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(54) Title: REPOPULATION OF TESTICULAR SEMINIFEROUS TUBULES WITH FOREIGN CELLS

(57) Abstract

An animal harboring a non-native germ cell, its corresponding line, and the corresponding germ cells, are obtained by colonizing the testis (or testes) of a host animal with primitive cells followed by raising and/or breeding the host.

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REPOPULATION OF TESTICULAR SEMINIFEROUS TUBULES WITH FOREIGN CELLS

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Field Of The Invention:

The present invention relates to animals harboring a non-native germ cell, to corresponding animal lines and germ cells, and to methods for obtaining the same.

Discussion Of The Background:

There have been many attempts to influence differentiation of developing cells by modifying the genotype of an embryo and then observing its effect on the phenotypic development pattern in the progeny. These techniques included nuclear transfer (McGrath & Solter, Science (1983) 220:1300-1302) and cell-egg fusions (Graham, "Heterospecific Genome Interaction," 1969 Wistar Institute Press, pp. 19-35, ed. Defendi). Of the two, the former has had limited success.

Another approach might be to add a stem cell(s) to an early embryo and determine its effect on development. For example, one might imagine that a stem cell from bone marrow would contribute to the population of, and thus modify the differentiation of the evolving bone marrow cells in the host embryo. To pilot these experiments, older embryo cells were introduced into young embryos resulting in modest success with colonization (Moustafa & Brinster, J. Exp. Zool. (1972) 181:193).

These initial experiments were followed by studies using bone marrow stem cells and teratocarcinoma stem cells (also called embryonal carcinoma cells). There was evidence that both these cells colonized the developing embryo (Brinster, J. Exp. Med. (1974) 140:1049-1056). In the case of the embryonal carcinoma (EC) cell, the colonization was demonstrated dramatically by a change in the color of hairs in the coat of the mouse. This was an exciting result which stimulated a great deal of interest among scientists in the field, because it showed the possibility of colonizing an animal with non-embryo cells. This would provide a means to introduce new genetic information through the DNA of the colonizing cells.

The next year these results were confirmed and extended by two other laboratories (for work done in one of these laboratories, see Mintz & Illmensee, Proc. Nat. Acad. Sci. (USA) (1975) 72:3585-3589) and it was demonstrated that the introduced EC cells may colonize numerous tissues including germ cells (sperm and eggs). Thus, a gene that was mutated, modified, or added to the cell *in vitro* could eventually end up in sperm or eggs of an animal, creating a new genetic strain of mice.

Unfortunately EC cells colonized the germline poorly and a better cell line was sought. In 1981 two scientists, Gail Martin and Martin Evans, independently described a more efficient cell designated the embryonic stem (ES) cell (Martin, Proc. Nat. Acad. Sci. (USA) (1981) 78:7634; Evans & Kaufman, Nature (1981) 292:154). These cells colonize the germline better than EC cells. However, it seems likely that they arise from the same pool of primitive cells in the embryo and are quite similar in biological characteristics.

Embryonic stem cells can be modified *in vitro* (in culture flasks) by adding genes or changing endogenous genes and then

the modified cells introduced into a blastocyst where they participate in development and can become sperm. This technique allows very specific modification of the mouse genome and other species.

Techniques for obtaining non-human transgenic animals through the injection of DNA into eggs are also known. See e.g., Gordon et al, Proc. Nat. Acad. Sci. (USA), (1980) 77:7380-7384. These techniques however, as well as the use of ES cells noted above, are very labor intensive.

Summary Of The Invention:

Accordingly, it is an object of this invention to provide a facile method for obtaining animals harboring a biologically functional non-native germ cell, and for obtaining corresponding resultant germ cells.

It is another object of this invention to provide a facile method for obtaining new animal lines, and for obtaining corresponding resultant germ cells.

It is another object of this invention to provide animals harboring a biologically functional non-native germ cell, their progeny, and corresponding resultant animal lines and germ cells.

It has been discovered by the inventors that the above objects and other objects which become apparent from the description of the invention given hereinbelow are satisfied by the following method. The testis (or testes) of a male animal host is (are) repopulated with at least one primitive cell (e.g. a totipotent stem cell) which is not native to the host. The animal may then be bred to obtain a novel animal

line in which every cell of the descendant animal is genetically non-native to the original host animal.

Brief Description Of The Figures

Figures 1 and 2 are photographs of cross-sections of a seminiferous tubule from the testes of an adult mouse. Figure 2 shows intense blue (dark) staining in all mature stages of spermatogenesis, which occupy the center of the tubule. Control Figure 1 does not. This demonstrates the survival, differentiation and development to later stages of spermatogenesis of foreign cells (blue/dark) transferred to the testis of an animal in accordance with the invention.

Figure 3 is a photograph of a cross-section of a testes, showing blue (dark) stained tubules, demonstrating, as in Figure 2, survival, differentiation and development of transferred cells. Figure 4 is a higher magnification view of the tubules shown in Figure 3. Figures 5 and 6 likewise show blue (dark) stained tubules.

