



(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention
of the grant of the patent:
08.12.1999 Bulletin 1999/49

(51) Int Cl.⁶: **C12N 15/00, A01K 67/027,
C12N 5/10, C07K 14/00,
C12N 15/12, C12N 15/85**

(21) Application number: **94913174.2**

(86) International application number:
PCT/GB94/00848

(22) Date of filing: **21.04.1994**

(87) International publication number:
WO 94/24274 (27.10.1994 Gazette 1994/24)

(54) **ISOLATION, SELECTION AND PROPAGATION OF ANIMAL TRANSGENIC STEM CELLS**

ISOLIERUNG, SELEKTION UND VERMEHRUNG VON TIERISCHEN TRAUSGEN-STAMMZELLEN

ISOLATION, SELECTION ET PROPAGATION DE CELLULES SOUCHES D'ANIMAUX
TRANSGENIQUES

(84) Designated Contracting States:
**AT BE CH DE DK ES FR GB GR IE IT LI LU MC NL
PT SE**

• **MOUNTFORD, Peter, Scott Stem Cell Sciences
Melbourne, VIC 3004 (GB)**

(30) Priority: **21.04.1993 GB 9308271**

(74) Representative: **Schlich, George William et al
Mathys & Squire
100 Gray's Inn Road
London WC1X 8AL (GB)**

(43) Date of publication of application:
07.02.1996 Bulletin 1996/06

(73) Proprietor: **UNIVERSITY OF EDINBURGH
South Bridge, Edinburgh EH8 9YL (GB)**

(56) References cited:
**EP-A- 0 235 113 WO-A-90/01541
WO-A-91/01140 WO-A-92/11355**

(72) Inventors:
• **SMITH, Austin, Gerard
AFRC Centre Genome Research
West Mains Road Edinburgh EH9 3JQ (GB)**

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Description

[0001] This invention relates to methods of isolating and/or enriching and/or selectively propagating animal stem cells, genetically modified animal cells and animals for use in said method, transgenic animals providing a source of such cells and selectable marker constructs for producing genetically modified cells and transgenic animals.

[0002] Stem cells are progenitor cells which have the capacity both to self-renew and to differentiate into mature somatic tissues.

[0003] Embryonic stem cells are the archetypal stem cell, being capable of differentiating to form the whole gamut of cell types found in the adult animal. Such stem cells are described as pluripotent as they are capable of differentiating into many cell types. Other types of stem cells, for example bone marrow stem cells and epidermal stem cells, persist in the adult animal. These stem cells have a more restricted capacity for differentiation.

[0004] In general, when required for research purposes or for medical use, stem cells have to be isolated from tissue samples by various fractionation procedures, but even after careful segregation of cell types, these stem cell preparations consist of mixed cell types and while enriched for stem cells, include high proportions of differentiated cells which are not categorised as stem cells.

[0005] Furthermore, most stem cells cannot be grown readily in culture and when attempts are made to culture stem cells, the cells being cultured (which ordinarily contain a mixed population of cell types) grow at different rates and stem cells rapidly become overgrown by non-stem cell types. An exception is that embryonic stem cells from two specific strains of mice (129 and Black 6) can be cultured *in vitro*. Thus established lines of embryonic stem cells can be obtained by culturing early (3½ day) embryonic cells from murine strain 129 and Black 6, or hybrids thereof.

[0006] WO-A-92/11355 describes a method for culturing and transforming human stem cell-containing compositions. EP-A-0235113 describes immortalization of primary cells using oncogenes. WO-A-90/01541 describes use of leukaemia inhibitory factor in maintenance of embryonic stem cells. WO-A-91/01140 describes a mammalian cell lacking a major histocompatibility complex antigen for use in generation of chimaeras. None of this art, however, describes selective culture of stem cells.

[0007] There has developed a pressing need to isolate and maintain *in vitro* embryonic stem cells from other murine strains and more especially from other species including other laboratory animals (e.g. rats, rabbits and guinea pigs), domesticated animals (e.g. sheep, goats, horses, cattle, pigs, birds, fish, etc.) and primates. Similarly, numerous medical applications for other pluripotent cells such as haematopoietic stem cells also demand their isolation and culture *in vitro*.

[0008] However hitherto the problems associated with producing cultures of stem cells including the problem of producing cell populations of a satisfactorily low degree of heterogeneity and the problem of overgrowth in culture of non-pluripotent cells have not been solved. A particular problem associated with the continuing presence of certain differentiated cell types is that these can cause elimination of stem cells from the culture by inducing their differentiation or programmed cell death.

[0009] We have now developed a technique by which the aforementioned problems can be overcome.

[0010] According to one aspect of the invention there is provided a method of isolating and/or enriching and/or selectively propagating desired animal stem cells, which comprises maintaining a source of said cells under culture conditions conducive to cell survival, characterised in that the source of cells includes stem cells containing a selectable marker which is capable of differential expression in (a) desired stem cells of said source and (b) cells of said source other than the desired stem cells, whereby differential expression of said selectable marker results in preferential isolation and/or survival and/or division of the desired stem cells containing the said selectable marker.

[0011] In the context of this invention, the term "animal cell" is intended to embrace all animal cells, especially of mammalian species, including human cells.

[0012] Examples of stem cells include both unipotent and pluripotent stem cells, embryonic stem cells, gonadal stem cells, somatic stem/progenitor cells, haematopoietic stem cells, epidermal stem cells and neuronal stem cells.

[0013] In carrying out the method of the invention, the source of cells may include pluripotent cells having a positive selectable marker and expression of the said marker is used to permit isolation and maintenance of the pluripotent cells. Alternatively, the source of cells may include a negative selectable marker which is expressed in cells other than the desired pluripotent cells and is used selectively to deplete the source of cells of cells other than the desired pluripotent cells.

[0014] The selectable marker may, for example, be a foreign gene, a cellular gene or an antibiotic resistance gene such as for example the bacterial neomycin resistance gene.

[0015] Alternatively the selectable marker may be a growth stimulating gene, for example an immortalising gene, an oncogene or a gene coding for the polyoma or SV40 T antigens or derivatives thereof, or the selectable marker may be a gene coding for a growth factor or a growth factor receptor or a signal transducing molecule or a molecule that blocks cell death.

[0016] In one particular embodiment the isolation and/or enrichment and/or selective propagation of the desired

pluripotential cells is dependent on the presence of cells other than the desired pluripotential cells and the simultaneous maintenance of both cell types is dependent on expression of a selectable marker, in one or the other cell population, which is capable of rescuing cells that do not express the marker but which neighbour cells which do themselves express the marker. In this instance, the selectable marker may, for example, be the hypoxanthine phosphoribosyl transferase (HPRT) gene.

[0017] In another embodiment the selectable marker may be a gene encoding a product which is toxic per se, or a toxic gene product which is conditionally active in combination with a suicide substrate. An example of such a gene product is a herpes simplex virus thymidine kinase (HSV-TK) in combination with ganciclovir.

[0018] Expression of the selectable marker may be achieved by operatively inserting the selectable marker into an expression construct prior to introduction to the cell source, in which case expression of the selectable marker can result from the introduction of either a stable or transiently integrated construct. Alternatively, expression of the selectable marker results from operatively inserting the selectable marker into an endogenous gene of the cell source.

[0019] Various means of introducing the selectable marker may be employed, including introduction into the cells by transfection, lipofection, injection, ballistic missile, viral vector or by electroporation.

[0020] The source of the cells may be a single cell such as a fertilized oocyte, or it may comprise a mixture of cells, such as cells derived from an embryo, blood or somatic tissue of a normally bred or transgenic animal or cell line. In the latter case the selectable marker may be incorporated into the transgenic animal's genome.

[0021] Most preferably, in carrying out the method of the invention a gene or gene fragment operatively linked to and regulating expression of the selectable marker is/are associated with a pluripotential stage of cellular development. Such a gene or gene fragment may be active in pluripotential cells of the developing embryo, especially in the inner cell mass and/or primitive ectoderm, or may be active in adult stem cells.

[0022] In preparing a source of cells for use in accordance with the invention one of the following protocols may advantageously be adopted:

- introducing into a source of cells containing stem cells, a selectable marker construct, wherein said selectable marker construct is adapted to operatively link to an endogenous gene which provides said differential expression, or
- introducing into a source of cells containing stem cells, a selectable marker construct, wherein said selectable marker construct has been previously linked to one or more gene fragments which provide said differential expression.

[0023] The genetic marker preferably comprises a selectable marker operatively linked to a promoter which is differentially active in the desired pluripotent cells (e.g. primitive ectoderm). By "selectable marker" is meant a selectable gene which may be a foreign gene or a cellular gene which is not naturally expressed, or such a gene which is naturally expressed, but at an inappropriate level, in the target cell populations. This gene in use acts as a selection marker by adapting the phenotype of the target cell population in such a way that cells with the so-adapted phenotype may be enriched or depleted under particular culture conditions.

[0024] In the case where stem cells are embryonic cells it is preferred that the selectable marker is operatively linked to a promoter which is differentially active in stem (e.g. primitive ectoderm, primordial germ cells) and non-stem cells. Promoter and other cis-regulatory elements may be included in the expression construct prior to introduction into the cells or by targeting promoter-less constructs into specific genes by site specific recombination.

[0025] A wide variety of gene products may be relied upon for selective isolation and propagation of the desired stem cells, including markers which are designed to protect the desired cells from the effects of an inhibiting factor present in the culture medium. In this instance, the inhibiting factor can, for example, be an antibiotic substance which inhibits growth or reproduction of cultured cells, not expressing the gene (i.e. cells other than the desired cells). The selectable marker (e.g. HPRT) may also provide protection both for the desired cells in which it is expressed as well as other closely associated cells by means of metabolic rescue.

