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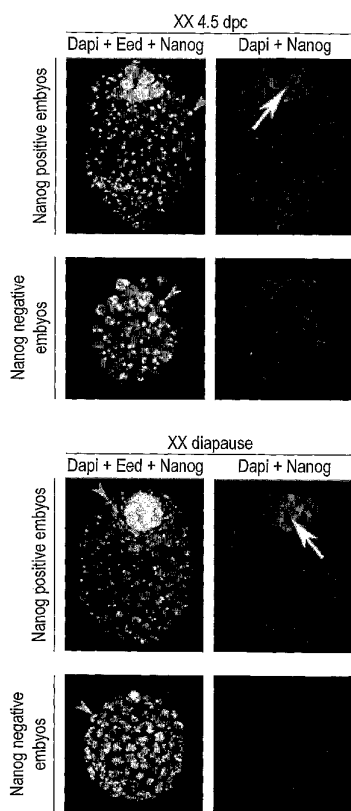
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(54) Title: REPROGRAMMING AND GENETIC MODIFICATION OF CELLS



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(57) Abstract: Methods for reprogramming and optional genetic modification of cells are provided. A pluripotent genome is obtained from a differentiated genome by fusing a pluripotent cell with a differentiated cell in the presence of Nanog or a MEK inhibitor. A cell is genetically modified by providing first and second cells, each containing chromosomes, fusing the first cell and the second cell, and culturing the fused cell so as to obtain a diploid cell containing at least one chromosome from the first cell and at least one chromosome from the second cell. A method of cell fusion comprises fusing a first cell and a second cell in the presence of Nanog or a MEK inhibitor. Cells obtained thereby and their uses are also described.



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Reprogramming and Genetic Modification of Cells

Field of the Invention

- 5 The present invention relates to reprogramming and genetic modification of cells, in particular cells that are to be reprogrammed. The invention relates especially to reprogramming of cells to a pluripotent state, and to genetic modification of a cell, e.g. to remedy a genetic defect, prior to such reprogramming.

10 Background to the Invention

- During embryo development pluripotent cells differentiate into many distinct cell types. These are defined by the specific subsets of genes that are either activated or repressed, the epigenome. Significantly, a differentiated epigenome can be reverted to pluripotency by nuclear transfer into enucleated oocytes and through cell fusion with
- 15 either ES or EG cells. In ES and EG-T cell hybrids it has been shown that the inactive X, silent imprinted genes and a silent Oct4-GFP reporter transgene of T cells are reactivated (Tada *et al*, 1997 and 2001). Moreover, ES and EG-T cell hybrids were shown in vivo to contribute to all the three primary germ layers, demonstrating pluripotency properties (Tada *et al*, 1997; 2001). Other studies have also shown that
- 20 nuclei of the central nervous system, of bone marrow and of splenocytes exhibit pluripotent potential after fusion with ES cells (Matveeva *et al*, 1998; Terada *et al*, 2002; Ying *et al*, 2002). Moreover, analysis of the chromatin status of the somatic genome in ES-differentiated cell hybrids showed acquisition of ES cell genome properties (Kimura *et al*, 2004). Together, these results showed that hybrids do not
- 25 simply contain the union of the two genomes, the likelihood is that the somatic donor genome has undergone full reprogramming.

- Although viable animals have been generated through somatic cell nuclear transfer (SCNT) into enucleated oocytes the success rate is extremely low, with most of the
- 30 embryos dying at early stages of development (reviewed in Hochedlinger and Jaenisch, 2003). A major contributing factor is likely to be errors in the reprogramming of the

donor genome (Humpherys et al, 2001, 2002; Kang et al, 2001, 2002; Xue et al, 2002; Santos et al, 2003). Moreover, in a proportion of cases, early cloned embryos showed incomplete reactivation of the pluripotency associated gene Oct4, suggesting that most cloned embryos fail to establish a truly pluripotent embryonic population (Bortvin et al, 5 2003). Supporting this notion the generation of adult mice from B cell nuclei is not possible without an extra step that involves the derivation of ES cells from the cloned embryos (Hochedlinger and Jaenisch, 2002). Thus, improving the efficiency of epigenetic reprogramming is desirable.

10 Reprogramming of the genome also occurs in vivo. Primordial germ cells (PGCs) entering the genital ridges at 10.5dpc-11.5dpc undergo genome wide demethylation, reactivation of the silent X chromosome and erasure of imprints (Hajkova et al, 2002; Sato et al, 2003; Tam et al, 1994). Recently, it was reported that reprogramming is a feature of early embryos (Mak et al, 2004; Okamoto et al, 2004). In these studies it 15 was shown that imprinted X chromosome inactivation occurs in all the cells of the late morula and blastocyst. At these stages, the inactive X chromatin showed, along with other epigenetic marks, enrichment of H3-K9 dimethylation and H3-K27 trimethylation. This was a surprising result as the inactive X is a marker of cell differentiation and its state is stable and heritably transmitted, being only reversible in 20 pluripotent cells (Wutz and Jaenisch, 2000). Importantly, the inactive X was shown to erase the epigenetic marks and reactivate, at around 4.5 dpc, in the epiblast (Mak et al, 2004; Okamoto et al, 2004). Unexpectedly, analysis of the epigenetic dynamics of the X chromosome in XX somatic nuclei transplanted into mouse oocytes showed noticeable differences to normal embryos (Bao et al, 2005). The inactive X epigenetic 25 mark trimethyl H3-K27 is not erased by the oocyte, persisting up to the epiblast formation when X reactivation occurs. This suggests that oocytes and epiblast have distinct properties in terms of genome reprogramming. The factors underlying reprogramming are not known. These must however be present in the cells of the blastocyst that will give rise to the pluripotent epiblast, as well as in ES cells that are 30 responsible for X reactivation in ES-differentiated cell hybrids. Likewise, these factors must also be present in PGCs migrating into the genital ridges and in EG cells.

Nanog is a unique homeodomain-containing protein present in pluripotent mammalian cells and essential for early development. Coincident with formation of pluripotent cells in the blastocyst, the previously inactivated paternal X chromosome in female
5 XX embryos becomes reactivated. Reactivation is confined to those cells that express Nanog and does not occur in *Nano* null embryos.

Nanog expression in the early embryo marks the future pluripotent epiblast cells and is rapidly downregulated during ES cell differentiation and embryo development
10 (Chambers et al, 2003; Mitsui et al, 2003). In addition, Nanog has the unique properties, when overexpressed in ES cells, of allowing self-renewal in the absence of the otherwise essential factors Lf and serum (BMP-4) (Chambers et al, 2003; Ying et al, 2003). Moreover, Nanog is expressed in the pluripotent EG cells and in the PGCs, in which reprogramming is occurring (Chambers et al, 2003; Yamaguchi et al, 2005).
15 In vitro characterization of Nanog null ES cells and isolated Inner Cell Masses (ICMs) showed that these fail to retain an undifferentiated state, differentiating into endoderm like cells (Mitsui et al, 2003).

Comparison analysis of WT and *Nanog*^{-/-} 4.5 dpc and diapause female embryos
20 showed that X chromosome reactivation only occurs in WT embryos, in Nanog expressing cells.

WO 03/064463 describes use of Nanog in combination with LIF to reprogram a cell or nucleus. However, in comparative examples described herein, expression of Nanog
25 was found to be insufficient to induce reprogramming.

For cell therapy purposes it is desired to genetically modify cells, especially to modify pluripotent cells and derive progeny therefrom. In concert with nuclear reprogramming, genetic modification of the cells is known using e.g. vectors. Genetic modification of
30 cells produced by SCNT has been used, for example, in the treatment of immunodeficiency (Rideout et al, 2002). But these methods can leave artefacts in the

genome. Cell therapy products should be diploid cells, but it is known that fusion of diploid cells yields tetraploid cells or cells in which segregation of chromosomes is uneven, yielding a variety of different aneuploid cell types (Matveeva et al, 1998). This would be unacceptable for regulatory authorities.

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It would thus be desirable to increase the efficiency of nuclear reprogramming. But as described the current efficiency is low. It would also be desired to introduce genetic modification into reprogrammed cells and derive diploid progeny therefrom.

- 10 An object of the present invention is to ameliorate or at least provide an alternative to the above problems. An object of specific embodiments of the invention is to provide improved reprogramming methods and cells obtained thereby. A further object of specific embodiments of the invention is to provide a method for genetic modification of cells, optionally followed by reprogramming of the modified cells and derivation of
- 15 progeny thereof.

Summary of the Invention

According to the invention, Nanog expression and / or inhibition of MEK is used for reprogramming of cells.

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Detailed description of the invention

According to the invention there is provided a method of obtaining a pluripotent genome from a differentiated genome, comprising:-

- 25 fusing a pluripotent cell with a differentiated cell; and overexpressing Nanog in the fused cell.

Nanog can be overexpressed by overexpressing Nanog in the pluripotent cell prior to the fusing step, overexpressing Nanog in the differentiated cell prior to the fusing step or overexpressing Nanog in the fused cell, i.e. after the fusing step. Nanog is suitably

30 overexpressed by introducing into a cell a genetic construct which expresses Nanog.

Nanog can also be overexpressed by introducing into the cell, or culturing the cell in the presence of, a medium component which increases Nanog expression in the cell.

In a particular embodiment of the invention the method comprises:-

- 5 fusing a pluripotent cell with a differentiated cell to form a fused cell; wherein at least one of the pluripotent cell, the differentiated cell and the fused cell is treated with a MEK inhibitor.

Both the pluripotent cell and the differentiated cell can be treated with a MEK
10 inhibitor.

It has advantageously been found that the use of Nanog, via direct overexpression or via overexpression using e.g. a MEK inhibitor, in this way increases the efficacy of obtaining a cell with pluripotent properties. It is believed that treatment with a MEK
15 inhibitor results in upregulation of expression of Nanog. The inventors have also advantageously found that following further culture a pluripotent, diploid cell can be obtained, containing genetic information from the differentiated cell. In methods of the invention, a fused cell is maintained in culture and spontaneously loses chromosomes until its chromosome complement reduces and reverts to diploid.
20 Generally, at least one or more chromosomes from the differentiated cell contribute to the diploid state of the resultant, pluripotent cell. Hence, a pluripotent cell can be obtained and used to derive differentiated progeny thereof including cells and tissue starting from a differentiated cell containing a desired gene or a desired chromosome or other desired genetic material.

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The pluripotent cell is suitably an embryonic stem (ES) cell, an embryonic carcinoma (EC) cell or an embryonic gonadal (EG) cell, and in specific embodiments of the invention, described in more detail below, cell fusions have successfully resulted in a pluripotent genome by fusing an ES cell with a differentiated cell.

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The differentiated cell can be a somatic cell and in general it is believed that substantially all somatic cells are suitable for use in application of the invention described herein. The differentiated cell can also be a stem cell and, again, it is believed that the application of the invention is not restricted to any particular stem
5 cells, but extends generally to stem cells, in particular neural stem cells, haematopoietic stem cells etc. In use of a particular example of the invention, reprogramming has successfully been carried out when the differentiated cell is a neural stem cell.

10 Nanog can be provided via various different means. In one embodiment, Nanog is provided by overexpressing Nanog in the pluripotent cell, and in a separate embodiment Nanog is provided by expressing Nanog in the differentiated cell.

It is also optional for Nanog to be expressed directly in both of or either of the
15 pluripotent cell and the differentiated cell, conveniently by transfecting one or more of the cells with a vector containing a nucleotide type sequence encoding Nanog together with a suitable promoter.

It is preferred that the methods of the present invention result in Nanog being
20 overexpressed in the pluripotent cell, and in practice expression levels of from two times up to fifteen times the endogenous ES cell level are believed suitable, preferably three to ten times, with about five times the normal level being used in one or more of the examples.

25 The invention is believed to be of application generally to mammalian cells, including murine, human, porcine, ovine, bovine and caprine and in all cases it is preferred that the pluripotent cell and the differentiated cell are of the same species. It is further preferred that the pluripotent cell and the differentiated cell are both mouse cells or both human cells.

It has hitherto been described that following fusion of ES cells there is unequal segregation of chromosomes (Matveva et al 2005). However, in accordance with the present invention, reprogramming methods comprise culturing the fused cell so as to obtain a diploid cell. It has hence advantageously and surprisingly been found that, following the teachings of the invention, chromosome segregation is not, in fact, unequal but can be carried out so as to obtain cells which contain a diploid chromosome complement. Analysis of these cells typically shows that they contain a mixture of chromosomes derived from the pluripotent cell and also chromosomes derived from the differentiated cell, i.e. a mix of chromosomes.

10

Thus, in a particularly preferred embodiment of the invention, fusing of the pluripotent cell and the differentiated cell results in a tetraploid cell and the method comprises obtaining progeny of the tetraploid cell and identifying such progeny which, for example by spontaneous chromosome loss, are or have become diploid. In practice, a significant proportion of the cells obtained are diploid. Without wishing to be bound by any theory, it seems both the case that a large proportion of these cells spontaneously revert to diploid status and also that during culture of the cells, preferably continued in the presence of Nanog, there is a survival advantage for truly diploid cells. Thus, a diploid cell population can be obtained with chromosomes derived from each of the two starting material cells.

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The invention thus offers the opportunity to derive a cell, whether a somatic cell or a stem cell or other, from a differentiated cell by carrying out the reprogramming step as described and, thereafter, culturing a diploid, pluripotent cell obtained as progeny of the fused cell, and deriving a differentiated cell from the diploid, pluripotent cell.