Detailed Description Of The Preferred Embodiments

According to the present invention, primitive cells are introduced into prepared seminiferous tubules of animal testis (or testes). There, the introduced primitive cells develop into mature spermatozoa. This colonization is possible because the lumen of the seminiferous tubule is an immunologically privileged site. Evidence for this is the absence of endogenous antibody specific to sperm, and the presence of T-cells that are tolerant to spermatozoa in the circulating blood of males. Furthermore, injection of sperm into the tissues of a male generally provokes an immunological response.

Cells which can develop into more differentiated cells of two or more types are used as the primitive cells. Thus one of two types of cells can be used: (1) totipotent cells, which are cells having the potential to differentiate into any cell type, including germ cells; and (2) pluripotent cells, which are cells capable of differentiating into two or more types of cells, e.g., bone marrow stem cells, liver stem cells, kidney stem cells, etc....

An illustrative procedure for repopulating the testis (or testes) of a host is as follows. A male host animal is prepared by destroying the native germ cell population in the seminiferous tubules by a method that leaves intact (i.e., biologically functional) the supporting cells, including the Sertoli cells. Suitable known methods include physical means (e.g. radiation[1], heat[2], etc.), chemical means (e.g. cadmium[3], Busulfan®[4], etc.), but any other known techniques to selectively destroy native sperm cells can be used. Preferably radiation is used, with Busulfan@ being most preferred.

A typical Busulfan® treatment is illustrated as follows. Forty milligrams of Busulfan® are dissolved in 10 ml of dimethylsulfoxide, to which 10 ml of water are then added. An aliquot of this solution is injected intra-peritoneally into a

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[1]Withers et al. Radiation Res. (1974) 57:88-103.

[2]Gasinka et al, Neoplasma (1990) 37(3):357-366.

[3]Parizek, J. Reprod. Fert. (1960) 1:294-309..

[4]1,4-Butanediol dimethylsulfonate esters; e.g., 1,4-bis(methanesulfonoxy)butane; 1,4-di(methanesulfonyloxy)butane; 1,4-di(methylsulfonoxy)butane; methanesulfonic acid tetramethylene ester; tetramethylene bis(methane sulfonate). See Bucci et al, Mutation Res. (1987) 176:259-268.

mouse in an amount sufficient to administer a dose of 1 mg of Busulfan® per 25 g of mouse weight (Bucci et al, Mutation Res. (1987) 176:259-268). Over the course of several weeks, testicular biopsies are examined histologically to verify the absence of spermatogonia and the sperm which would have resulted from them. The male is then allowed to recover, and tested to ascertain that there is no remaining spermatogenesis by breeding and/or testicular biopsy.

Alternatively, one can employ a male host that genetically has low numbers or no sperm in its testes, such as inbred strains of mice that produce offspring with seminiferous tubules depleted of sperm cells. For example, the sperm cells of so-called W-mutant mice are deficient for an enzyme necessary for their survival and proliferation. Homozygotes and compound heterozygotes for certain alleles of this gene are so deficient for this enzyme that they are completely devoid of sperm cells when adult. C57BL/6J mice segregating the W^v and W^{44} alleles, WB/REJ mice segregating the W allele, and 129/SV mice segregating the W^{54} allele can be maintained and bred toward this end. For example, a C57BL/6J $W^v/+$ animal bred to a WB/REJ W/+ will yield a proportion of hybrid W/W^v males, which when mature will totally lack sperm cells. The testes of these hybrids may accept donor cells from C57BL/6J mice or WB/REJ mice without histocompatibility problems.

If necessary, tolerance in the recipient can be induced by transferring cells corresponding to those primitive cells being transferred to the testis (or testes) of the host, to the thymus of the host before transfer of the primitive cells to the testis (or testes). Thus if spermatogonia is the primitive cell being transferred to the testis (or testes) of

the host, spermatogonia may be first transferred to the thymus of the host to induce tolerance. See, Posselt et al, Science (1990) 249:1293-1295.

Alternatively, cells from an animal of the same strain as the recipient host can be used, or an immunodeficient host can be used. For an example of the latter, inbred strains of mice (e.g. SCID mice or nude mice) bred to act as recipients of sperm cells can be used. As an example of the former 129/SV mice segregating the c and c^h allele for albinism can be maintained. Homozygotes and compound heterozygotes for these alleles are easily distinguished by coat color. A Busulfan®-treated homozygote for the one allele can receive donor cells from a homozygote for the other allele. If the recipient animal thereafter gives rise to offspring showing the donor-type coat color marker, this is evidence that the donor cells gave rise to functional sperm.

The primitive cells used in accordance with the invention can come from other individuals (including both the same and other species) or *in vitro* culture. Examples of primitive cells that can be used include totipotent stem cells, embryonal carcinoma cells, embryonic stem cells, sperm cells from other males (e.g. juvenile males with high levels of primitive sperm cell types), primordial germ cells, other primitive cells, etc.. Primitive sperm cells from seminiferous tubules, embryonic stem cells grown in culture, or primitive cells from body organs are prime candidates. The use of female (XX) cells is also within the scope of the present invention.