[0026] Alternatively the selectable marker may selectively permit the growth of stem cells. In this instance the marker may encode a growth factor, a growth factor receptor, a transcription factor, an immortalising or an oncogenic product (e.g. temperature sensitive simian virus 40 T antigen).

[0027] Alternatively, the selectable marker may be a cell surface antigen or other gene product which allows purification or depletion of expressing cells for example by panning or fluorescence-activated cell sorting (FACS). The invention thus enables stem cell populations to be obtained/maintained having a satisfactory degree of homology.

[0028] Alternatively the selectable marker may be a conditionally toxic gene for instance herpes simplex virus thymidine kinase [HSV-TK]. In this instance expression of the selectable marker is directed to cells other than the desired cells and not to stem cells. Cells other than the desired phenotype may be selectively depleted by addition of a lethal substrate (e.g. ganciclovir).

[0029] The genetic marker may be introduced into the source of cells by a variety of means, including injection, transfection, lipofection, electroporation or by infection with a viral vector.

[0030] Further, the source of cells may be produced by transfection extemporaneously, or the source of cells may be derived from a transgenic animal, e.g., the founder transgenic animal or an animal at least one ancestor of which has had the aforementioned genetic marker introduced into its genetic complement. In such transgenic animals it is possible for the marker to pass down the germ line and eventually results in the production of progeny, from the tissues of which (especially from the embryonic tissue) the required source of cells can be derived.

[0031] Thus according to further aspects of the invention, there is provided an animal cell capable of being cultured to form a mixture of cells including desired stem cells and cells other than the desired stem cells, characterised in that all cells in the said mixture of cells contain a selectable marker and in that in the said mixture of cells, under appropriate selective culture conditions, differential expression of the selectable marker in (a) the desired stem cells and (b) cells other than the desired stem cells enables selective survival or growth of the desired stem cells to occur, so as to enable isolation and/or enrichment and/or propagation of desired stem cells.

[0032] The invention further provides an animal cell capable of being cultured under selective culture conditions so as to grow as stem cells, characterised in that said cells contain stem cells containing a genetic marker, whereby a gene product associated with the genetic marker is produced and which under said culture conditions causes selective survival and/or division of the desired stem cells to occur.

[0033] The animal cells according to this aspect of invention are preferably characterised by possessing the preferred characteristics described above.

[0034] The invention also provides a method of preparing a transgenic animal comprising obtaining a desired stem cell according to the method of the invention, excising the selectable marker from the desired stem cell and generating the transgenic animal therefrom.

[0035] The invention further provides a method of preparing a transgenic animal, said animal comprising a selectable marker capable of differential expression in (a) desired stem cells and (b) cells other than desired stem cells, the method comprising:

- providing a blastocyst;
- providing animal cells according to the invention;
- introducing the animal cells into the blastocyst;
- transferring the blastocyst to a recipient; and
- allowing an embryo to develop to a chimaeric animal to enable germline transmission of the selectable marker.

[0036] A transgenic animal obtained in this latter way may have genetic characteristics such that it or its progeny, during embryonic development or later life, constitute a source of animal pluripotential cells as defined above for use in other aspects of the invention.

[0037] Vectors for use in producing an animal cell defined above form a further aspect of the invention.

[0038] Thus the invention further provides vectors for use in genetically modifying animal cells so as to produce transformed cells suitable for use as the source of cells for the method referred to above, said vector comprising a first genetic component corresponding to said selectable marker and a second genetic component which in the genetically modified animal cells (1) results in the said differential expression of the selectable marker from either a transiently or stably integrated construct or (2) enables site-directed integration of the selectable marker into a specific gene so as to provide operative coupling of the selectable marker with targeted endogenous gene regulatory elements.

[0039] Such vectors may be in the form of expression vectors in which said second genetic component includes control sequences which are differentially activated (a) in stem cells and (b) in cells other than the desired stem cells.

[0040] The invention covers vectors which when used to transform animal cells become integrated into the animal genome as well as vectors which do not become so integrated.

[0041] The expression vectors referred to above may comprise a DNA sequence coding for the afore-mentioned selectable marker operatively linked to a genetic control element, or sequence enabling targeting of a promoterless marker to an endogenous gene which is expressed differentially in the said stem cells and in cells other than the desired stem cells.

[0042] For the generation of pluripotential embryonic stem cells the expression constructs preferably comprise a DNA sequence coding for said selectable marker operatively linked or targeting to a genetic control element(s) which is associated with a stage of embryonic development prior to differentiation of pluripotential embryonic cells. Most preferably the genetic control elements derive from a gene specifically active in the inner cell mass of the mouse blastocyst, in primitive ectoderm, and in primordial germ cells of the early embryo.

[0043] In more detail, the present invention has resulted in the development of expression constructs which direct specific expression of selectable markers in stem cells and not in differentiated cell types. Having introduced an expression construct by transfection or via the generation of transgenic animals, stem cells present within mixed cell

populations can be isolated by culturing in the presence of the selection agent *in vitro*, or by otherwise manipulating the culture conditions.

[0044] One example of a gene which displays a suitably restricted stem cell expression pattern and therefore may provide suitable "stem cell specific" regulatory elements for the expression of a selectable marker in accordance with the invention is the Oct4 gene.

[0045] Octamer binding transcription factor 4 is a member of the POU family of transcription factors (reviewed by Schöler, 1991). Oct4 transcription is activated between the 4- and 8-cell stage in the developing mouse embryo and it is highly expressed in the expanding blastocyst and then in the pluripotent cells of the egg cylinder. Transcription is down-regulated as the primitive ectoderm differentiates to form mesoderm (Schöler *et al.*, 1990) and by 8.5 d.p.c. (days post coitum) is restricted to migrating primordial germ cells. High level Oct4 gene expression is also observed in pluripotent embryo carcinoma and embryonic stem cell lines, and is down-regulated when these cells are induced to differentiate (Schöler *et al.*, 1989; Okamoto *et al.*, 1990).

[0046] Selectable marker genes under the control of the Oct4 promoter may, according to the invention, be applied to the isolation of embryonic stem cell lineages. Furthermore, reports describing low level Oct4 expression in some adult tissues (Takeda *et al.*, 1992) may extend the utility of these expression constructs beyond embryonic stem cells to include other stem cells essential to tissue homeostasis and repair in other systems including the haematopoietic system. In the event that Oct4 is not expressed in somatic stem cells, other transcriptional regulatory elements, such as those associated with the haematopoietic stem cell specific antigen CD34, may be utilised in a similar manner.

[0047] Two specific approaches are provided according to the invention for generating the desired spatial and temporal restrictions in transgenic expression. The first approach is through the generation of transgenic animals in which a partially characterised Oct4 gene promoter fragment (Okazawa *et al.*, 1991) is employed to drive stem cell specific transcription of the selectable marker. An appropriate selectable marker is the neomycin phosphotransferase gene which confers resistance to the antibiotic G418. An alternative is to utilise a selectable marker which is associated with the production of a gene product which can counteract a deficiency in a metabolite, e.g. the hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene in HPRT-deficient cells (Hooper *et al.*, 1987). This approach may be advantageous in situations where stem cells require continuous support from closely associated differentiated cells. In this instance direct cell contact will permit metabolic rescue of the neighbouring support cells by the stem cells despite the lack of selectable marker gene expression in the support cells.

[0048] The second approach utilises the endogenous Oct4 gene locus, and therefore the associated Oct4 gene regulatory elements, to link resistance marker gene expression as closely as possible with the endogenous Oct4 gene expression profile. This may be accomplished by high efficiency gene trap targeted mutagenesis of the Oct4 gene in embryonic stem cells. This approach provides more tightly regulated control of selectable marker gene expression by avoiding random integration site effects which often result in unpredictable expression patterns of randomly integrated constructs.

[0049] The invention will now be described in more detail in the following Example, with particular reference to the accompanying drawings of which Figure 1 illustrates the structure of plasmid Oct-1-Neo- β S, Figure 2 illustrates the structure of plasmid Oct-4-Neo- β fos and Figure 3 illustrates the structure of the plasmid Oct4-tgvec.

EXAMPLE 1

1. Isolation of OCT4 Promoter Sequences:

[0050] We screened a strain 129 mouse genomic lambda library with a 330 bp 5'Oct4 cDNA fragment. Several clones were isolated and screened by restriction analysis and Southern blot hybridization. A 1.4 kb Bam HI-Hind III fragment containing the Oct4 promoter element (Okazawa *et al.*, 1991) was isolated from clone 1 and ligated into pBluescript II KS(-) (Stratagene) to generate pOct4 (5' genomic).

2. Construction of Plasmids:

[0051] To generate the Oct4-Neo promoter constructs an engineered Neomycin resistance gene (neo), designed to provide an Nco I restriction site at the translation initiation codon, was isolated from pLZIN (Ghattas *et al.*, 1991) as a 1.1 kb Xba I-Sph I fragment encompassing encephalomyocarditis virus internal ribosome entry site sequence (EMCV-IRES, Ghattas *et al.*, 1991) and 5'-Neo coding sequences and cloned into pSP72 (Promega Biotech). The Kpn I-Nco I EMCV-IRES sequence was replaced with a 1.3 kb Oct4 promoter fragment isolated from pOct4 (5'genomic) by Kpn I and subsequent partial Nco I restriction digest. Neo3'-coding, rabbit β -globin gene (intron) and SV40 polyadenylation sequences were isolated as a 1.7 kb Sph I fragment from 6P-IRESNeo- β S and ligated into the Sph I site to generate Oct4-Neo- β S (Figure 1). To generate the Oct4-Neo- β fos construct (Figure 2), an Oct4-Neo- β S Bam HI fragment incorporating the Oct4 promoter, neo gene and the rabbit β -globin intron was inserted 5' to a human c-fos genomic sequence.