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The invention additionally provides a method of genetically modifying a cell, comprising:-

- providing a first cell containing chromosomes,
- providing a second cell containing chromosomes,
- fusing the first cell and the second cell to form a fused cell, and

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culturing the fused cell so as to obtain a diploid cell containing at least one chromosome from the first cell and at least one chromosome from the second cell.

Optionally, Nanog is overexpressed in the first cell, the second cell and / or the fused cell. For example, at least one of the first cell, the second cell and the fused cell can be treated with a MEK inhibitor. The first cell and the second cell are preferably both diploid, and can be both mouse cells or both human cells.

The thus-obtained cell contains chromosomes derived from each of the first cell and the second cell, and thus the method can be used to combine genetic material from two different sources. After obtaining the resulting diploid cell this can be tested to identify which particular combination of chromosomes have ended up in the diploid cell and, as and when necessary, selection carried out to identify a cell having a particularly desired combination of chromosomes.

15

In operation of the method, it is preferred that one of the first cell and the second cell is pluripotent. The other of the first cell and the second cell is optionally pluripotent, and can be a somatic cell, optionally a stem cell. In examples below, the other cell is a neural stem cell.

20

Selection of a cell with a particular combination of chromosomes is achievable according to the invention. Thus, an embodiment of the invention comprises identifying a first cell having a desired copy of a gene and an undesired copy of the same gene which is to be modified, identifying a second cell having a modified form of the undesired gene, fusing the first cell and second cell, culturing the fused cell, and identifying a progeny of the fused cell containing both the desired copy of the gene and the modified form of the undesired gene. In this way, the combination of genes from the first and second cells yields a cell free of a genetic defect, free of the undesired gene. Techniques are readily available enabling cells which do or do not contain the desired combination of chromosomes / genes to be identified and separated,

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so that an isolated cell or a pure population of engineered cells free of the defect is or are obtained.

The invention is usefully exploited for curing a genetic defect in the first cell wherein
5 the undesired gene constitutes a gene having a defect and the modified form of the gene in the second cell constitutes the cure.

Another embodiment of the invention comprises the fusion of a first cell engineered to alter the expression of a normal endogenous gene with a second cell containing normal
10 copies of the endogenous gene, culturing the fused cell, and identifying a progeny of the fused cell containing both a normal copy of the gene and the engineered form of the gene. This invention is usefully exploited for treating a disease by making a compensatory change in the expression of a gene.

15 It is also optional for the cure or modified gene to be engineered into the second cell, and this may be appropriate if there is not available a second cell with the right combination of genes for fusion with the first cell. Hence a method of the invention in which a suitable second cell is created may comprise genetically modifying the second cell prior to fusion with the first cell so as to introduce into the second cell the
20 modified form of the undesired gene. As described in relation to other aspects of the invention, it has been found that the resultant cell, though initially or potentially aneuploid, generally tetraploid, can undergo chromosomal segregation to become a diploid cell at an acceptable rate, yielding cells useful e.g. for ongoing culture, derivation of progeny and cell therapy.

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In a further aspect the invention provides a method of cell fusion, comprising:-
fusing a first cell and a second cell in the presence of a MEK inhibitor.

The first cell is preferably pluripotent, and may be an ES, EC or EG cell. The second
30 cell can also be pluripotent, but is preferably a somatic cell, more preferably a stem cell. Nanog can be provided by expressing Nanog in the first cell and/or second cell, as

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described elsewhere herein. The first cell, the second cell and / or the fused cell can be cultured in the presence of the MEK inhibitor, again as described elsewhere herein.

It is desired to use the method to obtain a diploid cell, and in carrying out the methods
5 it has been found that the initial cell, which has an initial combination of chromosomes from both cells reverts to diploid status at a high frequency. A method of the invention comprises fusing the first and second cells to yield an aneuploid cell and culturing the fused cell to obtain diploid progeny thereof.

10 Cells are also provided by the invention, being cells obtained according to a method of any aspect of the invention. The invention provides additionally methods of cell therapy using the cells and use of a cell according to the invention in manufacture of a medicament for use in therapy, preferably cell therapy. A method of treatment of the invention comprises administering to a patient an effective amount of cell of the
15 invention.

A further method of treatment comprises administering to a patient an effective amount of a genetically modified cell of the invention.

20 Treatment can suitably comprise:-

- identifying a patient needing a genetically modified cell,
- identifying the genetic modification required to treat the patient, and
- preparing a genetically modified cell according to the method of any embodiment of the invention.

25

Still further, the invention provides use of Nanog in reprogramming a cell obtained by a fusion of a pluripotent cell and a somatic cell. The pluripotent cell is typically an ES cell, and the somatic cell is typically a stem cell, and in a specific embodiment is a neural stem cell

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The invention also provides use of a MEK inhibitor in reprogramming a cell obtained by a fusion of a pluripotent cell and a somatic cell. The pluripotent cell is typically an ES cell, and the somatic cell is typically a stem cell, and in a specific embodiment is a neural stem cell.

5

The invention also provides use of Nanog in an improved method of somatic cell nuclear transfer to obtain a pluripotent cell. Hence, a method of nuclear transfer of the invention comprises transferring a somatic cell nucleus into a recipient cell to form a nuclear transfer cell, and overexpressing Nanog in the nuclear transfer cell.

10 Reprogramming of the donor, somatic cell nucleus is thereby achieved with improved efficiency.

In an embodiment, the invention also provides use of a MEK inhibitor in an improved method of somatic cell nuclear transfer to obtain a pluripotent cell. Hence, a method
15 of nuclear transfer of the invention comprises transferring a somatic cell nucleus into a recipient cell to form a nuclear transfer cell, and treating the nuclear transfer cell with a MEK inhibitor. Reprogramming of the donor, somatic cell nucleus is thereby achieved with improved efficiency.

20 The recipient cell is generally an oocyte, preferably enucleated and also preferably of the same species as the donor.

In preferred embodiments of the present invention, the MEK inhibitor is a MEK1 inhibitor. Examples of suitable MEK inhibitors, already known in the art, include the
25 MEK1 inhibitor PD184352, and those discussed in Davies et al (2000).

Ultimate progeny of these and other aspects of the invention can include cloned non-human animals (human cloning forms no part of the invention) and pluripotent cell cultures. Hence, in further embodiments of the invention there is provided a method of
30 obtaining a live non-human animal, comprising a method of cell fusion or nuclear

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transfer of the invention and a method of obtaining a culture of pluripotent stem cells, comprising a method of cell fusion or nuclear transfer of the invention.

The donor somatic cell is more preferably a stem cell, and in particular a neural stem
5 cell. Nanog is provided as described herein, by expressing it in the donor somatic cell. A MEK inhibitor can be used, again as described elsewhere herein. The MEK inhibitor upregulates expression of Nanog as described herein. It is preferred that Nanog is overexpressed in the donor somatic cell, such as by being expressed at a level greater than the normal, endogenous level of Nanog expression in ES cells, for example at a
10 level that is from 2 to 15 times the level of endogenous expression of Nanog in an ES cell.

An embodiment of this invention is to use a somatic donor cell with a gene defect for nuclear transfer, then correct the gene defect in the resulting pluripotent cell by
15 methods known in the art for use in treatment of a disease. This embodiment is believed to be of application generally to mammalian cells, including murine, human, porcine, ovine, bovine and caprine and in all cases it is preferred that the recipient oocyte and the somatic donor cell are of the same species.

20 In preferred embodiments of the present invention, the cells are pre-treated with a MEK inhibitor.

Reference to Nanog herein refers to the family of polypeptides described in WO 03/064463, including the preferred polypeptides described, and hence refers e.g. to a
25 polypeptide which is a pluripotency determining factor having from 200 to 400 amino acids. Specifically, a mouse pluripotency determining factor is represented by SEQ ID NO 2 therein, a human pluripotency determining factor by SEQ ID NO 4 therein, a rat pluripotency determining factor by SEQ ID NO 6 therein, and a macaque pluripotency determining factor by SEQ ID NO 8 therein, the contents of WO 03/064463 being
30 fully referred to and incorporated herein by reference.

- Nanog refers also to a factor which maintains a cell in a pluripotent state, acts intracellularly and comprises a homeodomain, in particular a homeodomain that has at least 50% sequence identity with the homeodomain from SEQ ID NO: 2, 4, 6 or 8 therein or, in relation to a factor for cells of a given species, one that has at least 50%
- 5 sequence identity with the homeodomain of pluripotency determining factor of the same species. Generally, a homeodomain is around 60 amino acids in length and the factor comprises a homeodomain in which any 20 amino acid fragment has at least 35% sequence identity with the homeodomain of SEQ ID NO: 2, 4, 6 or 8.
- 10 Nanog further refers to isolated polypeptides which include (a) polypeptide molecules comprising an amino acid sequence as set out in SEQ ID NO: 2, 4, 6 or 8; (b) naturally occurring variants of (a); (c) orthologues of (a) or (b), and (d) biologically active and diagnostically or therapeutically useful fragments, analogues and derivatives thereof.
- 15 Nanog additionally includes the polypeptides of SEQ ID NO: 2, 4, 6 and 8 (in particular the mature polypeptide) as well as polypeptides which have at least 50% similarity (preferably at least 50% identity) to the polypeptide of SEQ ID NO: 2, 4, 6 or 8 and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of SEQ ID NO: 2, 4, 6 or 8 and still more preferably at
- 20 least 95% similarity (still more preferably at least 95% identity) to the polypeptide of SEQ ID NO: 2, 4, 6 or 8 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids. "Similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid
- 25 substitutes of one polypeptide to the sequence of a second polypeptide. Various different approaches are known for the calculation of sequence similarity and identity. Generally, a suitable way to perform these calculations is to run database searches using a program such as Smith-Waterman, BLAST or FASTA, and use one or preferably two or even three similarity tables. The Blosum and PAM (Point Accepted
- 30 Mutation) matrices are suitable amino acids similarity matrices for database searching and sequence alignment. If Smith-Waterman or FASTA is used then it is relevant to

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ensure the open gap penalty is large enough, and if the initial runs do not uncover any homologous sequences it can be appropriate to try a different algorithm - this is particularly true when starting with one of the heuristic algorithms, BLAST or FASTA.

5 In examples of the invention set out below in more detail, neural stem cells are used as the somatic cell, i.e. as a source of differentiated epigenome. Neural stem (NS) cells were expandable and could be manipulated genetically (Conti et al, 2005). These can be derived from embryonic and adult brain and from ES cells. NS cells form a homogeneous population that expresses morphological and molecular features of
10 radial glial cells in a reproducible manner. In addition, transplantation assays showed that these cells are not transformed, even after prolonged expansion (Conti et al, 2005). Nanog overexpression in ES cells was shown to enhance reprogramming of the NS cell genome after ESxNS cell fusion. In addition, Nanog expression is necessary for the reprogramming of NS cells as shown by the failure of *Nanog*^{-/-} ES cells to
15 generate ES-NS cell hybrids. However, in our tests this property was rescued by the introduction of transgenic expression of Nanog in both *Nanog*^{-/-} ES cells and, to a lesser extent, in NS cells. Comparison of the ability of ES-ES and ES-NS FACS sorted hybrids to produce ES-like colonies suggested that overexpression of Nanog from ES cells is the only factor required for the reprogramming of the NS cell genome. In the
20 present study we analysed the results in the context of reprogramming and provide a system based on Nanog for the establishment of a pluripotent genome from a differentiated genome.

In further examples, set out in more detail below, overexpression of Nanog in
25 embryonic stem cells (ES) increased 200-fold the frequency of pluripotent hybrids obtained after fusion with neural stem (NS) cells. This cannot be explained by effects of Nanog on cell viability or clonogenicity. Forced expression of Nanog in NS cells did not convert them to ES cells indicating that factors additional to Nanog are necessary for reprogramming. However, Nanog overexpressing ES cells formed
30 pluripotent hybrids with NS cells at the same efficiency as ESxES fusions, in which

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there is no reprogramming. This implied that the level of Nanog is the only limiting factor for reprogramming the NS cell epigenome to pluripotency in hybrids.

The otherwise relatively normal but *Nanog* null ES cells failed to yield any pluripotent hybrids upon fusion with NS cells. Their capacity was fully restored by introduction of a Nanog transgene. These findings demonstrated that, although Nanog is dispensable for the maintenance of pluripotency, it plays a pivotal role in orchestrating formation of the pluripotent epigenome, both in naïve early embryo cells and in reprogramming of differentiated cell nuclei.

10

Also described below is the use of a MEK inhibitor for increasing the efficacy of obtaining a cell with pluripotent properties and upregulating the expression of Nanog in the cells. It has been shown that if cells are treated with a MEK inhibitor then this enhances reprogramming by cell fusion with a similar effect to that of using a Nanog trans gene.

15

In a further embodiment of the present invention, there is provided use of a MEK inhibitor in reprogramming a cell. Preferably, the cell is a somatic cell. In preferred embodiments, the cell is reprogrammed *in vitro*. Preferably, the cell is not a fused cell.

20

There is further provided a method of reprogramming a somatic cell, comprising treating the cell with a MEK inhibitor.

In this respect, the invention allows for reprogramming a somatic cell back to pluripotency, thus enabling derivation of pluripotent cells without cell fusion or nuclear transfer.