These primitive cells may be obtained in accordance with known procedures. For example, spermatogonia stem cells may

be obtained as follows. The testis of a neonatal animal is collected and the covering removed. The tubules are enzymatically-digested to produce a single-cell suspension which contains approximately 15% stem cells. Besides such spermatogonial stem cells found in neonatal and adult testis, there are several other known sources of suitable cells. Stem cells from a tissue culture, such as embryonic stem and embryonal carcinoma (teratocarcinoma) cells are easily utilized. Other desirable cells are primitive cells from organs which have the potential to regenerate, such as the liver, or lymphoid organs, such as the bone marrow.

In a preferred embodiment the cells and/or tubules are modified to facilitate the colonization as well as diversity of cells that are effective. For example, the primitive cells may be added with their corresponding Sertoli cells to facilitate population, or the surface of the primitive cells may be treated with phytohemagglutinin (PHA) to make the primitive cells more adherent (Mintz et al, Dev. Biol. 31:195-99). Alternatively the tubules can be subjected to a limited enzymatic digestion to render them more accessible to the transferred primitive cells.

In an optional embodiment of the invention, the primitive cells are modified genetically by a variety of known techniques[5] so that the genetic characteristics of the resulting spermatozoa can be predetermined. However the primitive cells which are used in accordance with the invention may also be native cells (including native cells possessing naturally induced mutations or variations) or

[5]See, e.g., Lovell-Badge, "Teratocarcinoma and Embryonic Stem Cells: A Practical Approach", IRL Pres (1987), ed., Robertson, or Capecchi, Science (1989) 24:1288-1292.

native cells possessing artificially induced mutations or variations.

The selected primitive cells are then introduced into the individual tubules. For example, a prepared male can be anesthetized and the testis (or testes) surgically exposed. By micromanipulation methods a thin glass needle is introduced into exposed tubules, one after another, and each tubule is injected with a solution containing the primitive cells being used to colonize the tubule. Alternatively, the primitive cells can also be introduced by injecting into other parts of the tubular system e.g. the lumen of the rete testes. To inject the rete testes one may use either fine stainless-steel needles or fine pulled-glass capillaries loaded with donor primitive cells. A micromanipulator is used to direct the tip of such an instrument to penetrate the rete testis. The cells are expelled and will back-fill the seminiferous tubules.

Other systems may also be suitably used for introduction of the cells. These include surgical techniques to sever the seminiferous tubules inside the testicular covering, with minimum trauma, which allow injected cells to enter the cut ends of the tubules. For example, a fine surgical thread is circled about a number of tubules, and then drawn tight, severing the tubules. A donor-cell suspension is then injected into the testis.

Alternatively neonatal testis (or testes), which are still undergoing development, can be used. Here, a surgical procedure to expose the neonatal testis (or testes) for injection of new cells is used. Neonatal mice are chilled on ice for anesthetization. The tiny testes are surgically exposed, and a small bladed-instrument is used to disrupt the

tubular architecture. Donor cells are injected, and become incorporated during repair. These cells then participate, with endogenous cells, if present, in the maturation of the testis.

As noted *supra*, the primitive cells entering the tubule are generally protected from destruction by the immunologically privileged environment of the internal lumen of the tubule. Cells that leak from the tubule are typically destroyed by the immune system of the host since the cells are foreign to the animal. In a preferred embodiment, a dissimilarity in histocompatibility of donor cells and recipient males is used to promote rapid destruction of leaked cell, but this is not absolutely necessary.

The destruction of cells outside the tubule lumen is desirable because primitive stem cells have a high propensity to undergo transformation into malignant growths. But if this undesirable property is eliminated from the primitive cells then destruction of primitive cells outside the site of colonization could be unimportant.

In an embodiment of the invention, one can use animal strains tolerant towards cells from antigenically different animals of the same or different species. For example, nude mice, having no thymus-derived cells, and SCID mice (Bosma et al, Ann. Rev. Imm. (1991) 9: 323-50), having low levels of both B-cells and T-cells, can be used as recipients.

Results of the introduction of the primitive cells are monitored after recovery of the males from the implantation procedure. As is known, depending on the species of the animal used, the period for spermatogenesis is approximately

30 to 60 days, and another 10 to 15 days is needed for epididymal maturation of spermatozoa. See, e.g., "Reproduction in Domestic Animals", 4th ed., Acad. Press (1991), ed. P. Cupps. Therefore, depending on the species used, males that have received primitive cells in accordance with the invention can be examined beginning about two months after cell transfer.

The present invention is applicable to any species of animals, including human, in which the male has testes, including but not limited to transgenics. The invention is also not limited to mammalian species. It can be used to provide animals and animal lines of many types with a single, or many, novel genetic modification(s) or novel characteristic(s). The animals to which the present invention can be applied include animals, such as rodents (e.g., mice, rats, etc...), which can be modified to permit their use in cellular diagnosis or assays. The present invention may also be advantageously applied to farm animals such as domesticated ruminants or fowl (e.g., cattle, chickens, turkeys, horses, swine, etc...), to imbue these animals with advantageous genetic modification(s) or characteristic(s).

Once an initial fertilization event is achieved and the resulting offspring is fertile, the animal line with its novel genetic modification or characteristic is established, with the novel genetic modification or characteristic being present in both male and female offspring. Thus, in accordance with the invention one may produce an animal harboring, in its testes only, a biologically functional germ cell which is not native to that animal by repopulating its testicular seminiferous tubules. This (parent) animal can produce

progeny. Every cell in the progeny is genetically non-native as compared to the parent animal.