EP 0 695 351 B1

This 1.7 kb genomic sequence (Bal I-Sph I) encodes human c-fos mRNA 3' coding and non-coding sequences previously associated with mRNA destabilization (Triesman, 1985), and, the c-fos polyadenylation sequence.

[0052] The Oct4-neo construct (Oct4-tgtvec) is designed for targetted integration into the Oct4 gene (Figure 3). The Oct4 targetting construct contains 1.7kb of 5'Oct4 gene sequence and 4.2kb of 3'Oct4 gene sequence. Following homologous recombination this construct incorporates a lacZ-neomycin fusion gene (β geo, encoding a bifunctional protein, Freidrich and Soriano, 1991) into the first intron of the Oct4 gene. Splicing from the splice donor sequence of the first exon-intron boundary to the integrated IRES- β geo sequence is facilitated by the inclusion of a murine engrailed-2 splice acceptor sequence (Skarnes et al., 1992) immediately 5' to the IRES- β geo sequence. Translation of the β geo cistron of the Oct4- β geo fusion transcript is facilitated by the inclusion of the EMCV-IRES immediately 5' to the β geo coding sequence.

3. ES cell transfection and colony selection:

[0053] Mouse 129 ES cells (line CGR-8) were prepared and maintained in the presence of Differentiation Inhibiting Activity (DIA) or Leukemia Inhibitory Factor (LIF) as described by Smith (1991). Plasmid DNA for transfection was linearised by Sal I digest, ethanol precipitated and resuspended at 10-14 mg/ml in PBS. Following 10 hours culture in fresh medium, near confluent ES cells were dispersed by trypsinisation, washed sequentially in culture medium and PBS, and resuspended at 1.4×10^8 /ml in PBS for immediate transfection. Routinely, 0.7ml of cell suspension was mixed with 0.1 ml DNA containing solution and electroporated at 0.8 kV and 3.0 μ FD using a Biorad Gene Pulser and 0.4 cm cuvettes. Transfections were plated on gelatinised tissue culture dishes at $5-8 \times 10^4$ /cm² in growth medium for 16 hours prior to the addition of selection medium containing 200 μ g/ml (active) G418 (Sigma). Single colonies were picked 8-10 days post-transfection and transferred in duplicate into 24 well tissue culture plates for further expansion in growth medium containing 200 μ g/ml G418.

[0054] Clonal cell lines bearing the Oct4-Neo- β S and Oct4-Neo- β fos constructs (referred to as Oct4-Neo cell lines) were grown for two days, washed twice with PBS and the medium replaced with fresh G418 medium with or without DIA. Cell lines which grew normally in the presence of DIA but did not survive in the absence of DIA were selected for expansion and further analysis.

[0055] Clonal cell lines bearing the Oct4-tgtvec targetting construct (referred to as Oct4-targetted cell lines) were expanded in duplicated 24 well plates. Once confluent, one series of cells were frozen for storage while the remainder were analysed by Southern analysis.

4. Further characterisation of Oct4-Neo and Oct4-targetted cell lines:

[0056] Selected Oct4-Neo cell lines were assayed for ES cell growth and differentiation in DIA supplemented or non-supplemented medium at various G418 concentrations. Cells were plated at 1×10^4 /cm² in 12 well tissue culture plates in the various media preparations and cultured for 6 days. Fresh medium was applied every 2 days until day 6 when cells were fixed and stained as previously described (Smith, 1991.) Oct4-targetted cell lines positive by genomic Southern analysis were analysed by lacZ staining and growth and differentiation in DIA supplemented or non-supplemented medium in 200 μ g/ml G418.

5. Production of embryoid bodies from Oct4-Neo cell lines:

[0057] Embryoid bodies were generated by the hanging drop method (Hole and Smith, in press) and maintained in suspension culture for 2, 4, 6 or 8 days in the presence or absence of G418. Control embryoid bodies were generated from the parental cell line CGR-8 in the absence of G418. Embryoid bodies were then collected and transferred to gelatinised tissue culture dishes to enable adherence and expansion of the aggregates for analysis of contributing cell types. All embryoid bodies were maintained for 4 days in the absence of DIA and G418 prior to inspection.

6. Production of chimeras from Oct4-Neo and Oct-4 targetted cell lines:

[0058] Selected Oct4-Neo cell lines were cultured in the absence of G418 for 7 days prior to embryo injection as previously described (Nichols et al., 1990). Briefly, blastocysts for injection were collected 4 d.p.c. from C57BL/6 donors, injected with 10-20 cells and allowed to re-expand in culture prior to transfer to the uteri of pseudopregnant recipients. Chimeras were identified by the presence of patches of sandy coat colour on the C57BL/6 background. Male chimeras were test bred for transmission of the Oct4-Neo transgene. Transgenic mice were then crossed onto different genetic backgrounds.

7. Results

[0059] The Oct4-Neo- β S construct generated approximately 50 colonies/106 cells transfected while the Oct4-Neo- β fos construct generated approximately 10 fold fewer colonies. Three clones were selected on the basis of their differential survival in medium containing G418 (200 μ g/ml) in the presence or absence of DIA. All three cell lines displayed apparently normal growth rates in DIA-supplemented G418 containing media and died when cultured in the absence of DIA in G418 medium. Cultures maintained in DIA supplemented G418 medium grew as essentially pure ES cells while cultures maintained in DIA supplemented medium in the absence of G418 grew as mixed cultures of ES cells and differentiated progeny closely resembling those of the parental CGR-8 line. Thus G418 selection eliminates differentiated cell types and allows propagation of pure stem cell populations. The three cell lines selected were designated Oct4-Neo- β S18, Oct4-Neo- β S21 and Oct4-Neo- β fos11. These cell lines have been introduced into host blastocysts and resulting chimaeras may be test bred. Similar results were obtained with ES clones targeted with the Oct4-tgvec construct. In addition, histochemical staining of these cultures for β -galactosidase activity confirmed that expression of β geo was restricted to undifferentiated stem cells (Mountford et al, 1994).

[0060] Embryoid bodies were generated from the Oct4-Neo cell line Oct4-Neo- β fos11 to examine the effect of G418 selection on mixed cell aggregates and to test the utility of the selection system in isolating ES cells from mixed cell populations. Embryoid bodies generated with both the experimental cell line (Oct4-Neo- β fos11) and the parental cell line (CGR-8) and cultured in the absence of G418 were composed almost entirely of differentiated cells with few if any ES like cells. In contrast, visual analysis of the expanded colonies revealed that the Oct4-Neo- β fos11 embryoid bodies cultured in the presence of G418 contained high proportions of ES cells. The feasibility of isolating stem cells from differentiating systems is thus confirmed.

8. Summary

[0061] ES cells capable of germ line transmission have previously been established from only 2 inbred strains of mice, 129 and C57BL/6. Combining the Oct4-neomycin selection scheme with established of ES cell isolation and propagation procedures (Evans and Kaufman, 1981; Martin, 1981; Nichols et al., 1990; Yoshida et al, 1994) should enable ES cell line derivation from previously non-productive mouse strains and other mammalian species in which Oct4 is differentially expressed.

[0062] Selection against non-stem cell phenotypes in mixed cell populations may be advantageous for several reasons. Firstly, selection against differentiated cells in mixed populations provides a method for extensive stem cell enrichment. Secondly, selective removal of differentiated cells prevents their overgrowth in the cultures. Thirdly, elimination of differentiated cells may enhance stem cell self-renewal due to the loss of differentiation inducing activity associated with differentiated cells.

EXAMPLE 2

RESCUE AND RECOVERY OF PLURIPOTENTIAL STEM CELLS FROM ES CELL EMBRYOID BODIES

40 **Methods**

1. Cell Culture

[0063] ES cells were routinely maintained in medium supplemented with Differentiation Inhibiting Activity (DIA) as described by Smith (1991). Embryoid bodies were formed by aggregation of ES cells in the absence of DIA. The aggregates were produced by plating dissociated ES cells in 10 μ l or 30 μ l drops of medium at a density of 100 cells/drop. Arrays of drops were plated on the lid of a 10cm tissue culture dishes using a multipipettor. This was then inverted over the base of the dish, which contained 10ml of water in order to maintain humidity, and the hanging drops were cultured at 37°C in a 7% CO₂ atmosphere.

2. Histology and β -Galactosidase Staining

[0064] Embryoid bodies were fixed in Bouin's solution and embedded in agar. Paraffin sections were then prepared by standard procedures and stained with haematoxylin and eosin. Alkaline phosphatase staining of embryoid body outgrowths was carried out using Sigma Kit 86-R. Histochemical staining for β -galactosidase was performed with Xgal as described (Beddington et al, 1986).

Results

3. Cell Lines and Selection System

5 [0065] Fos11 is a derivative of the ES cell line CGR8 which has been transfected with the Oct4neofos construct. Fos11 cells express low levels of G418 resistance under control of the Oct4 proximal promoter element, but differentiated progeny show no expression of the transgene and are therefore sensitive to G418. OKO160 and OKO8 are derivatives of the ES cell lines CGR8 and E14TG2a respectively in which an IRES- β geoA cassette has been introduced into one allele of the Oct4 gene by homologous recombination as described. OKO cell lines express high levels
10 of β geo in the undifferentiated state and therefore stain strongly with Xgal and are G418-resistant. Differentiated progeny lose expression of β geo and become negative for Xgal staining and sensitive to G418. In monolayer cultures, Fos11 and OKO cells are maintained as pure ES cell populations by culture in the presence of DIA and selection in G418. Under conditions which favour differentiation, however, such as low density and absence of DIA (Smith, 1991), G418 selection results in the complete elimination of these cultures over 3-5 days. Rb40 cells are a derivative of CGR8
15 which are constitutively resistant to G418 due to expression of neoR directed by the human β -actin promoter.