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Reference to a MEK inhibitor herein refers to MEK inhibitors in general. Reference is also made to MEK1 inhibitors, for example P184352 (Davies et al, 2000). P184352 has been found to have a high degree of specificity and potency when compared to other known MEK inhibitors.

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The invention is now illustrated in specific embodiments set out in the following examples and accompanied by drawings in which:-

- 5 Fig. 1 shows Nanog expression is necessary for X reactivation in vivo;
- Fig. 2 shows Nanog enhances ES cell ability to generate ES-differentiated cell hybrid colonies;
- Fig. 3 shows the effect of PEG in cell viability and differentiation;
- Fig. 4 shows an analysis of NS epigenome in ES-NS cell hybrids;
- 10 Fig. 5 shows FACS analysis of fusion mixtures;
- Fig. 6 shows Nanog is necessary for the generation of ES like ES-NS hybrid colonies;
- Fig. 7 (Supplementary Figure 1) shows a chromosome count of hybrid clones;
- Fig. 8 (Supplementary Figure 2) shows that ES-NS hybrids exhibit pluripotency after deletion of transgenic Nanog; and
- 15 Fig. 9 shows the effect of PD184352 in the formation of pluripotent ES-NS hybrid colonies.

In more detail, Figure 1 shows that Nanog expression is necessary for X reactivation in vivo. (A-D) Immunofluorescence for Eed and Nanog of female (XX) 4.5 dpc and
 20 diapause embryos resulted from crosses between Nanog heterozygous mice. Panels (A, B) exhibit complete confocal projections of analysed embryos. Arrows indicate Nanog positive cells. (C, D) Higher magnification of A and B nanog positive embryos. Note absence of Eed large foci from Nanog positive cells (white arrowheads). Panels exhibit partial confocal projections of analysed embryos. Red arrowheads point to examples of
 25 cell nuclei exhibiting large Eed focus (inactive X). Embryo nuclei were counterstained with Dapi. (E, F) Tables summarizing the scores for presence (with Xi) or absence (with no Xi) of Eed large focus in Nanog positive cells of 4.5 (E) and diapause (F) embryos.

30 Figure 2 shows Nanog enhances ES cell ability to generate ES-differentiated cell hybrid colonies. (A-F) Illustrated are plates containing hybrid colonies stained with

Leishmans. Scored colonies of fusions involving RH and RHN ES cells with ES O4GiP (A), NS O4GiP (embryo) (B), NS TGFP (D), NS O4GiP (Adult) (E) and MEF TGFP (F) exhibited red and green fluorescence (see right column example). Scored EF4 x NS O4GiPGFPIH (C) colonies exhibited green fluorescence only. All scored colonies displayed ES morphology and scores were performed while cells were in culture. (G) Analysis of the levels of Nanog, Oct4 and Sox2 in RH, RHN, EF4, EF4cre3, ES O4GiP, NS O4GiP (embryo) and NS TGFP cells. α -tubulin was used as loading control. Note that ES O4GiP cells were grown under puromycin selection, which is driven by Oct4 regulatory sequences, therefore representing a relatively pure ES cell population.

Figure 3 shows the effect of PEG in cell viability and differentiation. (A) Bar chart indicating number of ES cells attached to 100mm plates 24 hours after PEG treatment of RH and RHN cells. 10^6 cells were plated. (B)- Illustrated are 96-well plates containing RH and RHN colonies stained with Alkaline phosphatase (AP). PEG treated and untreated cells were allowed to sit for 4 hours in culture plates before being trypsinized and single cell FACS sorted into 96 well plates. Cells were then cultured for 12 days. (C) Table summarizing 96 well plate data.

Figure 4 shows an analysis of NS epigenome in ES-NS cell hybrids. (A-D) Immunofluorescence for trimethyl H3-K27 ($\text{me}_3\text{H3-K27}$) (A) and Ubiquityl H2A (ubH2A) (B) and RNA FISH for Xist (C) and Oct4 (D) in XX NS TGFP, XY RH, XY RHN, XXXY RH-NS TGFP and XXXY RHN-NS TGFP cells. Yellow arrowheads indicate presence of $\text{me}_3\text{H3-K27}$, ubH2A or Xist RNA nuclear body in XX NS TGFP cells. Percentage of cells displaying these markers is indicated in yellow. White arrowheads indicate presence of Oct4 or Xist RNA nascent transcripts. Percentage of cells exhibiting expected allelic expression is indicated in white. All nuclei were counterstained with Dapi. (E) RT-PCR analysis of Blbp, Olig2 and Oct4 in NS TGFP, NS O4GiP, RH, RHN, RH-NS TGFP (clone 1 and 2) and RHN-NS TGFP (clone 1 and 2). Gapdh was used as a ubiquitously expressed control. cl - clone

Figure 5 shows FACS analysis of fusion mixtures. (A-C) Illustrated are FACS plots detecting both red and green fluorescence of RHxNS TGFP, RHNxNS TGFP, RHNxES O4GiP, RHNxMEF TGFP and RHNxT TGFP. (A) mock fusion providing a gate control for the analyses and FACS sorting of the hybrid population; (B) fusion mixture 24 hours after PEG treatment; (C) Purity check of FACS sorted hybrids gated in B. (D) Hybrids sorted in B were plated and the formed colonies were scored as percentage of colonies per plated hybrid. These scores take into account the purity of the FACS sorted cells. Scores from a replicated experiment are indicated in (E).

Figure 6 shows Nanog is necessary for the generation of ES like ES-NS hybrid colonies. (A,B) Illustrated are plates containing hybrid colonies stained for Alkaline phosphatase of fusions between RCxNS O4GiP, RCA-/-xNS O4GiP, RCA-/-NZxNS O4GiP, RCA-/-xNS O4GiP NP, RCB-/-xNS O4GiP, RCB-/- NZxNS O4GiP, RCB-/-xNS O4GiP NP cells. Scores are indicated under each plate. (D) Analysis of the levels of Nanog, Oct4 and Sox2 in ES O4GiP, RC, RCA-/-, RCB-/-, RCA-/-NZ, RCB-/-NZ, NS O4GiP and NS O4GiP NP cells. α -tubulin was used as loading control. (E) RT-PCR analysis of Blbp, Olig2, GFP and Oct4 in ES O4GiP, NS O4GiP NP and NS O4GiP cells. Gapdh was used as a ubiquitously expressed control. (F) Effect of PEG in the viability of RC, RCB-/- and RCB-/- NZ cells. Bar chart indicates number of ES cells attached to 100mm plates 24 hours after PEG treatment. 2×10^6 cells were plated.

Figure 7 (Supplementary Figure 1) shows a chromosome count of hybrid clones. Examples show metaphase spreads of RH-NS O4GiP (A), RHN-NS O4GiP (B), RH-NS TGFP (C) and RHN-NS TGFP (C). Metaphase chromosomes were counted in 8 to 11 cells for 3 clones of each hybrid cell line and are indicated under each illustration. cl-clone.

Figure 8 (Supplementary Figure 2) shows ES-NS hybrids exhibit pluripotency after deletion of transgenic Nanog. (A) Schematic representation of EF4 and Neural Stem (NS) O4GiP RB cells. EF4 cells contain a constitutively expressing Nanog transgene flanked by lox P sites, which allows the deletion of Nanog using cre recombinase that

will then lead to constitutive expression of GFP. NS O4GiP RB contains a GFP transgene driven by Oct4 promoter sequences and a constitutively expressing RED fluorescent transgene. Grey boxes indicate IRES sequences. (B) EF4-NS RB O4GiP hybrids exhibiting green (GFP) and Red (dsRED) fluorescence. These markers are expressed from the donor NS cell genome. (C) EF4-NS RB O4GiP cells after Cre deletion of Nanog. Note increased GFP expression. (D) Illustration shows embryoid body (EB) differentiation of EF4-NS RB O4GiP cre hybrids. (E) RT-PCR analysis of T-brachyury expression in EF4-NS O4GiP RB cre EBs at 3, 5, 7 and 8 days (d) of differentiated (diff.). Undifferentiated hybrids and the parental/donor cell lines were also analysed. Gapdh was used as a ubiquitously expressed control. (F) Immunofluorescence for α -actinin (blue) of plated EBs.

Figure 9 shows analysis of the effect of PD184352 in the formation of pluripotent ES-NS hybrid colonies. (A-C)- FACS analysis for red and green fluorescence of RHxNS TGFP fusions. (A) Fusion mixture 24 hours after PEG treatment; (B) Purity check of FACS sorted hybrids gated in A. (C) Hybrids sorted in A were plated and the formed colonies were scored as percentage of colonies per plated hybrid. These scores take into account the purity of the FACS sorted cells. (D) Summary of data. (E) Examples of hybrid colony morphology.

20

Example 1

Nanog expression in the early embryo is necessary and correlates with progressive X reactivation

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X-chromosome reactivation is known to occur in vivo in the female embryo epiblast at around 4.5 dpc (Mak et al, 2004; Okamoto et al, 2004). To investigate a putative involvement of Nanog in in vivo reprogramming we examined female *Nanog*^{-/-} embryos at 4.5 dpc by immunofluorescence for both Eed and Nanog. Eed is a marker of the inactive X chromosome in pre-implantation embryos (Silva et al, 2003; Mak et al, 2004; Okamoto et al, 2004). Of 25 embryos resulting from crosses between

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Nanog^{+/-} mice, 6 lacked *Nanog* staining, four of which were female. *Nanog* negative embryos showed in all the nuclei of the embryo presence of a single large Eed focus, with the exception of apoptotic and rare poorly Eed stained cells (Figure 1A). Female *Nanog* positive embryos exhibited a variable number of *Nanog* positive ICM cells (Figure 1E). In contrast to the rest of the embryo, a proportion of these cells showed no Eed focus, which is consistent with X reactivation (Figure 1C,E). In general *Nanog*^{-/-} embryos were smaller. To determine if this would be due to a developmental delay we analysed 6.5 dpc embryos in diapause (Figure 1B). Of 31 embryos examined, 8 lacked *Nanog* staining, of which 3 were female. As with the 4.5 dpc embryos these also exhibited the presence of an Eed focus in all cells. On the contrary, female *Nanog* positive embryos did not reveal an Eed focus on almost all the cells positive for *Nanog* (Figure 1D-F).

In conclusion, this data shows that *Nanog*^{-/-} female embryos fail to reactivate the silent X-chromosome, suggesting a role for *Nanog* in epigenetic reprogramming.

Example 2

ES cells overexpressing *Nanog* have enhanced ability to generate ES like ES-differentiated cell hybrid colonies

To investigate whether *Nanog* is involved in reprogramming we used cell fusion of ES cells with other ES, NS, Mouse Embryonic Fibroblasts (MEF) and Thymocytes (T) and determined the ability of WT and *Nanog* manipulated cells to produce ES-like ES-differentiated cell hybrid colonies. Pluripotent hybrids have previously been produced using 3 different methods of cell fusion, namely electrofusion, polyethylene glycol (PEG) mediated and spontaneous cell fusion by co-culture (Matveeva et al, 1998; Terada et al, 2002; Ying et al, 2002). Here we used PEG induced fusion to generate cell hybrids. Initially we used RH ES cells that express constitutively the red fluorescence and the hygromycin resistance genes, and a RH ES cell derivative, RHN, which overexpresses *Nanog*. These were fused to both ES and NS cells carrying a

transgene containing the regulatory sequences of the mouse Oct4 gene (Yeom et al, 1996) driving GFP and puromycin resistance (O4GiP) (Ying et al, 2002). This transgene is expressed exclusively in pluripotent cells, and thus reactivates only if the NS cell genome reprograms, after ES-NS cell fusion. The NS cells were derived from
5 14.5 dpc foetal forebrains. In order to generate comparable results among different fusion pairs, the fusion procedure was performed simultaneously using the same parameters. As a result of fusing ES cells with either ES or NS cells, drug resistant red and green fluorescent colonies appeared on culture plates. These invariably displayed ES morphology but with enlarged nuclei. Independently of Nanog overexpression,
10 fusion between ES cells generated plates containing high numbers of hybrid colonies (Figure 2A). In contrast, ES x NS cell fusions had distinct results. RHN x NS O4GiP fusions, compared to RH x NS O4GiP fusions, exhibited a significantly higher number of colonies, approximately 200 fold difference (Figure 2B).

15 To confirm the increased ability of Nanog overexpressing ES cells in generating ES-NS cell hybrid colonies, we performed experiments using floxed Nanog ES cells (EF4) and a cre deleted derivative, EF4 cre3 (Chambers et al, 2003). These were fused to NS O4GiP derivative cells carrying a second transgene, CAG-egfp-Ires-Hygro (GFPIH) that conferred constitutive Hygromycin drug resistance. The EF4 cell line was
20 previously shown to self renew in the absence of LiF and the EF4 cre3 cell line can produce chimeras after blastocyst injection (Chambers et al, 2003). Consistently with the above results, EF4 x NS cell fusions produced a high number of drug resistant colonies whereas EF4 cre3 x NS cell fusions failed to produce drug resistant colonies (Figure2C).