Both the parent animal and its progeny provided by the present invention have very varied uses" including uses in agriculture and biomedicine, including human gene therapy. An illustrative agricultural use of the present invention relates to increasing the breeding potential of a valuable stud animal. In this use, a testicular biopsy from a valuable stud animal is used to obtain stem cells for transfer into the treated (with either Busulfan®, radiation, etc.) testes of a recipient animal. The recipient animal thereafter produces sperm which are genetically from the valuable stud and natural matings with this recipient male provide an alternative to artificial insemination with ejaculates from the valuable stud. This illustrative technique is particularly useful as insurance against an illness, injury or the death of a valuable stud animal.

Another illustrative application of the present invention is its application to create (chimeric) animals useful in either biomedicine or agriculture. The present invention provide an advantageous complementation to existing transgenic techniques.

Existing transgenic techniques, when applied to animals other than laboratory mice, are often hampered by the difficulty of recovering embryos, differing characteristics in embryos of different species, a lack of knowledge about the specifics of reproductive timing in the given species, the economics of current techniques, etc. The present invention permits the difficulty and expense of embryological transgenic work to be by-passed.

In accordance with the present invention, spermatogonia stem cells can be genetically modified and then transferred to recipient testes. The valuable genetic traits present in the resultant germ cells can be passed onto the (transgenic) progeny of the recipient stud. This particular application of the present invention is particularly important to the genetic engineering of large agricultural animals.

The present invention also has applications in gene therapy, including human gene therapy. For example, a patient with a deleterious genetic trait could undergo a testicular biopsy. Isolated stem cells can be genetically modified to correct the deleterious trait. The patient then undergoes a treatment to remove the remaining germ cells from his testes, for example by specific irradiation of the testes. His testes (now devoid of germ cells) can then be recolonized by his own, genetically-corrected, stem cells. The patient can then father progeny free from the worry that he would pass on a genetic disease to his progeny.

Conventional cell markers, such as surface antigens or internal enzymes, can be employed in the transferred primitive cells to facilitate detecting their presence in biopsy specimens of the testes. The presence in the ejaculate of spermatozoa with the characteristics of the marker is a reliable indication of success. Thus, in accordance with a preferred embodiment, at least one genetic marker is preferably used to distinguish the introduced cells from residual sperm that might arise from the host male. For example, transgenic mice strains that can produce a characteristic stain in sperm cells which serve as donor marker cells can be used. The promoter from a gene active in developing sperm cells (Zfy-1 or a homolog) is used to drive

expression of the gene for the bacterial enzyme beta-galactosidase (lac Z) in transgenic mice. In the presence of the reagent X-gal, sperm cells from these transgenic mice stain blue, unlike those from normal mice. Thus, when acting as donors, the cells can be easily distinguished from host cells, serving as a marker for analysis.

Co-insertion of both a gene of interest and a marker gene into stem cells to be used as donors may be carried out in accordance with the following illustration. The Zfy-1/beta-galactosidase construct described above is mixed in solution with a genetic construct of interest to either co-inject into eggs to produce transgenic mice to provide eventual donor cells or transfect into tissue culture cells to be used as donors. A proportion of the resulting animals or cell lines will contain both constructs, providing donor cells with an easily-detectible marker as well as the gene of interest.

Since colonization usually only takes place in some tubules, low numbers of spermatozoa can result. However, any male in which some transferred primitive cells have developed into mature spermatozoa is useful in accordance with the invention since a variety of conventional techniques exist to achieve fertilization in animals with low sperm numbers. These techniques include, but are not limited to: hormonal treatment, abstinence, artificial insemination, *in vitro* fertilization, zona drilling, and microintroduction of sperm into the egg.

The experiments described below, which are provided for purposes of non-limiting illustration of some embodiments of the invention, demonstrate the survival, differentiation and

development to later stages of spermatogenesis of cells transferred in accordance with the invention and show the cross-strain utility of the invention, and thereby provides strong evidence of the cross-species potential of the present invention.

Introduction:

Foreign potential stem cells for transfer were isolated from the testes of very young male mice, usually between 3 to 10 days of age. These cells carried a reporter or marker gene encoding the *E. coli* ß-galactosidase (lacZ) gene. This gene is not normally present in the mouse genome. However, if a cell contains the gene, it will make the enzyme ß-galactosidase. In the presence of the reagent X-gal, the cell will then stain blue. In addition, the transgene in these cells will only be active in late stages of spermatogenesis, in the round spermatid and later stages, because the lacZ structural gene is under control of a promoter or activating DNA sequence designated ZF. These late stages of spermatogenesis are not present in the neonatal testes. Therefore the transgene is not active in neonatal testes and these cells cannot be stained blue nor do any of the cells have the appearance of mature spermatozoa at this early age. As a result of this experimental procedure, the transferred cells must not only survive, but they must undergo differentiation and development to become late stages of spermatogenesis in order to stain blue. Furthermore, the transferred cell descendants can easily be distinguished from any endogenous sperm cells of the recipient host mouse (should any be present), because these endogenous cells will not stain blue.