4. Formation of Embryoid Bodies in the Presence and Absence of Selection against Differentiated Cells

20 [0066] Production of embryoid bodies by the conventional procedure (Doetschman et al, 1986) of detachment of clumps of cells followed by aggregation in bulk suspension culture results in a mixed population of aggregates, heterogeneous in both size and differentiation status. In order to obtain more uniform and consistent development, embryoid bodies in the present study were formed by aggregation of defined numbers of cells in individual drops of culture medium (see Methods). After 48 hours in hanging drop culture, the aggregates were transferred en masse into suspension culture in the presence or absence of G418.

25 [0067] Under G418 selection against differentiated progeny aggregates still formed from both Fos11 cells and the OKO clones. Although some dead cells appeared around the periphery of the aggregates, the bodies themselves increased in size during the culture period. Samples were harvested periodically from the bulk cultures and processed for histological examination. After several days embryoid bodies formed in the absence of selection were mostly cystic and contained a variety of morphologically differentiated cell types. Undifferentiated cells were rarely apparent. By
30 contrast, aggregates maintained under selection showed no indications of cellular specialisation and the bodies appeared to consist of solid balls of undifferentiated cells. The great majority of cells in these undifferentiated aggregates appeared healthy and viable and there was no evidence of necrosis, although occasional pyknotic nuclei, suggestive of apoptosis, were seen. Embryoid bodies formed in G418 were noticeably smaller than their counterparts formed in the absence of selection, however. This can be attributed to a combination of the lack of cyst development and the
35 removal of differentiated cells.

5. Persistence of Pluripotential Stem Cells in Embryoid Bodies formed under Selection against Differentiated Cells.

40 [0068] The absence of any undifferentiated aggregates in control cultures implied that it was unlikely that the effect of G418 was due to selection of a subpopulation of non-differentiating aggregates. In order to exclude definitively this possibility, however, and also to facilitate quantitative determination of the effects of G418 selection, a modified protocol was used which allows assessment of the behaviour of individual aggregates. Cultures were initiated in 30 μ l hanging drops in the presence or absence of G418 and maintained in drop culture for 7-8 days. Embryoid bodies were then transferred individually to gelatin-coated 96-well tissue culture plates and the media diluted 6-fold with media lacking
45 G418. The stem cell maintenance factor DIA was added at this stage to allow expansion of any undifferentiated ES cells which were present. The cultures were allowed to attach and outgrow for 48 hours then fixed and stained for alkaline phosphatase or for β -galactosidase as appropriate.

[0069] The data summarized in Table 1 show that in the absence of any selection undifferentiated stem cells are almost completely eliminated from embryoid bodies within 7 days of suspension culture. Outgrowths contained a variety
50 of morphologically differentiated cell types, but areas of cells with ES cell morphology were not observed. In the OKO cells expression of β -galactosidase is coupled to the stem cell-specific transcription factor Oct4 (Mountford et al, 1994) and therefore serves as a marker of undifferentiated cells. Isolated Xgal-staining cells were occasionally seen in OKO outgrowths, but clusters of staining cells were not detected under these conditions (but see Discussion).

[0070] The efficiency of embryoid body formation in G418 was identical to that in non-selected cultures, essentially
55 100%. In marked contrast to the untreated embryoid bodies, however, embryoid bodies established under continuous G418 selection gave rise to outgrowths consisting largely of ES cells. The undifferentiated nature of these cells was indicated by the characteristic morphology of ES cell colonies and by staining with alkaline phosphatase and was confirmed by Xgal staining of the OKO outgrowths.

[0071] Several outgrowths from embryoid bodies formed under selection were picked and transferred to 2cm wells. All of the colonies picked were readily expanded into mass cultures of undifferentiated cells. These cultures remained dependent on DIA and differentiated in similar fashion to parental ES cells when plated in non-supplemented media. Furthermore, these derivatives differentiated efficiently into multiple cell types on aggregation, confirming their pluripotency.

[0072] These findings demonstrate that the selective elimination of differentiated progeny results in the persistence of pluripotential stem cells in ES cell aggregates.

6. Stem Cell Extinction in Mixed Aggregates

[0073] The implication that differentiated progeny may be directly responsible for stem cell extinction in embryoid bodies was addressed further. The behaviour of OKO cells was assessed following formation of mixed aggregates with Rb40 ES cells which can differentiate in the presence of G418. Rb40 cells express neomycin phosphotransferase constitutively and G418 selection has no discernible effect on their differentiation, either in monolayer culture or in aggregates. Hanging drop cultures were established using a 3:1 ratio of OKO cells to Rb40 cells. Paraffin sections of mixed embryoid bodies revealed that they underwent extensive differentiation in both the absence and the presence of G418. The effective elimination of undifferentiated stem cells under both conditions was confirmed by Xgal-staining of outgrowths (Table 1).

[0074] This result provides direct evidence that the presence of differentiated progeny induces the elimination of pluripotential stem cells. This implies that certain differentiated stem cell progeny are a source of inductive signals which either instruct further differentiation of remaining stem cells or possibly induce them to enter apoptosis.

Conclusion

[0075] Aggregation induces ES cells to develop into differentiated structures known as embryoid bodies. Pluripotential stem cells rapidly become extinct in these embryoid bodies due to the efficient induction of differentiation and possibly also to selective cell death. However, if differentiated progeny are specifically eliminated from the aggregates using methods according to the invention, the stem cells persist and can be propagated.

[0076] The findings detailed above constitute a clear demonstration that through the use of a stem cell-specific selection system according to the invention it is possible to recover stem cells from conditions which would normally force their elimination by either differentiation or death.

Table 1.

Disappearance or Persistence of Oct-4 Expressing ES Cells in Embryoid Bodies.					
Culture	G418*	No. Drops	No. Outgrowths	No. Xgal +ve	% +ve Drops
OKO8	-	25	25	0	0
OKO8	+	25	24	24	96
OKO160	-	30	30	0	0
OKO160	+	30	30	30	100
OKO160:Rb40	-	30	29	0	0
OKO160:Rb40	+	30	30	0	0

*500µg/ml

EXAMPLE 3

PROCEDURES FOR ESTABLISHING EMBRYONIC STEM CELL CULTURES FROM MOUSE EMBRYOS

[0077] Lines of transgenic mice were established in which the neomycin phosphotransferase gene conferring resistance to G418 is expressed with the specificity of the Oct4 gene. The βS21 line harbour the Oct4neoβS transgene whilst in the OKO line the neo gene has been incorporated into the endogenous Oct gene via gene targeting with the Oct4-tgtvec construct. These mice were outcrossed for two generations with MF1 outbred albino mice and with inbred CBA mice. Neither of these mouse strains produce ES cells using standard procedures.

[0078] Four preferred procedures for isolating stem cells are described. In all cases the embryos are cultured in standard ES cell culture medium supplemented with either Differentiation Inhibiting Activity (Smith, 1991) or interleukin-

EP 0 695 351 B1

6 plus soluble interleukin-6 receptor (Yoshida et al, 1994). G418 is added at concentrations of 200µg/ml - 1mg/ml.

Procedure 1

5 [0079] Blastocysts are flushed on the fourth day of pregnancy. Groups of 4-10 blastocysts are cultured in 1cm tissue culture wells under G418 selection. Outgrowths are individually detached and dissociated with trypsin as described (Nichols et al, 1990) after 4-6 days in culture and replated in single wells. G418 selection is maintained. Colonies with the characteristic morphology of ES cells which appear in the cultures over the next 14 days are picked and expanded under continuous selection.

10

Procedure 2

[0080] As Procedure 1, except that blastocysts are put into implantation delay before harvesting by ovariectomy of the dams on the third day of pregnancy. Blastocysts are flushed 4 days after the ovariectomy.

15

Procedure 3

[0081] Post-implantation embryos between 5.5 and 7.5 days post-coitum are isolated and the primitive ectoderm separated by microdissection and/or protease digestion. The primitive ectoderm is gently dissociated into clumps of 20-50 cells which are then cultured as in Procedure 1.

20

Procedure 4

[0082] Embryos prepared as for Procedures 1, 2 or 3 are cultured in hanging drops under G418 selection for a period of 5-7 days before transfer to tissue culture wells and subsequent manipulation as in Procedure 1.

25

References

[0083]

30

- Beddington et al, 1989 - Development, 1989, 106, pp 37-46
Doetschman et al, 1985 - J Embryol Exp Morph, 1985, 87, pp 27-45
Evans and Kaufman, 1981 - Nature, 1981, 292, pp 154-156
Friedrich and Soriano, 1991 - Genes Dev, 1991, 5, pp 1513-1523
35 Ghattas et al, 1991 - Molecular and Cellular Biology, 1991, 11, no. 12, pp 5848-5859
Hooper et al, 1987 - Nature, 1987, 326, pp 292-295
Martin, 1981 - Proc Natl Acad Sci, 1981, 78, pp 7634-7638
Mountford et al, 1994 - Proc Natl Acad Sci, 1994, 91, pp 4303-4307
Nicholls et al, 1990 - Development, 1990, vol. 110, pp 1341-1348
40 Okamoto et al, 1990 - Cell, 1990, 60, pp 461-472
Okazawa et al, 1991 - EMBO J, 1991, 10, pp 2997-3007
Scholer et al, 1991 - TIG, October 1991, Vol 7, no. 10, pp 323-329
Scholer et al, 1990 - Nature 1990, 344, pp 435-439
Scholer et al, 1989 - EMBO J, 1989, 8, pp 2551-2557
45 Skarnes et al, 1992 - Genes Dev, 1992, 6, pp 903-918
Smith, 1991 - J Tiss Cult Meth, 1991, 13, pp 89-94
Takeda et al, 1992 - Nucleic Acids Research, 1992, 20, no. 17, pp 4613-4620
Triesman 1985 - Cell, 1985, 42, pp 889-902
Yoshida et al, 1994 - Mech Dev, 1994, 45, pp 163-171

50

Claims

1. A method of isolating and/or enriching and/or selectively propagating desired animal stem cells, which comprises maintaining a source of said cells under culture conditions conducive to cell survival, characterised in that the source of cells includes stem cells containing a selectable marker which is capable of differential expression in (a) desired stem cells of said source and (b) cells of said source other than the desired stem cells, whereby differential expression of said selectable marker results in preferential isolation and/or survival and/or division of the desired

55

stem cells containing the said selectable marker.