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To further characterize this phenomenon we used an independent NS cell line, TGFP, which expresses constitutively the fusion protein TAUGFP and the puromycin resistance gene (Pratt et al, 2000). These cells were fused to both RH and RHN ES cells. Yet again, contrasting results were obtained, with RHN x NS TGFP fusions
30 generating a high number of hybrid colonies whereas RH x NS TGFP fusions produced only one hybrid (Figure 2D). This phenomenon was also observed for ES x

NS (ES and adult derived) fusions, and to a lesser extent for ES x T and ES x MEF fusions (Figure 2E, F).

To examine this correlation in more detail, we compared the levels of Nanog, Oct4 and Sox2 between cell lines (Figure 2G). High levels of Nanog were detected in both RHN and EF4 ES cells whereas similar lower levels of Nanog were found for the remaining ES cell lines. In the case of EF4 and EF4 cre3 cell lines the difference in Nanog levels has been determined as 5 fold (Yates and Chambers, 2005). The levels of the pluripotency markers Oct4 and Sox2 did not differ among all ES cell lines used. As expected NS cells showed expression for Sox2.

Together, these results show that Nanog overexpression in ES cells enhances significantly the ability of these to generate ES-NS, but not ES-ES hybrid colonies, supporting a role for Nanog in reprogramming.

Example 3

PEG does not induce increased cell death or differentiation on RH compared to RHnanog ES cells

In order to assess a possible differential effect of PEG treatment on RH versus RHN ES cells we went on to analyse cell survival and differentiation. Cell survival was scored as number of ES cells attached to 100 mm plates 24 hours after PEG treatment. From 10^6 PEG treated RH and RHN ES cells plated, 6.6×10^5 and 5.3×10^5 cells were counted the following day respectively (Figure 3A). To verify possible induction of differentiation and changes in the ability of PEG treated ES cells to grow and form ES cell colonies, single cell FACS sorting of PEG treated and untreated RH and RHN ES cells was performed. Approximately 12 days after FACS sorting wells were stained with Alkaline-Phosphatase for undifferentiated ES cell status identification. The majority of plated untreated RH and RHN ES cells formed undifferentiated colonies (Figure 3 B,C), while PEG treated cells showed a reduction in the total number of

colonies. RH colonies were reduced by almost 50%, but the impact on RHN colony formation was much less. No increased differentiation was observed, with only 4 out of 39 RH colonies and 1 out of 73 RHN colonies being AP negative.

- 5 Overall this data suggests that PEG induces some degree of cell death in both RH and RHN ES cells, but does not induce cell differentiation. RHN cells were shown to be 2-fold more clonogenic after FACS and PEG treatment, which is insufficient to account for the observed 200 fold increase in hybrids.

10 **Example 4**

Hybrid cells segregate chromosomes and revert to diploidy at significant frequencies

- 15 To define the ES-NS cell hybrids we looked at the chromosomal content and to the identity of the NS cell epigenome. To analyse the former we looked at 12 independent ES-NS cell hybrid clones originated from fusions between RH x NS Oct4GiP, RHnanog x NS Oct4GiP, RH x NS TGFP and RHnanog x NS TGFP. After being passaged 5 times, the clones exhibited 80 chromosomes in the majority of metaphases
20 scored (Supplementary Figure 1).

Analysis of 6 ES-NS clones where selection was made exclusively for the NS cell genome showed presence of a diploid population that increased with the passage number. This is probably due to a growth advantage of diploid cells in relation to
25 tetraploid cells. Two of these clones were analysed at passage 12 and showed 30 and 50% diploid (40 chromosomes) content respectively. In addition, the cell population was made of two discrete populations, one with nearly 40 chromosomes and another with 80, suggesting that chromosomal segregation occurred in a single event. Further analyses will be required to evaluate the proportion of NS cell genome in these newly
30 reprogrammed diploid hybrids. Nevertheless, the data implies that diploid undifferentiated ES cells can be obtained from differentiated cells through cell fusion.

Example 5

NS genome acquires ES cell identity in ES-NS cell hybrids

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To evaluate the identity of the NS cell epigenome in ES-NS cell hybrids we looked at the status of the inactive X chromosome, and allelic expression of Oct4 and the Neural stem cell genes Blbp and Olig2. In contrast to XX pluripotent cells, XX differentiated cells have one inactive X chromosome. Among various cytological markers, the inactive X chromosome is found associated with Xist RNA and is enriched for trimethyl H3-K27 and ubiquitin H2A (Brown et al, 1992; Napoles et al, 2004; Silva et al, 2003). Concurrently with the differentiated status of NS cells, confocal analysis revealed one single nuclear body of Xist RNA, trimethyl H3-K27 and mono-ubiquitin H2A (Figure 4A-C). In addition, the inactive X was found hypoacetylated for H3-K9, hypomethylated for H3-K4 and enriched for the histone variant macroH2A (data not shown). Thus, if reprogramming occurs in XXXY ES-NS cell hybrids the inactive X should lose the silencing markers. This is what was observed, with the Xist RNA nuclear body being lost and instead, three pinpoint Xist signals characteristic of ES cells and corresponding to Xist nascent transcripts were detected (Figure 4C). Furthermore, immunofluorescence analysis of the chromatin status of ES-NS cell hybrids showed loss of trimethyl H3-K27 and mono-ubiquitinated H2A nuclear body (Figure 4A,B). RNA FISH analysis for Oct4 expression of XXXY ES-NS hybrids revealed the presence of 3 or 4 pinpoint signals per cell nucleus demonstrating reactivation of the Oct4 alleles of NS cell origin (Figure 4D). Further characterization of XXXY ES-NS hybrids included the expression analysis of genes, Olig2 and Blbp, which are expressed in NS cells and silenced in ES cells (Conti et al, 2005). In agreement with the above described results RT-PCR analysis revealed that both Blbp and Olig2 are switched off in ES-NS hybrid cells (Figure 4E).

To investigate pluripotency competence, hybrids from fusions between Nanog overexpressing ES and NS cells were allowed to differentiate, by embryoid body

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formation, and analysed for expression of the mesoderm marker T-brachyury (supplementary Figure 2). Taking into account that Nanog overexpression inhibits differentiation (Chambers et al, 2003) EF4-NS O4GiP RB hybrids were used. As described previously EF4 cells contain a floxed Nanog transgene which allows its deletion by the action of cre recombinase, while NS O4GiP RB express constitutively the red fluorescent protein and the blasticidin resistance gene (supplementary Figure 2A). Therefore, EF4-NS O4GiP RB hybrids exhibited red and green fluorescence from NS origin (supplementary Figure 2B). After cre recombinase treatment, loss of Nanog overexpression was observed as hybrids displayed increased green fluorescence (supplementary Figure 2C). These EF4-NS O4GiP RB cre hybrids were then allowed to differentiate by Lif withdrawal and Embryonic Body (EB) formation and analysed for T-brachyury expression (Supplementary Figure 2D, E). As expected, T-brachyury was not detected in the donor cells or in undifferentiated hybrids. However, and in accordance with pluripotency potential, it was expressed in differentiated hybrids. EB's were also plated, 1 to 3 per well, and analysed for presence of contracting cells. Approximately 70% (24/35) of wells showed beating cells and stained positively for the specific marker of skeletal and cardiac muscle cells α -actinin (supplementary Figure 2F).

Collectively, the data shows that for all the analysed markers, the NS genome acquires an ES cell identity in ES-NS cell hybrids. In addition, hybrids retained pluripotent capacity.

Example 6

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Nanog overexpression in ES cells is the only factor required for the reprogramming of NS cells after cell fusion

It is possible that ES cells overexpressing nanog could be more fusogenic than WT ES cells. To address this point, the cell populations RH x NS TGFP and RHN x NS TGFP, 24 hours after fusion, were analysed by FACS for the presence of cells exhibiting both

red and green fluorescence, primary hybrids. As expected, the number of double positive cells was relatively low. RH x NS TGFP and RHN x NS TGFP showed 0.17% and 0.1% double positive cells respectively (Figure 5B). In addition, we tested the ability of FACS sorted primary hybrids to form colonies. Hybrids were sorted for both
5 red and green fluorescence 24 hours after fusion. At this stage, reprogramming is likely to be in process, but not complete as it was previously shown that GFP expression, driven by Oct4 regulatory sequences, is first detected at around 48 hours after cell fusion (Tada et al, 2001; Do and Scholer, 2004). So, in addition to considerations such as cell cycle coordination, primary hybrids at this stage must
10 overcome epigenome differences to produce ES like colonies. Plated primary hybrids were selected in both hygromycin and puromycin 3 days after FACS sorting. Results from two independent experiments showed that sorted primary hybrids from RH x NS TGFP fusions did not produce hybrid colonies (Figure 5 D,E). In contrast, 34 and 11 hybrid colonies out of 2000 and 700 FACS sorted cells respectively, were obtained
15 from RHN x NS TGFP fusions (Figure 5 D,E).

These results raised the question of the relevance of Nanog overexpression in reprogramming. To address this, the number of RHN-NS TGFP colonies formed was compared to that of RHN-ES O4GiP, the latter being considered the reference unit for
20 colony formation per plated hybrid. Taking into account the proportion of hybrid colonies per total number of true double positive FACS sorted cells, RHN-NS TGFP and RHN-ES O4GiP primary hybrids appear to have the same ability to produce ES like colonies (Figure 5 D,E).

25 In conclusion, FACS sorting and analysis of ES-NS hybrids demonstrated that Nanog overexpression does not increase cell fusion but confirmed that it enhances the generation of ES like ES-NS hybrid colonies. It also indicates that Nanog could be the only limiting factor required for overcoming epigenetic differences in the generation of ES-NS hybrid colonies.

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Example 7

Neural cells as donor cells in reprogramming

- In somatic cell nuclear transfer (SCNT) the donor cell type used was shown to affect
5 ES cell derivation efficiency from blastocysts (Hoechedlinger and Jaenisch 2002;
Blelloch et al, 2004; Eggan et al, 2004; Inoue et al, 2005). In these studies, ES cells
were shown to give the best results suggesting that the differentiation status of the
donor cell is an important factor. NS cells are multipotent somatic stem cells (Conti et
al, 2005) therefore containing a somewhat plastic epigenome. In our experiments
10 RHN-NS primary hybrids were shown to produce ES like hybrid colonies at the same
efficiency as RHN-ES primary hybrids. To address whether this is due to a property of
the NS epigenome or to Nanog overexpression alone we analysed the ability of MEFs
and Thymocytes (T) primary hybrids to produce ES like colonies. MEFs and T cells
were fused to RH and RHN ES cells. Like for RH-NS, RH-MEFs and RH-T primary
15 hybrids failed to produce ES like colonies (data not shown). RHN-MEFs and RHN-T
primary hybrids were able to generate ES like colonies but at a much lower efficiency
(0.08% and 0.33% respectively) than RHN-ES and RHN-NS primary hybrids (Figure
5A-E).
- 20 These data indicate that the NS cell epigenome appears to be more susceptible to
reprogramming by fusion to Nanog overexpressing ES cells than its differentiated
counterparts.

Example 8 (Comparative)

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Nanog expression in NS cells is not sufficient for reprogramming

- In order to investigate whether Nanog expression is sufficient to reprogram the NS
genome into that of an ES cell, NS cells were transfected with Nanog (NS O4GiP NP).
30 These cells stably express Nanog and the protein levels are similar to those found in
ES cells (Figure 6D). In addition, cells proliferated normally and exhibited expression

of the NS cell markers Blbp and Olig2 (Figure 6E). However, there was no endogenous Oct4 or GFP expression driven from Oct4 regulatory sequences. When plated into standard GMEM or N2B27 ES medium, cells differentiated into astrocyte type cells as previously reported (Conti et al, 2005). Additional experiments using
5 trichostatin A and 5-Azacytidine, two powerful epigenetic destabilizers, also failed to reprogram Nanog expressing NS cells (data not shown).

Taken together these results suggest that outside an ES cell context, Nanog expression is not sufficient to induce reprogramming.

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Example 9

Nanog is necessary for the generation of ES like ES-NS cell hybrid colonies

15 Cultures of both Nanog null isolated ICMs and ES cells failed to retain an undifferentiated state (Mitsui et al, 2003). These results were interpreted as indicating a role for Nanog in the maintenance of the pluripotent state. However, using a conditional deletion strategy, we have derived *Nanog*^{-/-} ES like cells (RC^{-/-}) that express pluripotent markers, can be serially passaged and retain pluripotent potential.
20 The strategy involved the introduction of a floxed Nanog transgene into the ES cell followed by targeted deletion of both Nanog endogenous genes and finally cre excision of transgenic Nanog. Further evidence that RC^{-/-} cells are true ES cells comes from the fact that selection can be made for the activity of endogenous Nanog promoters driving G418 and hygromycin resistant genes, introduced by the targeting
25 vectors. This allowed the selection of a pure ES-like population in which the levels of both Oct4 and Sox2 were similar to other Nanog expressing ES cell populations (Figure 6D). To investigate whether Nanog is necessary for reprogramming, parallel fusions of either RC^{-/-} or its parental cell line RC were performed with NSO4GiP cells. RC^{-/-} were also fused with NS O4GiP expressing transgenic Nanog, NS O4GiP NP.
30 Finally, RC^{-/-} cells rescued with a Nanog expressing transgene (RC^{-/-} NZ) were fused to NS O4GiP. These experiments were performed using two independently targeted

- clones, RC-/- A and B. In order to obtain a meaningful readout of hybrid colony numbers, $2 \times 10^6:2 \times 10^6$ fusion mixtures were plated. As expected the fusion of the parental cell line, RC, with NSO4GiP generated hybrid colonies (Figure 6 A,B). In contrast RC-/- cells have completely lost this ability. RC-/- cells rescued with Nanog, RC-/- NZ, not only gain the ability of producing hybrid colonies but had it further enhanced in relation to RC cells. This result correlates with the presence of higher levels of Nanog in RC-/- NZ cells (Figure 6D). Interestingly, RC-/- cells were also able to generate hybrid colonies when fused to NSO4GiP NP, which express Nanog.
- 10 Together, these results suggest that the presence of Nanog expression is essential for the reprogramming of the NS genome after ESxNS cell fusion.