An example of a cross section of a seminiferous tubule from the testis of an adult control mouse not containing the transgene and treated with X-gal is shown in Figure 1. There are no cells that show blue or dark staining. In contrast, a cross section of a seminiferous tubule from the testis of an adult mouse containing the lacZ transgene and treated with X-gal is shown in Figure 2. There is intense blue (dark) staining in all the mature stages of spermatogenesis, which occupy the center of the tubule. Figure 1 and 2 represent examples of a negative and positive control, respectively. They are referred to below in the experimental descriptions.

The cells for transfer were obtained from neonatal mice (3 to 10 days old) at a time before they would show any stain because of their immature stage of differentiation. These cells are reporter cells and carry the transgene ZF-lacZ; they are obtained from the testes of hybrid mice that carry antigenic determinants from C57BL/6 and SJL mice. The cells were then injected into the seminiferous tubules of recipient or host mice at several sites on the testicular surface.

The recipient mice were of several types as follows:

1. Hybrid mice of C57BL/6 x SJL parent stock. These mice were immunologically tolerant of the donor cells and should not reject them. The host mice were prepared by treatment with Busulfan® to destroy their endogenous sperm cells.

2. Mice that carry the W-mutation. Homozygotes and compound heterozygotes for certain alleles of this gene are completely devoid of sperm cells in the adult. Therefore, any sperm cells in their seminiferous tubules must come from the

differentiation of transferred cells. Furthermore, these mice are immunologically incompatible with the donor cell strain of mice because the W-mice are of C57 background and would not be tolerant of the SJL antigens on the surface of cells containing the ZF-lacZ transgene.

3. Inbred mice of the 129/SV strain. These mice are immunologically incompatible with the donor cells because the cells contain C57BL/6 and SJL antigens and 129/SV mice would recognize both strains as foreign. Foreign cells would be rejected and destroyed by a mouse if present in a normal environment, such as a skin or organ graft. However, some parts of the testis, particularly the inner region of the seminiferous tubules, is considered to have a degree of immunological privilege (i.e. They do not reject foreign tissue as readily as other body locations. The uterus is the best example of an immunologically privileged location, since it does not reject the fetus carrying the male antigens).

The implementation and use of the invention has substantiated in the following examples, and the results obtained provided proof of several important aspects of the invention. The examples describing implementation of the invention are as follows.

Experimental:

1. Primitive stem cells or spermatogonia can be transferred from one animal into another host animal and the donor cells will survive, divide and differentiate. Cells were taken from neonatal testes of hybrid mice (C57BL/6 x SJL) containing the ZF-lacZ transgene, and these cells were microinjected into the seminiferous tubules of Busulfan®

treated hybrid (C57BL/6 x SJL) mice. The host testes were removed at various times (days to months) following the injection and examined for the presence of cells from the donor animal by analyzing the testes for the presence of lacZ staining. A number of animals were found that clearly demonstrated the success of the transfer as well as survival, division and differentiation of the donor cells. In Figure 3 is shown a cross section from one of these testes, and it is clear that a number of tubules are stained blue (dark). This is the same type staining seen in Figure 2 and, therefore, represents descendants of donor cells rather than regeneration of endogenous cells, which would not stain but would look like Figure 1. Furthermore, mature spermatozoa are seen in some tubules. Figure 4 is a higher magnification of one of the tubules from Figure 3. The mature spermatozoa are clearly seen as thin dark nuclei toward the center of the tubule (arrow). The blue stain (dark) is very obvious at this magnification. Survival, division and differentiation of donor cells must take place to achieve this result. Donor cells will not stain because they are immature; endogenous cells (should any regenerate) will not stain because they lack the transgene; and no mature spermatozoa were present among the donor cells. A few transferred stem cells divided and differentiated to fill this seminiferous tubule in the host animal.

2. In the second implementation of the invention, the same type cells described above, from hybrid mice and carrying the ZF-lacZ transgene, were transferred into the seminiferous tubules of W-mutant mice. These mice are devoid of endogenous sperm cells. The success of the procedure is demonstrated in Figure 5 which shows a cross section of a tubule that received donor cells. There is blue staining of cells in this cross section demonstrating their derivation from donor cells.

Furthermore, this mutant animal is incapable of generating sperm cells. In this embodiment of the invention the donor cells have colonized a completely sterile testis in an animal that is not immunologically tolerant of the donor mouse strain. The testis environment has provided an immunological protection as anticipated in the invention application. The examination of this testis was performed 120 days after donor cell transfer indicating that cell survival, division and differentiation continues in the host for a long period, probably until death.

3. In the third implementation of the invention, the same type donor cell was transferred into the seminiferous tubule of a 129/SV inbred mouse. The mouse had previously been treated with Busulfan® to destroy endogenous sperm cells. The result is shown in Figure 6. Again the donor cells (stained blue or dark) have colonized this tubule. The differentiation has not proceeded as far as in the examples above; perhaps, because the transfers were among the first performed while improvements were still underway. However, this example demonstrates tolerance of foreign cells in the tubule despite very strong immunological differences. The examination of this testis was performed 110 days after donor cell transfer, indicating a very long period of donor cell survival.