2. A method according to Claim 1 wherein the desired stem cells are selected from unipotential stem cells, pluripotential stem cells, embryonic stem cells, gonadal stem cells, somatic stem/progenitor cells, haematopoietic stem cells, epidermal stem cells and neuronal stem cells.
3. A method according to any preceding claim wherein the source of cells includes stem cells having a positive selectable marker and expression of said marker is used to permit isolation and/or enrichment and/or maintenance of the stem cells.
4. A method according to any preceding claim wherein expression of a negative selectable marker in cells other than the desired stem cells is used to selectively deplete the source of cells of cells other than the desired stem cells.
5. A method according to any preceding claim wherein the selectable marker is selected from a foreign gene, a cellular gene and an antibiotic resistance gene.
6. A method according to Claim 5 wherein the antibiotic resistance gene is the bacterial neomycin resistance gene.
7. A method according to any of Claims 1 to 5 wherein the selectable marker is a growth stimulating gene.
8. A method according to Claim 7 wherein the growth stimulating gene is an oncogene or derivatives thereof.
9. A method according to Claim 7 wherein the growth stimulating gene is SV40 large T antigen or a derivative of SV40 large T antigen.
10. A method according to Claim 7 wherein the growth stimulating gene is selected from a gene that codes for a growth factor, a gene that codes for a growth factor receptor, a gene that codes for a signal transducing molecule, and a gene that codes for a transcription factor.
11. A method according to any of Claims 1 to 5 wherein the selectable marker is an immortalising gene.
12. A method according to Claim 11 wherein the immortalising gene is selected from the polyoma large T gene, a gene that blocks cell death and the bcl-2 gene.
13. A method according to any of Claims 1 to 5 wherein the isolation and/or enrichment and/or propagation of the desired pluripotential cells is dependent on the presence of cells other than the desired pluripotential cells and the simultaneous maintenance of both cell types is dependent on expression of a selectable marker, in one or the other cell population, which is capable of rescuing cells neighbouring those cells which do not themselves express the marker.
14. A method according to Claim 13 wherein the selectable marker is selected from HPRT, a gene encoding a toxic product, a toxic gene product which is conditionally active in combination with a suicide substrate and a herpes simplex virus thymidine kinase (HSV-TK) gene.
15. A method according to any preceding claim wherein the cells contain two selectable markers.
16. A method according to any preceding claim wherein expression of the selectable marker is achieved by operatively inserting the selectable marker into an expression construct prior to introduction to the cell source.
17. A method according to any preceding claim wherein expression of the selectable marker results from the introduction of a stably integrated, episomally maintained or transiently maintained construct.
18. A method according to any preceding claim wherein expression of the selectable marker results from operatively inserting the selectable marker into an endogenous gene of the cell source.
19. A method according to any preceding claim wherein the selectable marker is introduced into the cells by transfection, lipofection, injection, ballistic missile, viral vector, electroporation or any other means.

EP 0 695 351 B1

20. A method according to any preceding claim wherein the source of cells is obtained by culture of a single cell or cell line; cells derived from a fertilized oocyte, or a transgenic animal or a non-transgenic animal; cells derived from an embryo, from blood or from somatic tissue; or a mixture of any of the aforementioned cells.
- 5 21. A method according to Claim 20 wherein the source of cells is obtained by culture of a cell or cells obtained from a transgenic animal.
22. A method according to any preceding claim wherein the selectable marker is operatively linked to a gene or gene fragment regulating expression which gene or gene fragment is differentially active in stem and non-stem cells.
- 10 23. A method according to any preceding claim wherein a gene or gene fragment operatively linked to and regulating expression of the selectable marker is/are associated with a pluripotential stage of cellular development.
24. A method according to Claim 23 wherein the gene or gene fragment is active in pluripotential cells of the developing embryo.
- 15 25. A method according to Claim 23 or 24 wherein the gene or gene fragment is active in primitive ectoderm.
26. A method according to any of Claims 22-25 wherein the gene or gene fragment is all or part of the Oct4 gene.
- 20 27. A method according to any of claims 22-26 wherein the gene or gene fragment is the Oct4 promoter.
28. A method according to any preceding claim wherein the selectable marker is the neomycin phosphotransferase gene.
- 25 29. A method according to any of Claims 22-28 wherein the gene or gene fragment is active in pluripotent haematopoietic cells.
- 30 30. A method according to Claim 29 wherein the gene or gene fragment is all or part of the CD34 gene.
31. A method according to any preceding claim which includes the step of introducing into a source of cells containing stem cells, a selectable marker construct, wherein said selectable marker construct is adapted to operatively link to an endogenous gene which provides said differential expression.
- 35 32. A method according to any of Claims 1 to 30 which includes the step of introducing into a source of cells containing stem cells, a selectable marker construct, wherein said selectable marker construct has been previously linked to one or more genes or gene fragments which provide said differential expression.
- 40 33. A method of selectively isolating and/or enriching and/or propagating desired animal stem cells, which comprises introducing into a source of cells containing stem cells, a selectable marker construct which operatively links to or has been previously linked to genes or gene fragments which provide differential expression of the selectable marker in (a) desired stem cells of said source and (b) cells of said source other than the desired stem cells and which under appropriate culture conditions enables selective isolation and/or enrichment and/or propagation of the desired stem cells containing the selectable marker.
- 45 34. A method according to Claim 33 wherein the selectable marker is operatively linked to a gene or gene fragment regulating expression, which gene or gene fragment is differentially active in stem and non-stem cells.
- 50 35. A method according to Claim 34 wherein the gene or gene fragment is the Oct4 promoter.
36. A method according to any of Claims 33-35 wherein the selectable marker is the neomycin phosphotransferase gene.
- 55 37. An animal cell capable of being cultured to form a mixture of cells including desired stem cells and cells other than the desired stem cells, characterised in that all cells in the said mixture of cells contain a selectable marker and in that in the said mixture of cells, under appropriate selective culture conditions, differential expression of the selectable marker in (a) the desired stem cells and (b) cells other than the desired stem cells enables selective survival or growth of the desired stem cells to occur, so as to enable isolation and/or enrichment and/or propagation

of desired stem cells.

38. An animal cell as claimed in Claim 37 wherein the selectable marker is an antibiotic resistance gene.

5 39. A vector for use in genetically modifying cells so as to be suitable for use in a method according to any of Claims 1 to 36, said vector comprising a first genetic component corresponding to said selectable marker and a second genetic component which in the genetically modified animal cells directly or indirectly results in the said differential expression of the selectable marker.

10 40. A vector as claimed in Claim 39 in the form of an expression vector in which said second genetic component includes control sequences which are differentially activated in (a) desired stem cells and (b) cells other than the desired stem cells.

41. A vector as claimed in Claim 40 wherein the control sequence is the Oct4 promoter.

15

42. A vector according to Claim 41 wherein the selectable marker is an antibiotic marker.

43. A vector according to Claim 42 wherein the antibiotic marker is neomycin phosphotransferase.

20

44. A vector according to any of Claims 39-43 which when used in the genetic modification of cells for use in the method of any of Claims 1 to 36 is not integrated into the genome.

45. A vector according to any of Claims 39-43 in which said second genetic component includes sequences which enable at least a portion of the first genetic component to be specifically integrated into the genome.

25

46. A vector according to any of Claims 39-45 which additionally includes recognition sequences, eg lox P or FRT sites, which allow subsequent excision of the integrated construct via site-specific recombination.

30

47. A method of preparing a transgenic animal comprising obtaining a desired stem cell according to the method of any of claims 1-36, excising the selectable marker from the desired stem cell and generating the transgenic animal therefrom.

48. A method of preparing a transgenic animal, said animal comprising a selectable marker capable of differential expression in (a) desired stem cells and (b) cells other than desired stem cells, the method comprising:

35

providing a blastocyst;

providing animal cells according to any of Claims 37-38;

introducing the animal cells into the blastocyst;

transferring the blastocyst to a recipient; and

40

allowing an embryo to develop to a chimaeric animal to enable germline transmission of the selectable marker.

Patentansprüche

45

1. Verfahren zur Isolierung und/oder Anreicherung und/oder selektiver Vermehrung gewünschter tierischer Stammzellen, welches das am Leben erhalten eines Ursprungs der Zellen unter Kulturbedingungen umfaßt, was dem Überleben der Zelle dienlich ist, dadurch gekennzeichnet, daß der Ursprung der Zelle Stammzellen aufweist, die einen auswählbaren Marker enthalten, der als unterschiedlicher Terminus in (a) gewünschten Stammzellen des Ursprungs und (b) Zellen des Ursprungs, die verschieden von den gewünschten Stammzellen sind, geeignet ist, wobei der unterschiedliche Terminus des auswählbaren Markers eine bevorzugte Isolierung und/oder ein bevorzugtes Überleben und/oder eine bevorzugte Teilung der gewünschten Stammzellen zur Folge hat, die den auswählbaren Marker enthalten.