Example 10

15 The expression of Nanog is increased by a MEK inhibitor

It was shown that PD184352, an inhibitor of MEK, increases the levels of Nanog in ES cells.

- 20 The effect of PD184352 in reprogramming was also investigated by determining the conversion of NS cells to pluripotency in the context of cell fusion.

- RH ES cells, which express constitutively the dsRed fluorescent protein and hygromycin resistance, were fused to foetal derived Neural Stem cells (NS TGFP) that express the fusion protein TauGFP linked via an IRES to puromycin resistance. In one of the fusions RH cells were treated for 3 days prior and after fusion with $3 \mu\text{M}$ PD184352. In the control no PD184352 was added. Treated and untreated primary hybrids were sorted 24 hours after fusion and then plated (Figure 9A-C). Hygromycin and puromycin selection were added to the ES medium 3 days later. Colonies expressing dsRed2 and GFP fluorescence and exhibiting ES cell morphology were scored (Figure 9D and E). Results showed that PD184352 enhanced ES-NS hybrid

colony formation by 45-fold. Interestingly, the percentage of hybrid colonies formed per plated hybrid in PD184352 treated RH cells was just 2-fold lower compared to Nanog overexpressing ES cells (2.25% vs 4%). This result shows that PD184352 enhances reprogramming in the cell fusion context. This effect is likely to be mediated
5 by the increased levels of Nanog in treated RH cells. Accordingly, if Nanog is endogenously expressed then the MEK inhibitor can be used to upregulate Nanog and achieve associated effects, such as increased reprogramming.

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The invention thus provides methods of reprogramming, genetic modification of cells
20 via cell fusion and combinations, progeny and uses thereof.

Claims

1. A method of obtaining a pluripotent genome from a differentiated genome, comprising:-
 - 5 fusing a pluripotent cell with a differentiated cell; and
overexpressing Nanog in the pluripotent cell, the differentiated cell and / or the resultant fused cell.
2. A method according to claim 1, comprising overexpressing Nanog in the
10 pluripotent cell and / or the differentiated cell.
3. A method according to any of claims 1 to 2, comprising expressing Nanog in the fused cell.
- 15 4. A method of obtaining a pluripotent genome from a differentiated genome, comprising:-
fusing a pluripotent cell with a differentiated cell to form a fused cell; wherein at least one of the pluripotent cell, the differentiated cell or the fused cell is treated with a MEK inhibitor.
20
5. A method according to any preceding claim, wherein the pluripotent cell is an embryonic stem (ES) cell, an embryonic carcinoma (EC) cell or an embryonic gonadal (EG).
- 25 6. A method according to any preceding claim, wherein the differentiated cell is a somatic cell.
7. A method according to claim 6, wherein the differentiated cell is a stem cell.
- 30 8. A method according to claim 6, wherein the differentiated cell is a neural stem cell.

9. A method according to any preceding claim, wherein the pluripotent cell and the differentiated cell are mouse cells.
- 5 10. A method according to any preceding claim, wherein the pluripotent cell and the differentiated cell are human cells.
11. A method according to any preceding claim, comprising culturing the fused cell so as to obtain a diploid cell.
- 10 12. A method according to any preceding claim, wherein fusing of the pluripotent cell and the differentiated cell results in a tetraploid cell and the method comprises obtaining progeny of the tetraploid cell and identifying such progeny which are diploid.
- 15 13. A method according to any preceding claim, further comprising culturing a diploid, pluripotent cell obtained as progeny of the fused cell, and deriving a differentiated cell from the diploid, pluripotent cell.
14. A method of genetically modifying a cell, comprising:-
- 20 providing a first cell containing chromosomes,
providing a second cell containing chromosomes,
fusing the first cell and the second cell to form a fused cell, and
culturing the fused cell so as to obtain a diploid cell containing at least one chromosome from the first cell and at least one chromosome from the second cell.
- 25 15. A method according to claim 14, wherein at least one of the first cell, the second cell and the fused cell are treated with a MEK inhibitor.
16. A method according to claim 14 or 15, wherein the first cell and the second cell
- 30 are both diploid.

17. A method according to claim 14, 15 or 16, wherein the first cell and the second cell are both mouse cells.
18. A method according to claim 14, 15 or 16, wherein the first cell and the second
5 cell are both human cells.
19. A method according to any of claims 14 to 18 wherein one of the first cell and the second cell is pluripotent and the other of the first cell and the second cell is optionally pluripotent.
- 10 20. A method according to claim 19 wherein the other cell is a somatic cell.
21. A method according to claim 19 or 20 wherein the other cell is a stem cell.
- 15 22. A method according to any of claims 19 to 21 wherein the other cell is a neural stem cell.
23. A method according to any of claims 14 to 22 comprising:-
identifying a first cell having (i) a desired gene and (ii) an undesired gene
20 which is to be modified,
identifying a second cell having a modified form of the undesired gene,
fusing the first cell and second cell,
culturing the fused cell, and
identifying a progeny of the fused cell containing both the desired gene and the
25 modified form of the undesired gene.
24. A method according to claim 23, for curing a genetic defect in the first cell wherein the undesired gene constitutes a gene having a defect and the modified form of the gene in the second cell constitutes the cure.

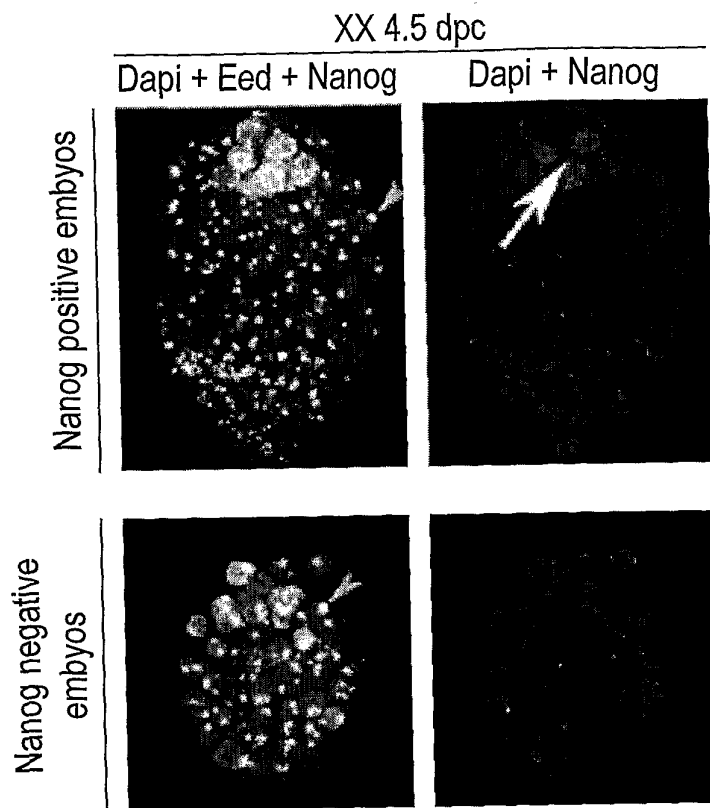
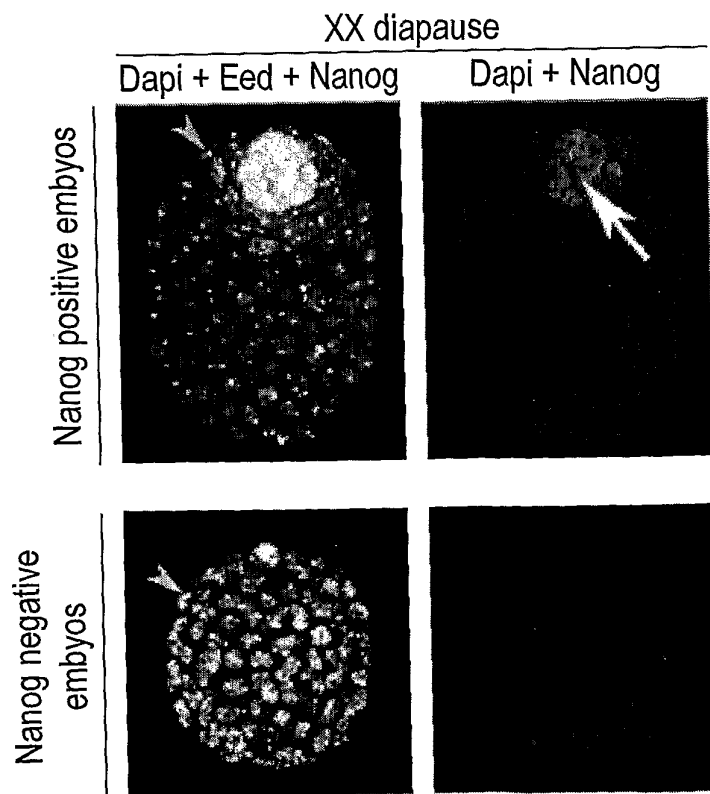
25. A method according to claim 24, comprising genetically modifying the second cell prior to fusion with the first cell so as to introduce into the second cell the modified form of the undesired gene.
- 5 26. A method according to any of claims 14 to 25 comprising culturing the cells in the presence of Nanog.
27. A method according to any of claims 14 to 26 comprising expressing Nanog in the first cell and/or the second cell.
- 10 28. A method of cell fusion, comprising:-
fusing a first cell and a second cell in the presence of Nanog.
29. A method of cell fusion, comprising:-
15 fusing a first cell and a second cell in the presence of a MEK inhibitor.
30. A method according to claim 28 or 29, wherein the first cell is pluripotent.
31. A method according to any of claims 28 to 30, wherein the first cell is an ES,
20 EC or EG cell.
32. A method according to any of claims 28 to 31, wherein the second cell is a somatic cell.
- 25 33. A method according to claim 32, wherein the second cell is a stem cell.
34. A method according to claim 33, wherein the stem cell is a neural stem cell.
35. A method according to any of claims 28 to 34 comprising expressing Nanog in
30 the first cell.

36. A method according to any of claims 28 to 35 wherein fusing the first and second cells yields an aneuploid cell and the method comprising culturing the fused cell to obtain diploid progeny thereof.
- 5 37. A method of obtaining a live non-human animal, comprising a method of cell fusion according to any of claims 28 to 36.
38. A method according to any preceding claim wherein the MEK inhibitor is a MEK1 inhibitor.
- 10 39. A method according to claim 38, wherein the MEK1 inhibitor is PD184352.
40. A method according to any preceding claim wherein the cells are treated with between around 0.1 μ M and around 25 μ M MEK inhibitor.
- 15 41. A method according to claim 40, wherein the cells are treated with between around 1 μ M and around 5 μ M MEK inhibitor.
42. A cell obtained according to a method of any preceding claim.
- 20 43. Use of a cell according to claim 42 in manufacture of a medicament for use in therapy.
44. Use according to claim 43 in cell therapy.
- 25 45. A method of treatment, comprising administering to a patient an effective amount of cell according to claim 42.
46. A method of treatment comprising administering to a patient an effective
30 amount of a genetically modified cell obtained according to any of claims 14 to 27.

47. A method of treatment according to claim 46, comprising:-
identifying a patient needing a genetically modified cell,
identifying the genetic modification required to treat the patient,
preparing a genetically modified cell according to the method of any of claims
5 14 to 27.
48. Use of Nanog in reprogramming a cell obtained by a fusion of a pluripotent cell
and a somatic cell.
- 10 49. Use according to claim 48, wherein the pluripotent cell is an ES cell.
50. Use according to claim 48 or 49, wherein the somatic cell is a neural stem cell.
51. Use of a MEK inhibitor in reprogramming a cell obtained by a fusion of a
15 pluripotent cell and a somatic cell.
52. Use according to claim 51, wherein the pluripotent cell is an ES cell.
53. Use according to claim 50 or 51, wherein the somatic cell is a neural stem cell.
20
54. A method of nuclear transfer, comprising transferring a somatic cell nucleus
into a recipient cell to form a nuclear transfer cell, and overexpressing Nanog in the
nuclear transfer cell.
- 25 55. A method according to claim 54, wherein the recipient cell is an oocyte.
56. A method according to claim 54, wherein the recipient cell is an enucleated
oocyte.

57. A method according to any of claims 54 to 56, comprising expressing Nanog at a level that is from 2 to 15 times the level of endogenous expression of Nanog in an ES cell.
- 5 58. A method of nuclear transfer, comprising transferring a somatic cell nucleus into a recipient cell to form a nuclear transfer cell, and treating the nuclear transfer cell with a MEK inhibitor.
59. A method according to claim 58, wherein the recipient cell is an oocyte.
- 10 60. A method according to claim 58, wherein the recipient cell is an enucleated oocyte.
61. A method of obtaining a live non-human animal, comprising a method of
- 15 nuclear transfer according to any of claims 54 to 60.
62. A method of obtaining a culture of pluripotent stem cells, comprising a method of nuclear transfer according to any of claims 54 to 60.
- 20 63. Use of a MEK inhibitor in upregulating expression of Nanog.
64. Use of a MEK inhibitor in reprogramming a cell.
65. Use according to claim 64, wherein the cell is a somatic cell.
- 25 66. Use according to claim 64 or 65, wherein the cell is reprogrammed *in vitro*.
67. Use according to any of claims 64 to 66, wherein the cell is not a fused cell.
- 30 68. A method of reprogramming a somatic cell, comprising treating the cell with a MEK inhibitor.