* * * * *

Having now fully described the invention, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth herein.

WHAT IS CLAIMED AS NEW AND DESIRED TO BE SECURED BY LETTERS PATENT OF THE UNITED STATES IS:

1. A non-human animal harboring, in its native seminiferous tubules, a biologically functional non-native germ cell.

2. The animal of Claim 1 wherein said animal is a mammal.

3. The animal of Claim 1 wherein said animal is a rodent.

4. The animal of Claim 1 wherein said animal is an agricultural animal.

5. An animal line obtained by breeding the animal of Claim 1 or progeny thereof.

6. The animal line of Claim 5 wherein said animal is a mammal.

7. A method for making a germ cell, comprising repopulating the testis of an animal with primitive cells which are not native to said animal.

8. The method of Claim 7, wherein said repopulating comprises:

(i) destroying the endogenous germ cell population in the seminiferous tubules of said animal, leaving supporting cells in said tubules biologically functional; and

(ii) colonizing said tubules with said primitive cells.

9. The method of Claim 8, wherein said step (i) comprises subjecting said tubules to a physical treatment.

10. The method of Claim 9, wherein said physical treatment is radiation treatment.

11. The method of Claim 8, wherein said step (i) comprises subjecting said tubules to a chemical treatment.

12. The method of Claim 11, wherein said chemical treatment is treatment with Busulfan®.

13. The method of Claim 8, wherein said supporting cells comprise Sertoli cells.

14. The method of Claim 7, wherein said primitive cells are totipotent stem cells.

15. The method of Claim 8, wherein said step (ii) comprises injecting a solution containing said primitive cells into said tubules or another part of the tubular system of said testes.

16. The method of Claim 15, wherein said animal is a farm animal.

17. A method for obtaining a germ cell comprising raising or breeding an animal of Claim 1 and collecting at least some of its germ cells.

18. A germ cell obtained from the animal of Claim 1.

19. Spermatozoa produced by a method comprising:

(i) repopulating the seminiferous tubules of an animal with primitive cells which are not native to said animal; and either

(iia) raising said animal, and

(iib) collecting at least some of the spermatozoa of said animal; or

(iiia) breeding said animal to obtain progeny, and

(iiib) collecting at least some of the spermatozoa of said progeny.

20. A method for making an animal harboring a biologically functional non-native germ cell comprising repopulating the seminiferous tubules of the testis of an animal with primitive cells which are not native to said animal.

21. The method of Claim 20 in which said seminiferous tubules contain no biologically functional endogenous germ cells.

22. A method of obtaining an animal line comprising breeding the animal harboring a non-native germ cell obtained in Claim 21.

23. The animal of Claim 2 wherein said mammal and said germ cell are of the same species.

24. The animal of Claim 2 wherein said mammal and said germ cell are of different mammalian species.

25. The animal of Claim 4 wherein said germ cells originate from a stud animal.

26. A method for the gene therapy of an animal in need thereof, comprising the steps of:

(i) destroying the endogenous germ cell population in the seminiferous tubules of said animal, leaving supporting cells in said tubules biologically functional; and

(ii) colonizing said tubules with stem cells free of the deleterious genetic trait(s) creating said need.

27. The method of Claim 26 wherein said animal is a human.

28. The method of Claim 26 wherein said animal is an agricultural animal.

29. A non-human animal harboring in its seminiferous tubules a biologically functional non-native germ cell, obtained by a process comprising the steps of:

(i) destroying the endogenous germ cell population in the seminiferous tubules of said animal, leaving supporting cells in said tubules biologically functional; and

(ii) colonizing said tubules with primitive cells which are non-native to said animal.