50

55

2. Verfahren nach Anspruch 1, bei dem die gewünschten Stammzellen aus einpotentialen Stammzellen, aus pluripotenten Stammzellen, aus embryonalen Stammzellen, aus gonadalen Stammzellen, aus somatischen Stammzellen/Vorläuferzellen, aus hämopoetischen Stammzellen, aus epidermischen Stammzellen und aus neuronalen Stammzellen ausgewählt werden.

3. Verfahren nach einem der vorangehenden Ansprüche, bei dem der Ursprung der Zellen Stammzellen enthält, die einen positiv selektierbaren Marker aufweisen, wobei der Terminus des Markers dazu verwendet wird, die Isolierung und/oder Anreicherung und/oder das am Leben erhalten der Stammzellen zu ermöglichen.
- 5 4. Verfahren nach einem der vorangehenden Ansprüche, bei dem der Terminus eines negativen auswählbaren Markers in Zellen, die verschieden von den gewünschten Stammzellen sind, dazu verwendet wird, den Ursprung der Zellen von Zellen, die verschieden von den gewünschten Stammzellen sind, ausgewählt zu entleeren.
- 10 5. Verfahren nach einem der vorangehenden Ansprüche, bei dem der auswählbare Marker aus einem fremden Gen, einem zellularen Gen und einem Gen für antibiotische Resistenz ausgewählt wird.
6. Verfahren nach Anspruch 5, bei dem das Gen für antibiotische Resistenz das bakterielle Gen für Neomycinresistenz ist.
- 15 7. Verfahren nach einem der Ansprüche 1 bis 5, bei dem der auswählbare Marker ein Wachstumsstimulierungsgen ist.
8. Verfahren nach Anspruch 7, bei dem das Wachstumsstimulierungsgen ein Onkogen oder ein Derivat von diesem ist.
- 20 9. Verfahren nach Anspruch 7, bei dem das Wachstumsstimulierungsgen das SV40 große T Antigen oder das Derivat des SV40 großen T Antigens ist.
10. Verfahren nach Anspruch 7, bei dem das Wachstumsstimulierungsgen aus einem Gen, das Schlüssel für den Wachstumsfaktor ist, aus einem Gen, das Schlüssel für einen Wachstumsfaktorrezeptor ist, aus einem Gen, das Schlüssel für ein Signalempfangs- und -weitergabemolekül ist, und einem Gen, das Schlüssel für einen Kopiefaktor ist, ausgewählt wird.
- 25 11. Verfahren nach einem der Ansprüche 1 bis 5, bei dem der auswählbare Marker ein Verewigungsgen ist.
- 30 12. Verfahren nach Anspruch 11, bei dem das Verewigungsgen aus dem Polyoma groß T Gen, einem Gen, das den Zelltod blockiert, und dem bcl-2 Gen ausgewählt wird.
13. Verfahren nach einem der Ansprüche 1 bis 5, bei dem die Isolierung und/oder Anreicherung und/oder Vermehrung der gewünschten pluripotentialen Zellen vom Vorhandensein von Zellen, die verschieden von den gewünschten pluripotentialen Zellen sind, abhängt, wobei das gleichzeitige am Leben erhalten beider Zelltypen vom Terminus des auswählbaren Markers in einer oder der anderen Zellpopulation abhängt, der zur Befreiung von Zellen imstande ist, die an solche Zellen angrenzen, die selbst nicht den Marker zeigen.
- 35 14. Verfahren nach Anspruch 13, bei dem der auswählbare Marker aus HPRT, einem Gen, das ein toxisches Produkt entschlüsselt, ausgewählt wird, einem toxischen Genprodukt, das in Verbindung mit einem Selbsttötungssubstrat und einem Herpes Simplex Virus Thymidinkinase (HSVTK) Gen bedingt aktiv ist.
- 40 15. Verfahren nach einem der vorangehenden Ansprüche, bei dem die Zellen zwei auswählbare Marker enthalten.
- 45 16. Verfahren nach einem der vorangehenden Ansprüche, bei dem der Terminus des auswählbaren Markers durch operatives Einsetzen des auswählbaren Markers in ein Terminuskonstrukt vor Einführung in den Zellursprung erhalten wird.
17. Verfahren nach einem der vorangehenden Ansprüche, bei dem der Terminus des auswählbaren Markers sich aus der Einführung eines stabil integrierten, episomal am Leben erhaltenen oder vorübergehend am Leben erhaltenen Konstrukts ergibt.
- 50 18. Verfahren nach einem der vorangehenden Ansprüche, bei dem sich der Terminus des auswählbaren Markers aus dem operativen Einsetzen des auswählbaren Markers in ein endogenes Gen des Zellursprungs ergibt.
- 55 19. Verfahren nach einem der vorangehenden Ansprüche, bei dem der auswählbare Marker in die Zellen durch Transfektion, Lipofektion, Injektion, "ballistic missile", viralen Vektor, Elektroporation oder jedes andere Mittel eingeführt wird.

- 5
20. Verfahren nach einem der vorangehenden Ansprüche, bei dem der Ursprung der Zellen durch eine Kultur einer einzigen Zelle oder Zellreihe erhalten wird, wobei die Zellen aus befruchteten Eimutterzellen oder einem transgenen oder einem nicht-transgenen Tier, aus Zellen eines Embryos, aus Blut oder aus körperlichem Gewebe oder einem Gemisch aller vorerwähnten Zellen gewonnen werden können.
- 10
21. Verfahren nach Anspruch 20, bei dem der Ursprung der Zellen durch eine Kultur einer Zelle erhalten wird oder von Zellen, die von einem transgenen Tier erhalten werden.
22. Verfahren nach einem der vorangehenden Ansprüche, bei dem der auswählbare Marker operativ an ein Gen oder an einen Genfragment-Steuerungsterminus angekoppelt ist, wobei das Gen oder das Genfragment in Stammzellen oder Nicht-Stammzellen unterschiedlich aktiv ist.
- 15
23. Verfahren nach einem der vorangehenden Ansprüche, bei dem ein Gen oder ein Genfragment operativ angekoppelt ist und der Steuerungsterminus des auswählbaren Markers mit einer pluripotentialen Stufe der zellularen Entwicklung verbunden ist.
24. Verfahren nach Anspruch 23, bei dem das Gen oder das Genfragment in Pluripotentialzellen von sich entwickelnden Embryos aktiv ist.
- 20
25. Verfahren nach Anspruch 23 oder 24, bei dem das Gen oder das Genfragment in einem einfachen (primitiven) Ektoderm aktiv ist.
26. Verfahren nach einem der Ansprüche 22 bis 25, bei dem das Gen oder das Genfragment vollständig oder Teil des Oct4 Gens ist.
- 25
27. Verfahren nach einem der Ansprüche 22 bis 26, bei dem das Gen oder das Genfragment der Oct4 Anreger ist.
28. Verfahren nach einem der vorangehenden Ansprüche, bei dem der auswählbare Marker das Neomycin-Phosphotransferase Gen ist.
- 30
29. Verfahren nach einem der Ansprüche 22 bis 28, bei dem das Gen oder das Genfragment in pluripotenten, hämopoetischen Zellen aktiv ist.
- 30
30. Verfahren nach Anspruch 29, bei dem das Gen oder das Genfragment insgesamt das D34 Gen ist oder ein Teil davon.
- 35
31. Verfahren nach einem der vorangehenden Ansprüche, welches den Schritt Einführen in einen Ursprung von Zellen, die Stammzellen enthalten, sowie ein auswählbares Markerkonstrukt umfaßt, wobei das auswählbare Markerkonstrukt dazu dient, operativ an ein endogenes Gen gekoppelt zu werden, das den unterschiedlichen Terminus schafft.
- 40
32. Verfahren nach einem der Ansprüche 1 bis 30, welches den Schritt Einführen in einen Ursprung von Zellen, die Stammzellen enthalten, sowie ein auswählbares Markerkonstrukt umfaßt, wobei das auswählbare Markerkonstrukt vorher an ein Gen oder mehrere Gene oder Genfragmente gekoppelt worden ist, die den unterschiedlichen Terminus schaffen.
- 45
33. Verfahren zur selektiven Isolierung und/oder Anreicherung und/oder Vermehrung gewünschter tierischer Stammzellen, welches die Einführung in einen Ursprung von Zellen, die Stammzellen enthalten, sowie ein auswählbares Markerkonstrukt umfaßt, das operativ an Gene oder Genfragmente koppelt oder vorher angekoppelt worden ist, die unterschiedliche Termini des auswählbaren Markers in (a) gewünschten Stammzellen des Ursprungs und (b) Zellen des Ursprungs, die verschieden von denen der gewünschten Stammzellen sind, schaffen, und die unter geeigneten Kulturbedingungen eine selektive Isolierung und/oder Anreicherung und/oder Vermehrung der gewünschten Stammzellen ermöglichen, die den auswählbaren Marker enthalten.
- 50
34. Verfahren nach Anspruch 33, bei dem der auswählbare Marker operativ an ein Gen oder einen Genfragment-Steuerungsterminus gebunden ist, wobei das Gen oder das Genfragment in Stammzellen und Nicht-Stammzellen unterschiedlich aktiv ist.
- 55