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*Fig. 1(A)**Fig. 1(B)*

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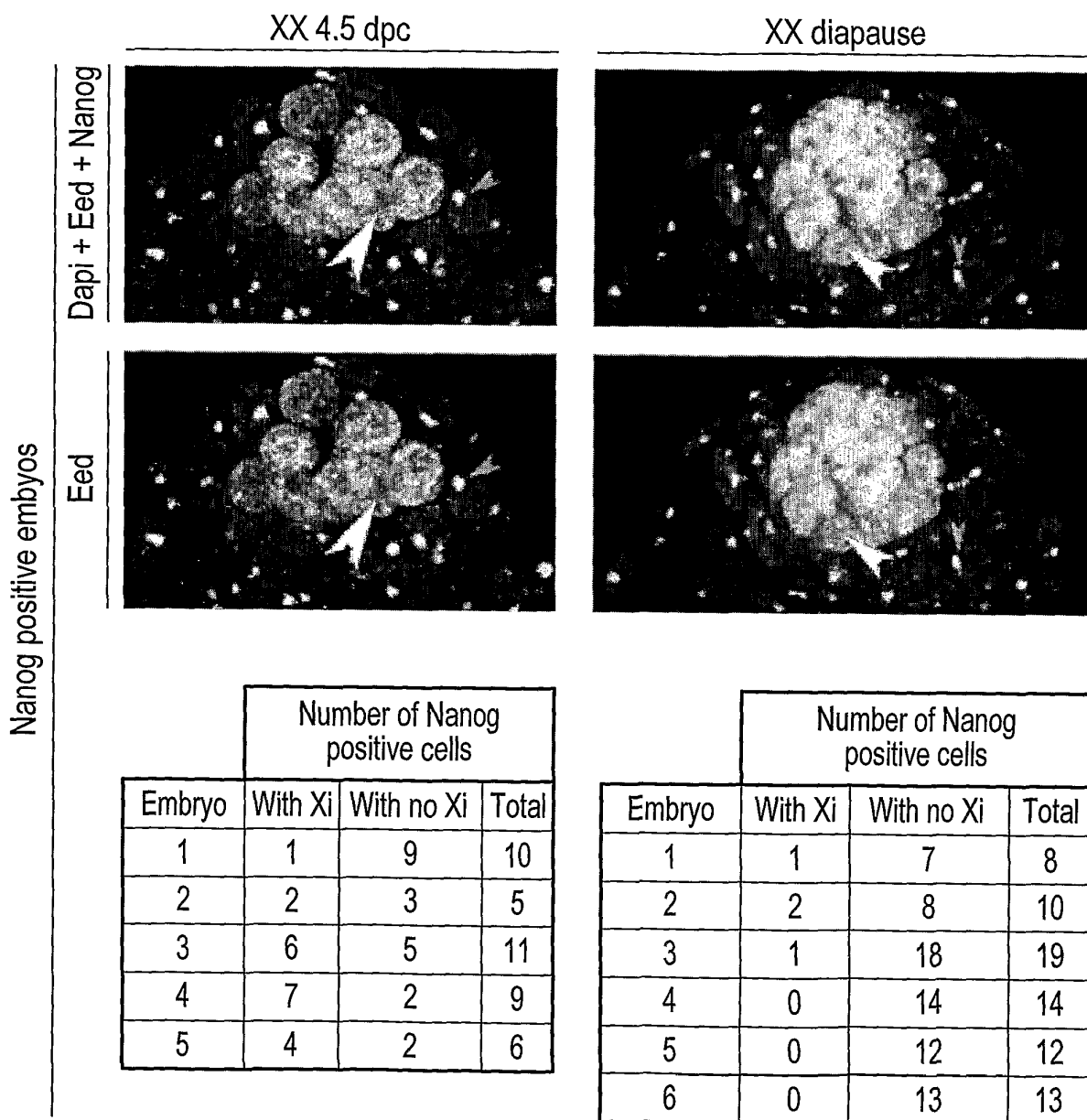


Fig.1(C)

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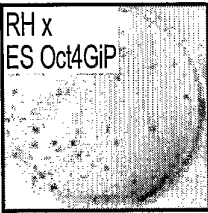
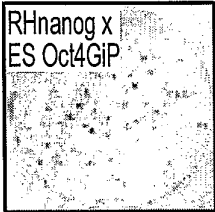
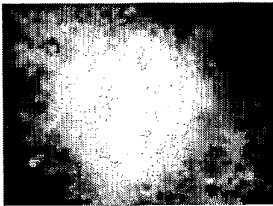
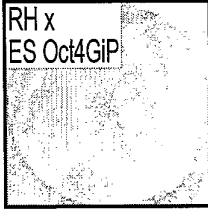
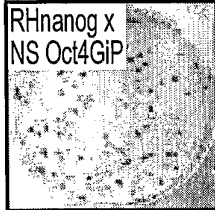
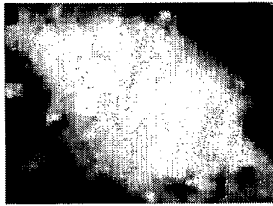
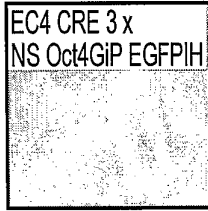
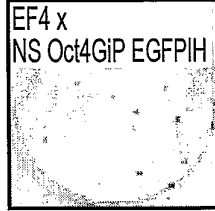
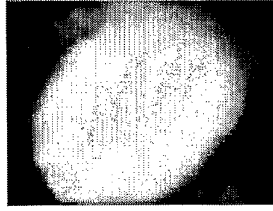
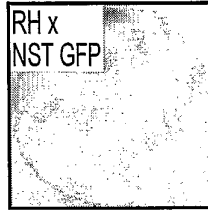
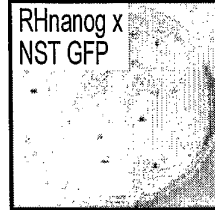
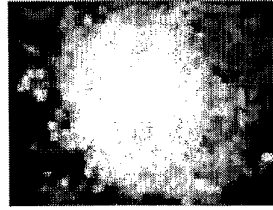
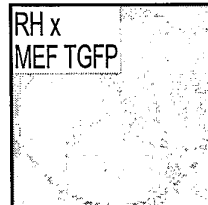
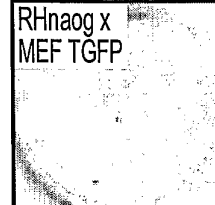
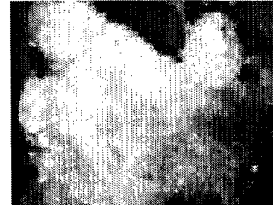
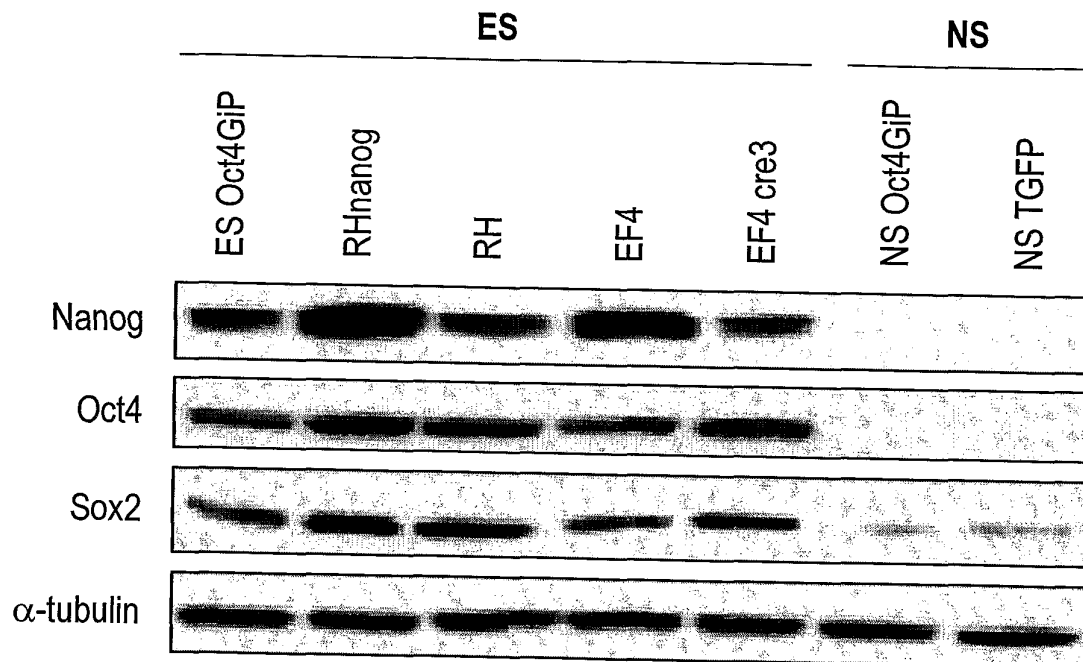
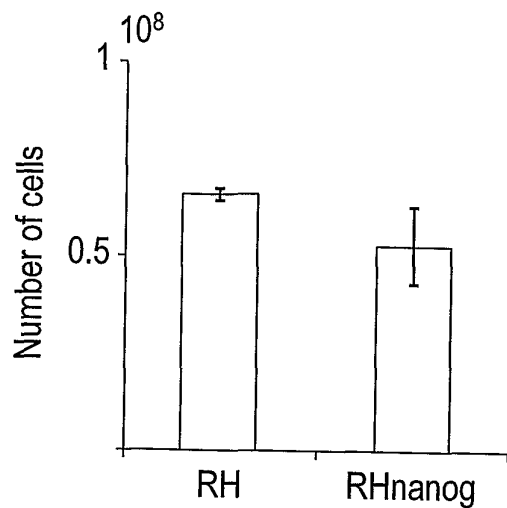
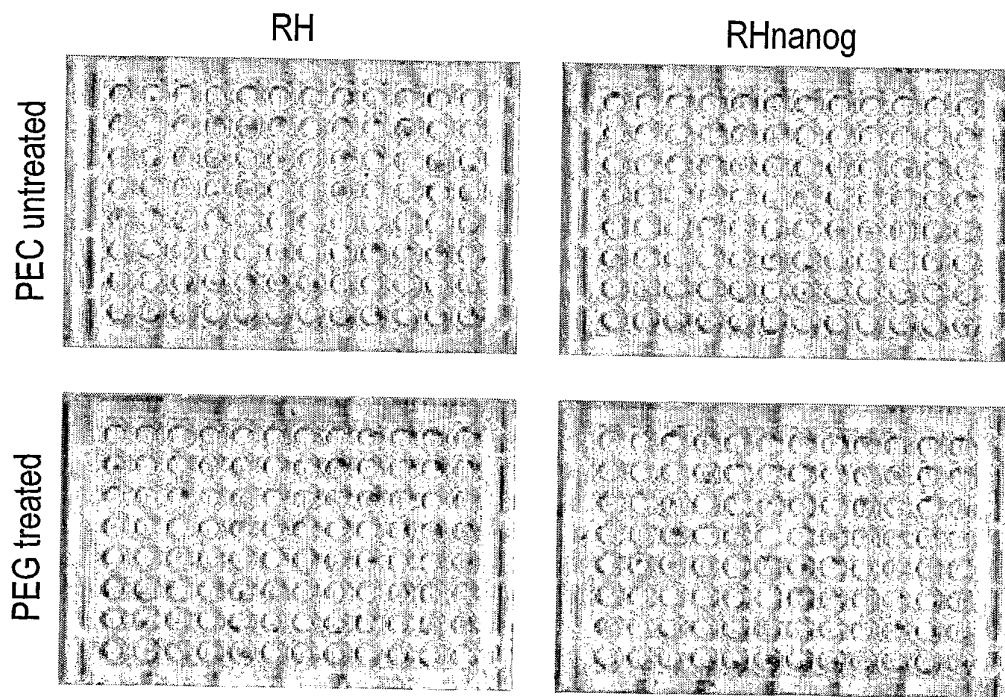
	<u>NO</u> overexpression of Nanog	<u>Colonies</u> Plates	over expression of Nanog	Example of scored hybrid colony
ES x ES 2.5x10 ⁵ : 2.5x10 ⁵ cells/plate		$\frac{313}{3} \vee \frac{575}{3}$		
ES x NS 5x10 ⁵ : 5x10 ⁵ cells/plate		$\frac{3}{4} \vee \frac{608}{4}$		
		$\frac{0}{3} \vee \frac{129}{3}$		
		$\frac{1}{4} \vee \frac{133}{4}$		
ES x MEF 10 ⁶ : 10 ⁶ Cells/plate		$\frac{0}{4} \vee \frac{27}{4}$		

Fig.2(A)

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*Fig.2(B)*

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*Fig.3(A)**Fig.3(B)*

	RH		RHnanog	
	- PEG	+ PEG	- PEG	+ PEG
AP +	62	35	80	72
AP -	2	4	0	1

Fig.3(C)

