGAAAGAAATAA
AAGCTGAAATGAATCATTCTCTCTACTATTATTCTGATATTTCACATTCTTAA
AATAAAGTGGTGATCCTAACTGACCTAAGACAGGGAATTTTTACTAGGATT
AAATGTCAGGAATTGTGAAAAAGTGAGTTTAAATGTATTTGGCTAAGGTGT
ATGTAAACTTCCGACTTCAACTG

PiggyBac

5' Inverted Tandem Repeat:
CCCTAGAAAGATAGTCTGCGTAAAATTGACGCATGCATTCTTGAAATATT
GCTCTCTCTTTCTAAATAGCGCGAATCCGTCGCTGTGCATTTAGGACATCTC
AGTCGCCGCTTGGAGCTCCCGTGAGGCGTGCTTGTCAATGCGGTAAGTGTC
ACTGATTTTGAACTATAACGACCGCGTGAGTCAAAATGACGCATGATTATC
TTTTACGTGACTTTTAAGATTTAACTCATACGATAATTATATTGTTATTTCAT
GTTCTACTTACGTGATAACTTATTATATATATATTTTCTTGTTATAGATATC
(minimal sequence is underlined and bold, i.e., first 35 bp)

3' Inverted Tandem Repeat:
TAAAAGTTTTGTTACTTTATAGAAGAAATTTTGAGTTTTTGTTTTTTTTTAAT
AAATAAATAAACATAAATAAATTGTTTGTTGAATTTATTATTAGTATGTAAG
TGTAAATATAATAAAACTTAATATCTATTCAAATTAATAAATAAACCTCGAT
ATACAGACCGATAAAACA**CATGCGTCAATTTTACGCATGATTATCTTTAA**
CGTACGTCACAATATGATTATCTTTCTAGGG (minimal sequence is
underlined and bold, i.e., first 35 bp)