35. Verfahren nach Anspruch 34, bei dem das Gen oder das Genfragment der Oct4-Aktivator ist.
36. Verfahren nach einem der Ansprüche 33 bis 35, bei dem der auswählbare Marker das Neomycin-Phosphotransferase Gen ist.
- 5
37. Tierische Zelle, die geeignet zum Anzüchten ist, um ein Zellengemisch zu bilden, das gewünschte Stammzellen und Zellen, die verschieden von den gewünschten Stammzellen sind, umfaßt, dadurch gekennzeichnet, daß alle Zellen in dem Gemisch einen auswählbaren Marker enthalten und daß in dem Zellgemisch unter geeigneten auswählbaren Kulturbedingungen unterschiedliche Termini des auswählbaren Markers in (a) den gewünschten Stammzellen und (b) Zellen, die verschieden von den gewünschten Stammzellen sind, ein ausgewähltes Überleben oder Wachsen der gewünschten Stammzellen ermöglichen, um die Isolierung und/oder die Anreicherung und/oder die Vermehrung der gewünschten Stammzellen zu ermöglichen.
- 10
38. Tierische Zelle nach Anspruch 37, bei der der auswählbare Marker ein Gen für antibiotische Resistenz ist.
- 15
39. Genetisches Material übertragendes Element (Vektor) zur Verwendung in genetisch modifizierenden Zellen, geeignet für die Verwendung in einem Verfahren nach einem der Ansprüche 1 bis 36, wobei der Vektor eine erste genetische Komponente entsprechend dem auswählbaren Marker und eine zweite genetische Komponente umfaßt, die in genetisch modifizierten tierischen Zellen direkt oder indirekt zu den unterschiedlichen Termini des auswählbaren Markers führen.
- 20
40. Vektor nach Anspruch 39 in Form eines Terminusvektors, bei dem die zweite genetische Komponente Steuersequenzen enthält, die unterschiedlich in (a) gewünschten Stammzellen und (b) Zellen, die verschieden von den gewünschten Stammzellen sind, aktiviert werden.
- 25
41. Vektor nach Anspruch 40, in dem die Steuersequenz der Oct4 Aktivator ist.
42. Vektor nach Anspruch 41, in dem der auswählbare Marker ein antibiotischer Marker ist.
- 30
43. Vektor nach Anspruch 42, in dem der antibiotische Marker Neomycin-Phosphotransferase ist.
44. Vektor nach einem der Ansprüche 39 - 43, der, wenn er bei der genetischen Modifikation von Zellen verwendet wird, zur Verwendung in dem Verfahren nach einem der Ansprüche 1 bis 36, nicht in das Genom integriert ist.
- 35
45. Vektor nach einem der Ansprüche 39 - 43, bei dem die zweite genetische Komponente Sequenzen enthält, die wenigstens einem Teil der ersten genetischen Komponente ermöglichen, spezifisch in das Genom integriert zu werden.
- 40
46. Vektor nach einem der Ansprüche 39 - 45, welcher zusätzlich Erkennungssequenzen, beispielsweise lox P oder FRT-Sltze, enthält, die ein nachfolgendes Ausschneiden des integralen Konstrukts über sitzspezifische Rekombination gestatten.
- 45
47. Verfahren zur Herstellung eines transgenen Tieres, umfassend: Erhalten einer gewünschten Stammzelle gemäß dem Verfahren nach einem der Ansprüche 1 - 36, Ausschneiden des auswählbaren Markers aus der gewünschten Stammzelle und Erzeugung eines transgenen Tieres daraus.
- 50
48. Verfahren zur Herstellung eines transgenetischen Tieres, wobei das Tier einen auswählbaren Marker enthält, der zu einem unterschiedlichen Terminus in (a) gewünschten Stammzellen und (b) Zellen, die verschieden zu den gewünschten Stammzellen sind, imstande ist, umfassend: Bereitstellung einer Blastozyste, Bereitstellung tierischer Zellen nach einem der Ansprüche 37 - 38, Einführen der tierischen Zellen in die Blastozyste, Übertragung der Blastozyste auf einen Rezipienten; und dem Tier zu gestatten, sich zu einem chimären Tier zu entwickeln, um eine Keimbahnübertragung des auswählbaren Markers zu ermöglichen.

55 **Revendications**

1. Procédé d'isolement et/ou d'enrichissement et/ou de propagation sélective de cellules souches animales voulues, qui comprend le maintien d'une source desdites cellules dans des conditions de culture conduisant à la survie des

EP 0 695 351 B1

cellules, caractérisé en ce que la source de cellules inclut des cellules souches contenant un marqueur sélectionnable qui est capable d'expression différentielle dans (a) des cellules souches voulues de ladite source et (b) des cellules de ladite source autres que les cellules souches voulues, de sorte que l'expression différentielle dudit marqueur sélectionnable entraîne l'isolement et/ou la survie et/ou la division préférentiels des cellules souches voulues qui contiennent ledit marqueur sélectionnable.

2. Procédé selon la revendication 1 dans lequel les cellules souches voulues sont choisies parmi les cellules souches unipotentes, les cellules souches pluripotentes, les cellules souches embryonnaires, les cellules souches gonadiques, les cellules souches/progénitrices somatiques, les cellules souches hématopoïétiques, les cellules souches épidermiques et les cellules souches neuroniques.
3. Procédé selon l'une quelconque des revendications précédentes dans lequel la source de cellules comprend des cellules souches ayant un marqueur sélectionnable positif et l'expression dudit marqueur est utilisée pour permettre l'isolement et/ou l'enrichissement et/ou le maintien des cellules souches.
4. Procédé selon l'une quelconque des revendications précédentes dans lequel l'expression d'un marqueur sélectionnable négatif dans des cellules autres que les cellules souches voulues est utilisée pour appauvrir sélectivement la source de cellules en cellules autres que les cellules souches voulues.
5. Procédé selon l'une quelconque des revendications précédentes dans lequel le marqueur sélectionnable est choisi parmi un gène étranger, un gène cellulaire et un gène de résistance à un antibiotique.
6. Procédé selon la revendication 5 dans lequel le gène de résistance à un antibiotique est le gène de résistance à la néomycine bactérien.
7. Procédé selon l'une quelconque des revendications 1 à 5 dans lequel le marqueur sélectionnable est un gène stimulant la croissance.
8. Procédé selon la revendication 7 dans lequel le gène stimulant la croissance est un oncogène ou des dérivés de celui-ci.
9. Procédé selon la revendication 7 dans lequel le gène stimulant la croissance est l'antigène T de grande taille de SV40 ou un dérivé de l'antigène T de grande taille de SV40.
10. Procédé selon la revendication 7 dans lequel le gène stimulant la croissance est choisi parmi un gène qui code un facteur de croissance, un gène qui code un récepteur de facteur de croissance, un gène qui code une molécule de transduction de signaux et un gène qui code un facteur de transcription.
11. Procédé selon l'une quelconque des revendications 1 à 5 dans lequel le marqueur sélectionnable est un gène immortalisant.
12. Procédé selon la revendication 11 dans lequel le gène immortalisant est choisi parmi le gène T de grande taille de polyome, un gène qui bloque la mort cellulaire et le gène bcl-2.
13. Procédé selon l'une quelconque des revendications 1 à 5 dans lequel l'isolement et/ou l'enrichissement et/ou la propagation des cellules pluripotentes voulues est dépendant de la présence de cellules autres que les cellules pluripotentes voulues et le maintien simultané des deux types de cellules est dépendant de l'expression d'un marqueur sélectionnable, dans l'une ou l'autre population de cellules, qui est capable de sauver les cellules voisines des cellules qui n'expriment pas elles-mêmes le marqueur.
14. Procédé selon la revendication 13 dans lequel le marqueur sélectionnable est choisi parmi HPRT, un gène codant un produit toxique, un produit de gène toxique qui est actif de manière conditionnelle en combinaison avec un substrat suicide et un gène de thymidine kinase du virus de l'herpès (HSV-TK).
15. Procédé selon l'une quelconque des revendications précédentes dans lequel les cellules contiennent deux marqueurs sélectionnables.
16. Procédé selon l'une quelconque des revendications précédentes dans lequel l'expression du marqueur sélection-

nable est obtenue par insertion opérationnelle du marqueur sélectionnable dans une construction d'expression avant l'introduction dans la source de cellules.

- 5 17. Procédé selon l'une quelconque des revendications précédentes dans lequel l'expression du marqueur sélectionnable résulte de l'introduction d'une construction intégrée de manière stable, maintenue de manière épisomique ou maintenue de manière transitoire.
- 10 18. Procédé selon l'une quelconque des revendications précédentes dans lequel l'expression du marqueur sélectionnable résulte de l'insertion opérationnelle du marqueur sélectionnable dans un gène endogène de la source de cellules.
- 15 19. Procédé selon l'une quelconque des revendications précédentes dans lequel le marqueur sélectionnable est introduit dans les cellules par transfection, lipofection, injection, missile ballistique, vecteur viral, électroporation ou tout autre moyen.
- 20 20. Procédé selon l'une quelconque des revendications précédentes dans lequel la source de cellules est obtenue par culture d'une seule cellule ou lignée cellulaire, de cellules dérivées d'un ovocyte fécondé, ou d'un animal transgénique ou d'un animal non transgénique, de cellules dérivées d'un embryon, du sang ou de tissu somatique, ou d'un mélange de cellules quelconques parmi les cellules mentionnées précédemment.
- 25 21. Procédé selon la revendication 20 dans lequel la source de cellules est obtenue par culture d'une cellule ou de cellules obtenues à partir d'un animal transgénique.
- 30 22. Procédé selon l'une quelconque des revendications précédentes dans lequel le marqueur sélectionnable est lié de manière opérationnelle à un gène ou fragment de gène régulant l'expression, lequel gène ou fragment de gène est actif de manière différentielle dans les cellules souches et dans les cellules autres que les cellules souches.
- 35 23. Procédé selon l'une quelconque des revendications précédentes dans lequel un gène ou fragment de gène lié de manière opérationnelle à et régulant l'expression du marqueur sélectionnable est/sont associés à un stade pluripotent du développement cellulaire.
- 40 24. Procédé selon la revendication 23 dans lequel le gène ou fragment de gène est actif dans les cellules pluripotentes de l'embryon qui se développe.
- 45 25. Procédé selon la revendication 23 ou 24 dans lequel le gène ou fragment de gène est actif dans l'ectoderme primitif.
26. Procédé selon l'une quelconque des revendications 22-25 dans lequel le gène ou fragment de gène constitue la totalité ou une partie du gène Oct4.
- 50 27. Procédé selon l'une quelconque des revendications 22-26 dans lequel le gène ou fragment de gène est le promoteur de Oct4.
28. Procédé selon l'une quelconque des revendications précédentes dans lequel le marqueur sélectionnable est le gène de la néomycine phosphotransférase.
- 55 29. Procédé selon l'une quelconque des revendications 22-28 dans lequel le gène ou fragment de gène est actif dans les cellules hématopoïétiques pluripotentes.
30. Procédé selon la revendication 29 dans lequel le gène ou fragment de gène constitue la totalité ou une partie du gène CD34.
31. Procédé selon l'une quelconque des revendications précédentes qui inclut l'étape d'introduction dans une source de cellules contenant des cellules souches d'une construction de marqueur sélectionnable, où ladite construction de marqueur sélectionnable est conçue pour se lier de manière opérationnelle à un gène endogène qui permet ladite expression différentielle.
32. Procédé selon l'une quelconque des revendications 1 à 30 qui inclut l'étape d'introduction dans une source de cellules contenant des cellules souches d'une construction de marqueur sélectionnable, où ladite construction de