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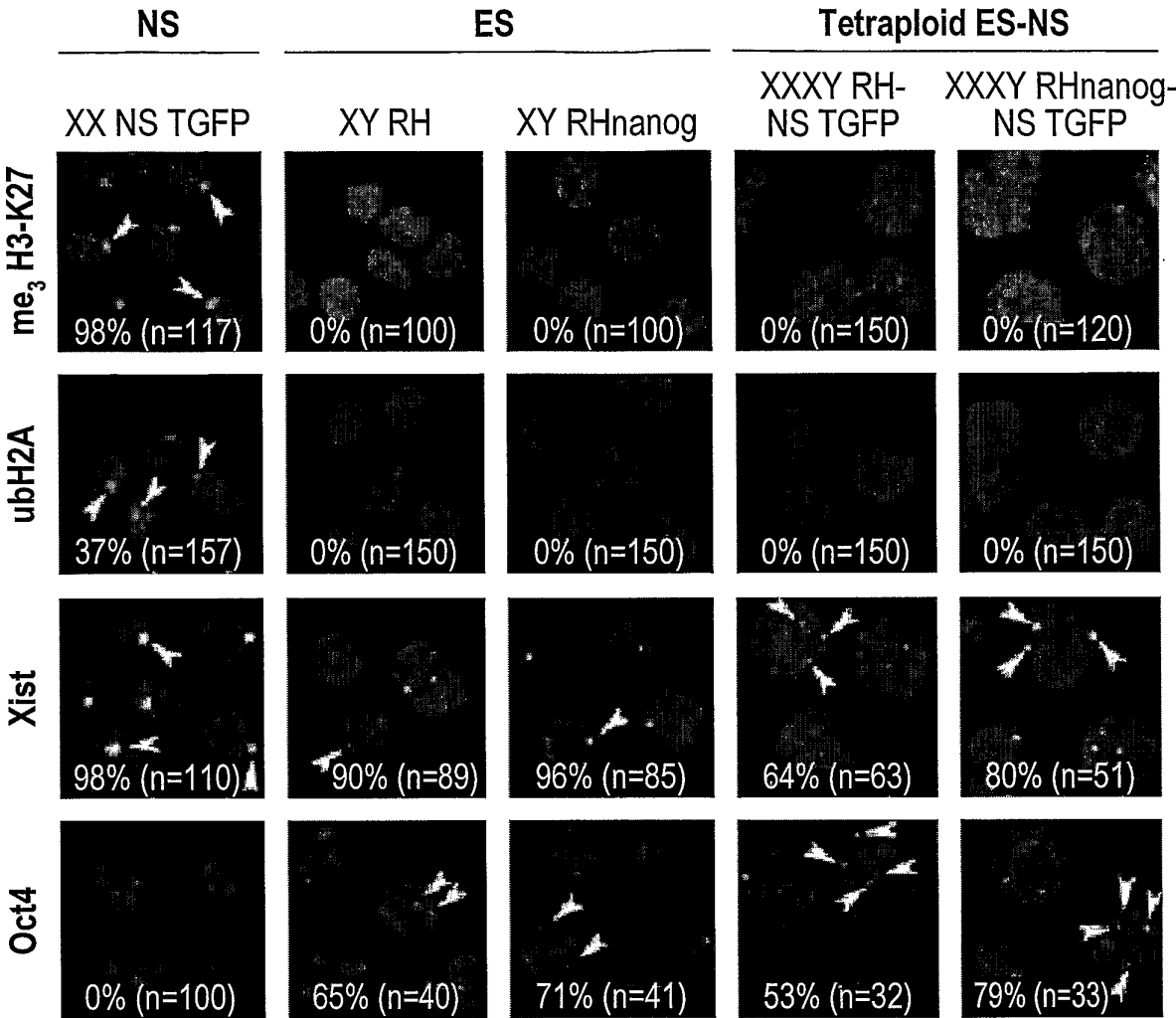


Fig.4(A)

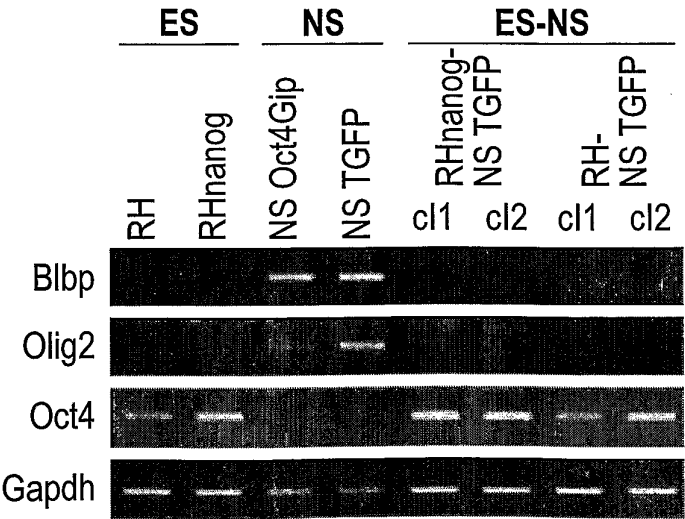
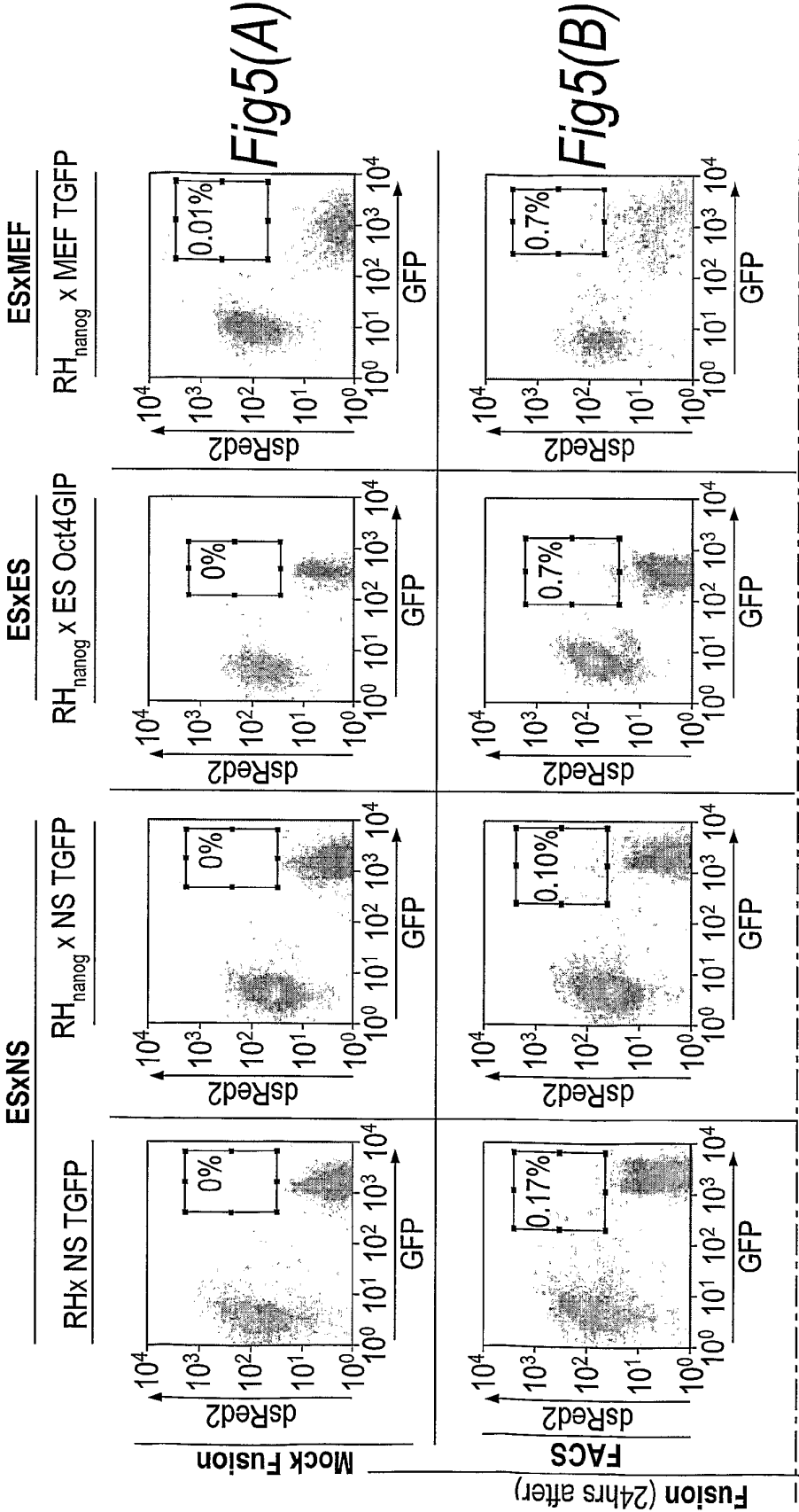
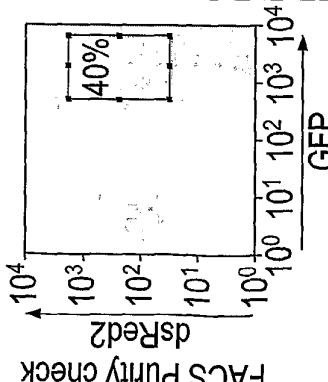
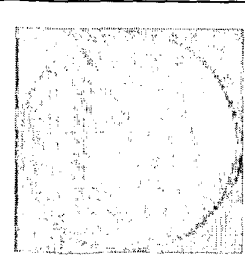
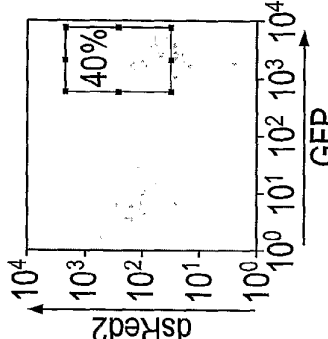
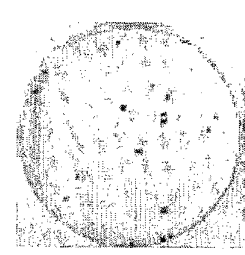
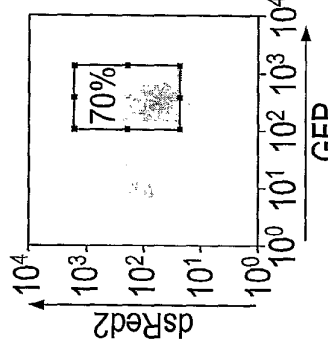
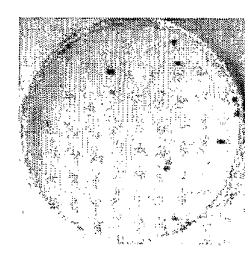
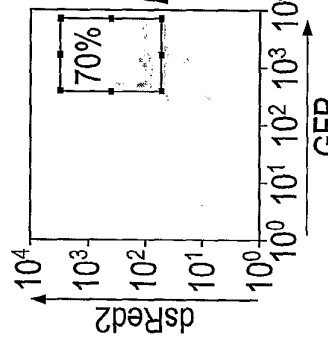
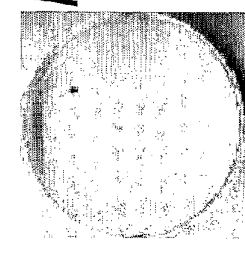
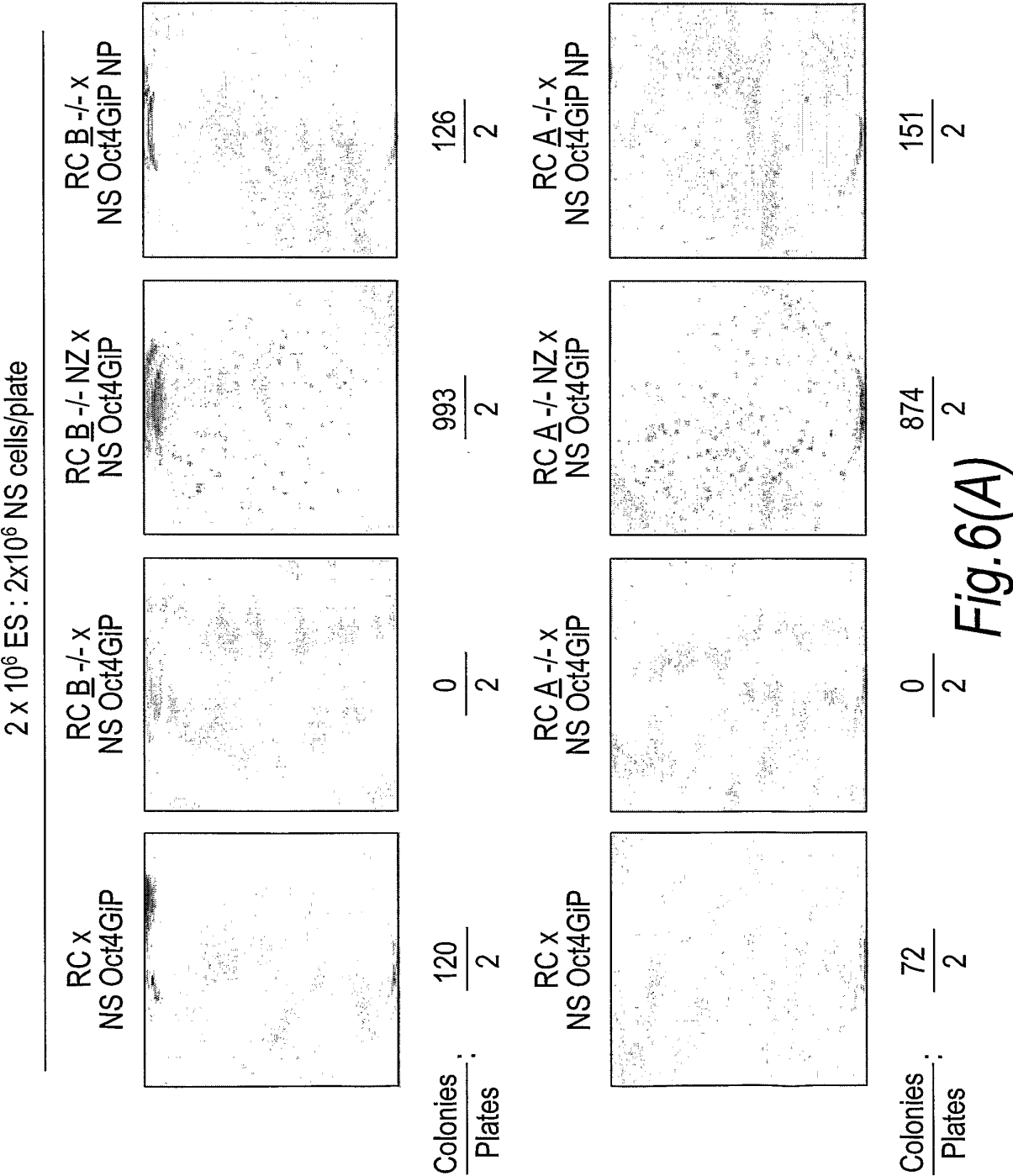