30. A mammal according to Claim 29.

31. A rodent according to Claim 29.

32. An agricultural animal according to Claim 29.

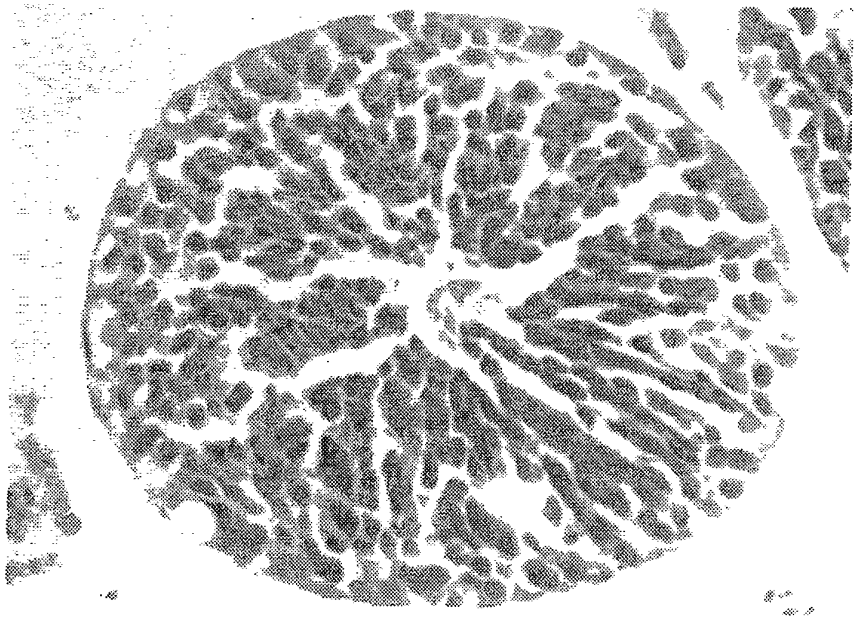

FIG. 1

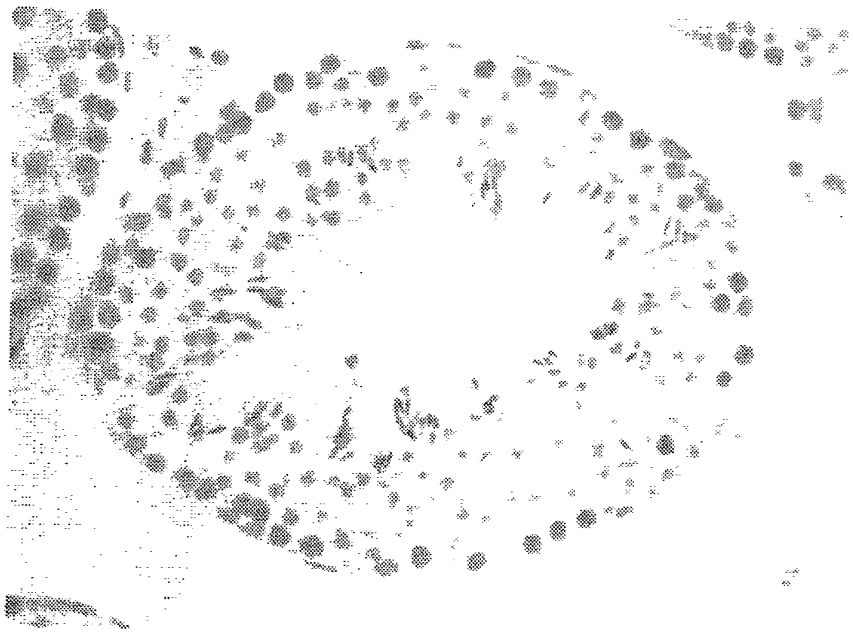

FIG. 2

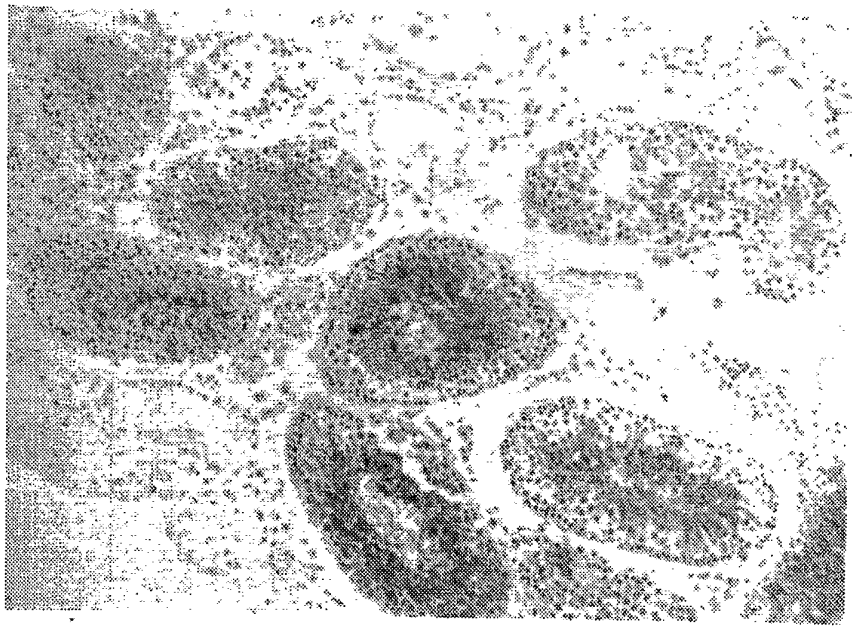

FIG. 3

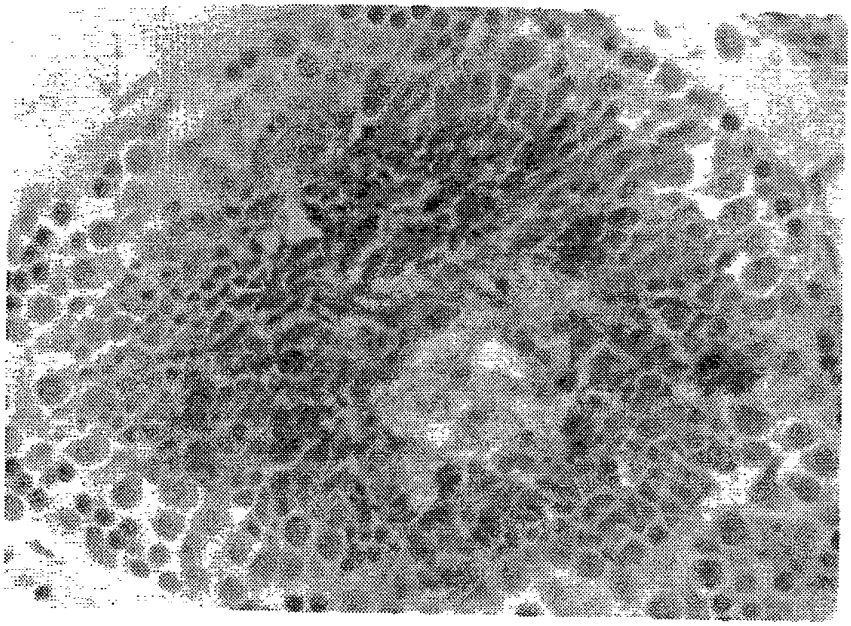

FIG. 4

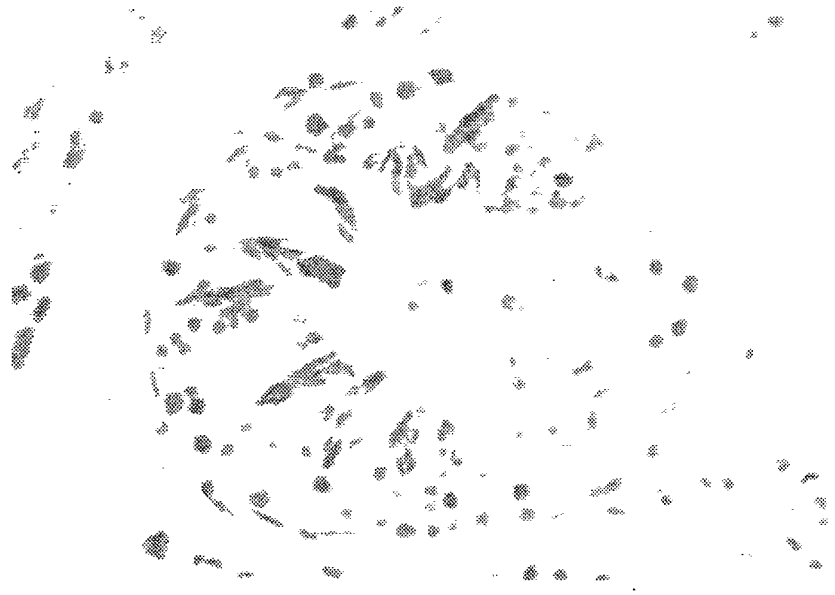

FIG. 5

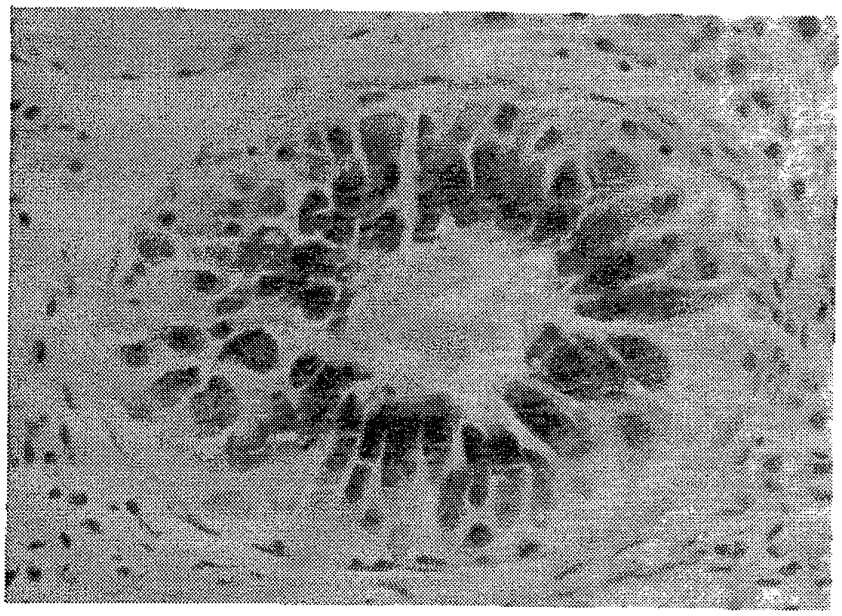

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/10368

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/00
US CL :800/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : NONE

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, transplantation germ cells

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Journal of Cellular Physiology, volume 139, issued 1989, Kuroda et al., "Differentiation of germ cells in seminiferous tubules transplanted to testes of germ cell-deficient mice of w/w and si/si genotypes", pages 329-334, see entire article.	1-3, 17-19 20-32
Y	Mutation Research, volume 249, issues 1991, Ehling et al., "Induction of specific locus and dominant lethal mutations in male mice by busulfan", pages 285-292, see entire article.	7-16, 19
Y	Mutation Research, volume 249, issued 1991, Generoso et al., "Comparison of two stocks of mice in spermatogonial response to different conditions of radiation exposure", pages 301-310, see entire article.	7-16,19
Y	Science, volume 244, issued 16 June 1989, Capecchi, "Altering the genome by homologous recombination", pages 1288-1292, see entire article.	1,4-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 07 JANUARY 1993	Date of mailing of the international search report 04 MAR 1993
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