marqueur sélectionnable a été préalablement liée à un ou plusieurs gènes ou fragments de gènes qui permettent ladite expression différentielle.

- 5
33. Procédé d'isolement et/ou d'enrichissement et/ou de propagation sélectifs de cellules souches animales voulues, qui comprend l'introduction dans une source de cellules contenant des cellules souches d'une construction de marqueur sélectionnable qui se lie de manière opérationnelle ou qui a été préalablement liée de manière opérationnelle à des gènes ou des fragments de gènes qui permettent l'expression différentielle du marqueur sélectionnable dans (a) des cellules souches voulues de ladite source et (b) des cellules de ladite source autres que les cellules souches voulues et qui, dans des conditions de culture appropriées, permet l'isolement et/ou l'enrichissement et/ou la propagation sélectifs des cellules souches voulues contenant le marqueur sélectionnable.
- 10
34. Procédé selon la revendication 33 dans lequel le marqueur sélectionnable est lié de manière opérationnelle à un gène ou fragment de gène régulant l'expression, lequel gène ou fragment de gène est actif de manière différentielle dans les cellules souches et les cellules autres que les cellules souches.
- 15
35. Procédé selon la revendication 34 dans lequel le gène ou fragment de gène est le promoteur de Oct4.
36. Procédé selon l'une quelconque des revendications 33-35 dans lequel le marqueur sélectionnable est le gène de la néomycine phosphotransférase.
- 20
37. Cellule animale capable d'être cultivée pour former un mélange de cellules incluant des cellules souches voulues et des cellules autres que les cellules souches voulues, caractérisée en ce que toutes les cellules dans ledit mélange de cellules contiennent un marqueur sélectionnable et en ce que, dans ledit mélange de cellules, dans des conditions de culture sélective appropriées, l'expression différentielle du marqueur sélectionnable dans (a) les cellules souches voulues et (b) des cellules autres que les cellules souches voulues permet à la survie ou à la croissance sélective des cellules souches voulues de se produire, de manière à permettre l'isolement et/ou l'enrichissement et/ou la propagation des cellules souches voulues.
- 25
38. Cellule animale selon la revendication 37 dans laquelle le marqueur sélectionnable est un gène de résistance à un antibiotique.
- 30
39. Vecteur destiné à être utilisé dans la modification génétique de cellules pour qu'elles puissent être utilisées dans un procédé selon l'une quelconque des revendications 1 à 36, ledit vecteur comprenant un premier composant génétique correspondant audit marqueur sélectionnable et un second composant génétique qui, dans les cellules animales modifiées génétiquement, entraîne directement ou indirectement ladite expression différentielle du marqueur sélectionnable.
- 35
40. Vecteur selon la revendication 39 sous forme d'un vecteur d'expression dans lequel ledit second composant génétique comprend des séquences de contrôle qui sont activées de manière différentielle dans (a) les cellules souches voulues et (b) les cellules autres que les cellules souches voulues.
- 40
41. Vecteur selon la revendication 40 dans lequel la séquence de contrôle est le promoteur de Oct4.
42. Vecteur selon la revendication 41 dans lequel le marqueur sélectionnable est un marqueur antibiotique.
- 45
43. Vecteur selon la revendication 42 dans lequel le marqueur antibiotique est la néomycine phosphotransférase.
44. Vecteur selon l'une quelconque des revendications 39-43 qui, quand il est utilisé dans la modification génétique de cellules destinées à être utilisées dans le procédé selon l'une quelconque des revendications 1 à 36, n'est pas intégré dans le génome.
- 50
45. Vecteur selon l'une quelconque des revendications 39-43 dans lequel ledit second composant génétique inclut des séquences qui permettent à au moins une partie du premier composant génétique d'être intégrée spécifiquement dans le génome.
- 55
46. Vecteur selon l'une quelconque des revendications 39-45 qui inclut en outre des séquences de reconnaissance, par exemple des sites lox P ou FRT, qui permettent l'excision subséquente de la construction intégrée par recombinaison spécifique de site.

47. Procédé de préparation d'un animal transgénique comprenant l'obtention d'une cellule souche voulue selon le procédé selon l'une quelconque des revendications 1-36, l'excision du marqueur sélectionnable de la cellule souche voulue et la production de l'animal transgénique à partir de celle-ci.

5 48. Procédé de préparation d'un animal transgénique, ledit animal comprenant un marqueur sélectionnable capable d'expression différentielle dans (a) des cellules souches voulues et (b) des cellules autres que les cellules souches voulues, le procédé comprenant:

la fourniture d'un blastocyste;

10 la fourniture de cellules animales selon l'une quelconque des revendications 37-38;

l'introduction des cellules animales dans le blastocyste;

le transfert du blastocyste à un receveur; et

le fait d'amener un embryon à se développer en un animal chimère pour permettre la transmission par la lignée germinale du marqueur sélectionnable.

15

20

25

30

35

40

45

50

55

Fig. 1

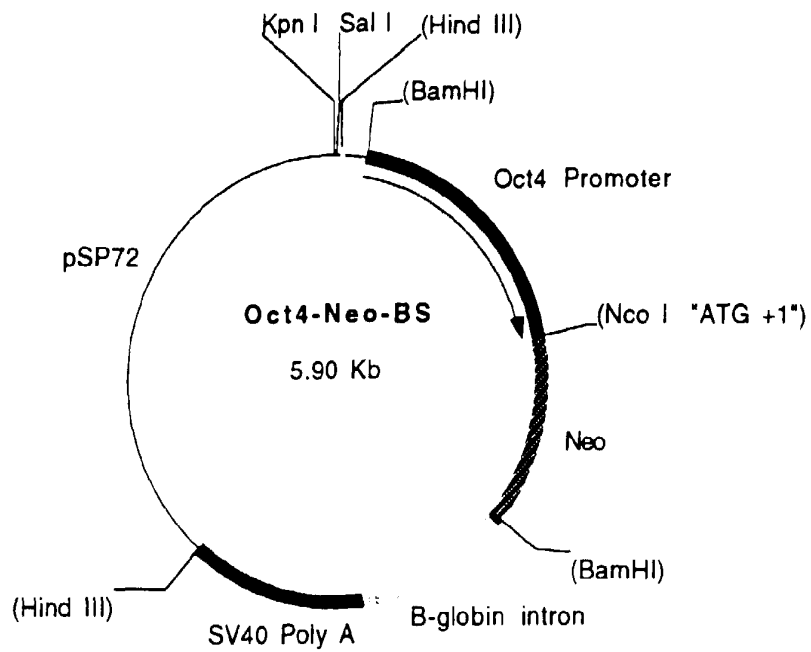


Fig. 2

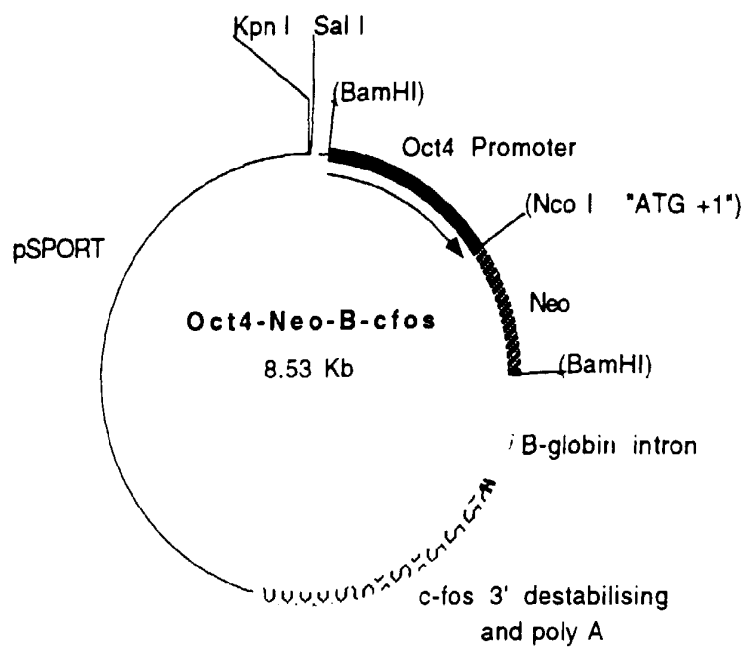


Fig. 3