Fig.4(B)



FACS Purity check  3000 plated cells 0 colonies	 Plated FACS Sorted cells	 2000 plated cells 34 colonies $(34 \cdot (0.4 \cdot 2000)) \cdot 100 = 4.25\%$	 700 plated cells 3.93%	 1500 plated cells 35.3 ± 12.7 colonies $(35.3 \cdot (0.7 \cdot 1500)) \cdot 100 = 3.37 \pm 1.2\%$	 700 plated cells 3.93%	 3500 plated cells (3 plates) 2 ± 1 colonies $(2 \cdot (0.7 \cdot 3500)) \cdot 100 = 0.08 \pm 0.04\%$	 1500 plated cells 0%	Fig5(C)	Fig5(D)	Fig5(E)	Fig5(F)
		% of colonies/ plated hybrid (replica experiment)									

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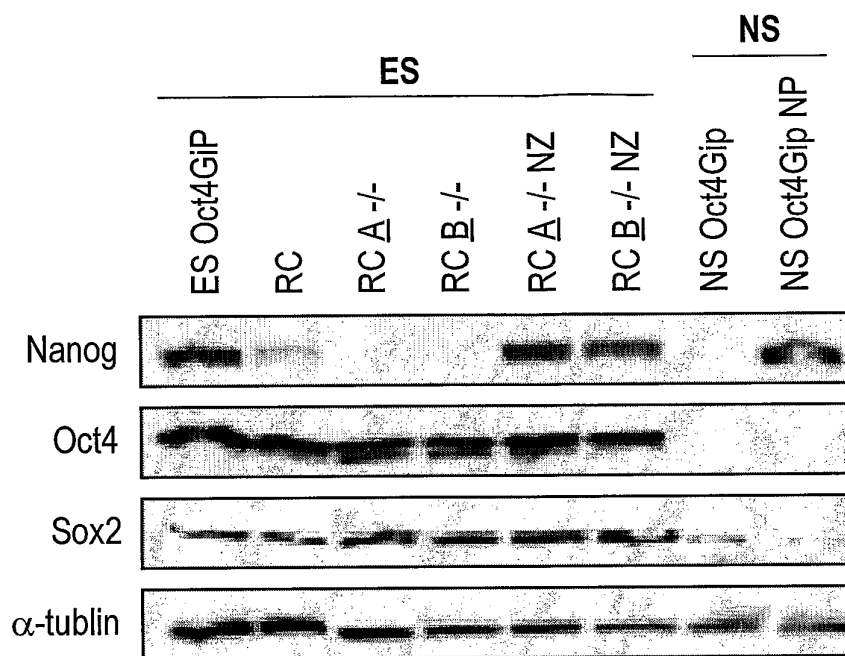


Fig.6(B)

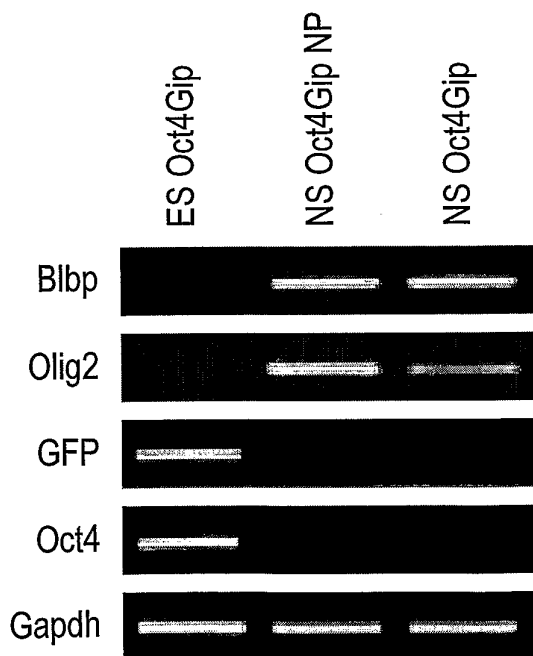


Fig.6(C)

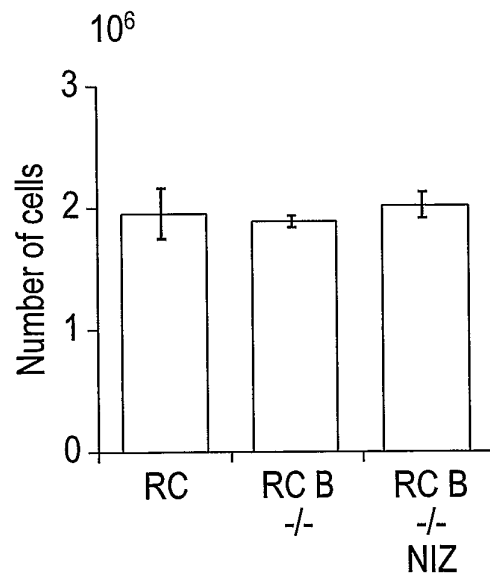
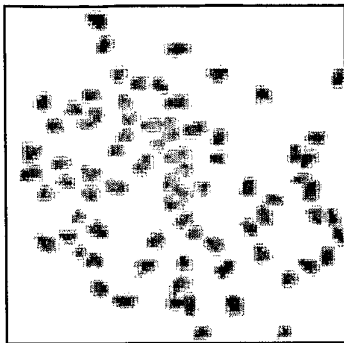


Fig.6(D)

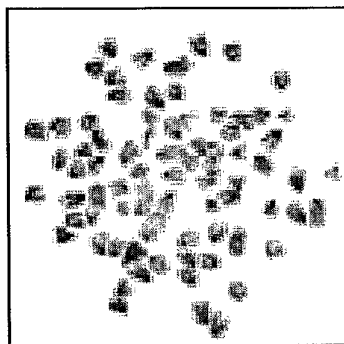
11/15

XXXY RHnanog-
NS TGFP



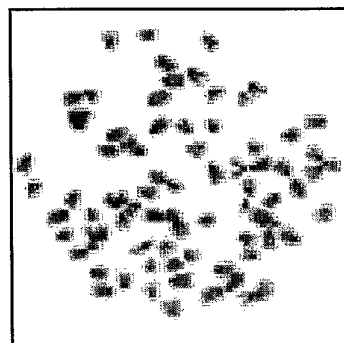
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XXXY RH-
NS TGFP



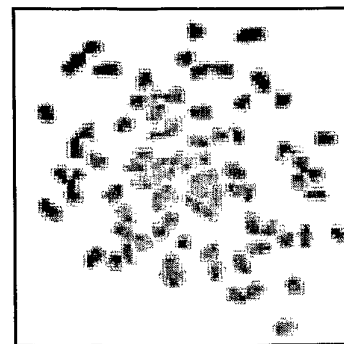
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XXXY RHnanog-
NS Oct4GFP



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XXXY RH-
NS Oct4GFP



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Fig. 7(D)

Fig. 7(C)

Fig. 7(B)

Fig. 7(A)

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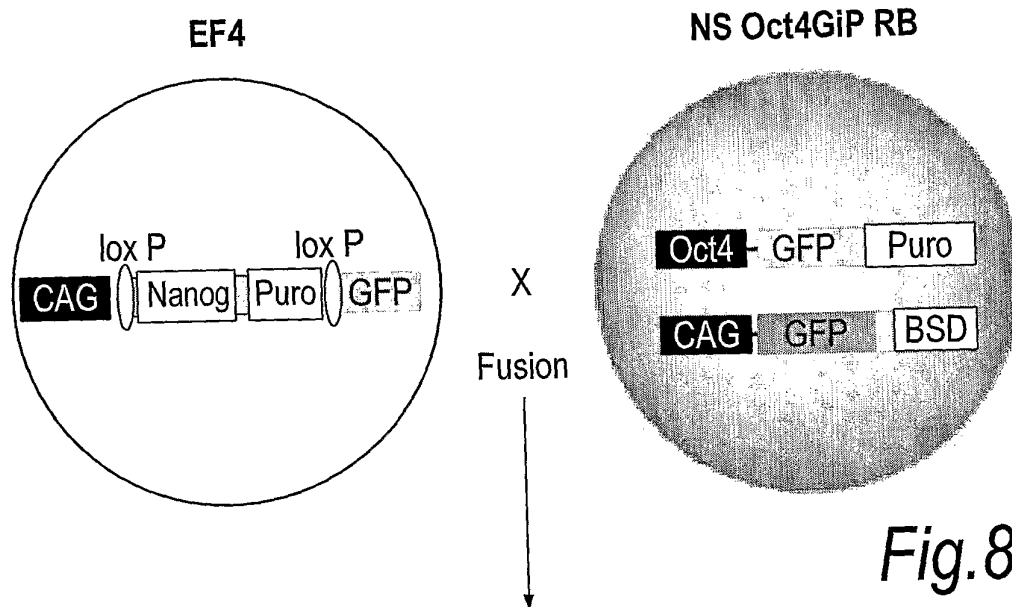


Fig.8(A)

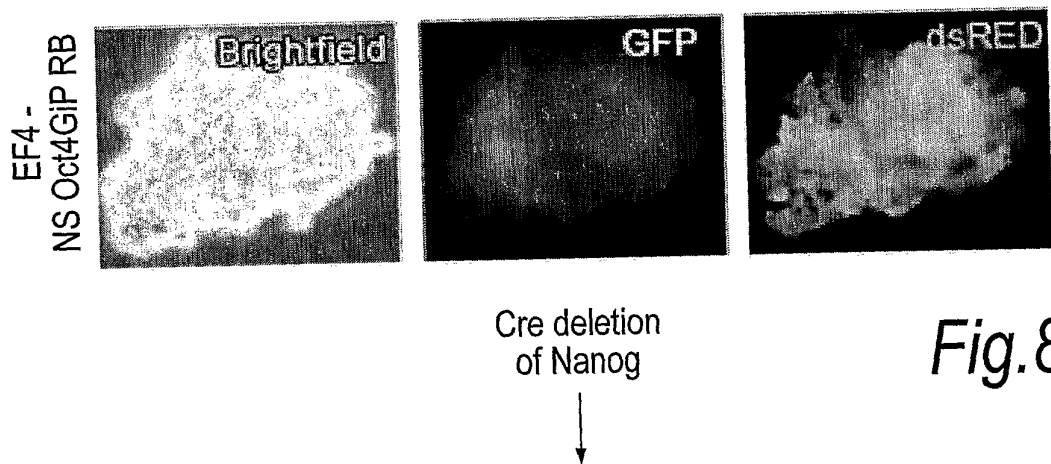


Fig.8(B)

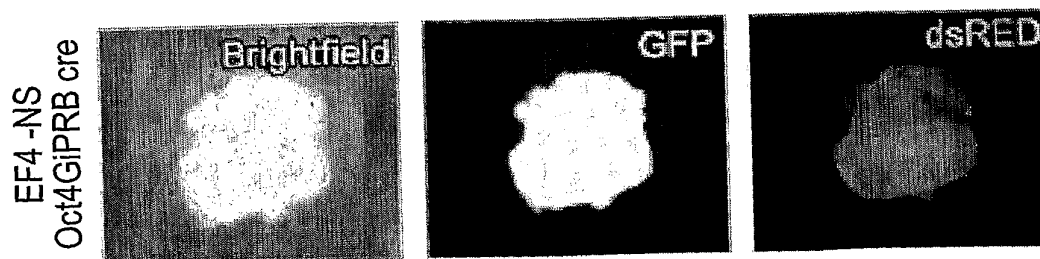


Fig.8(C)

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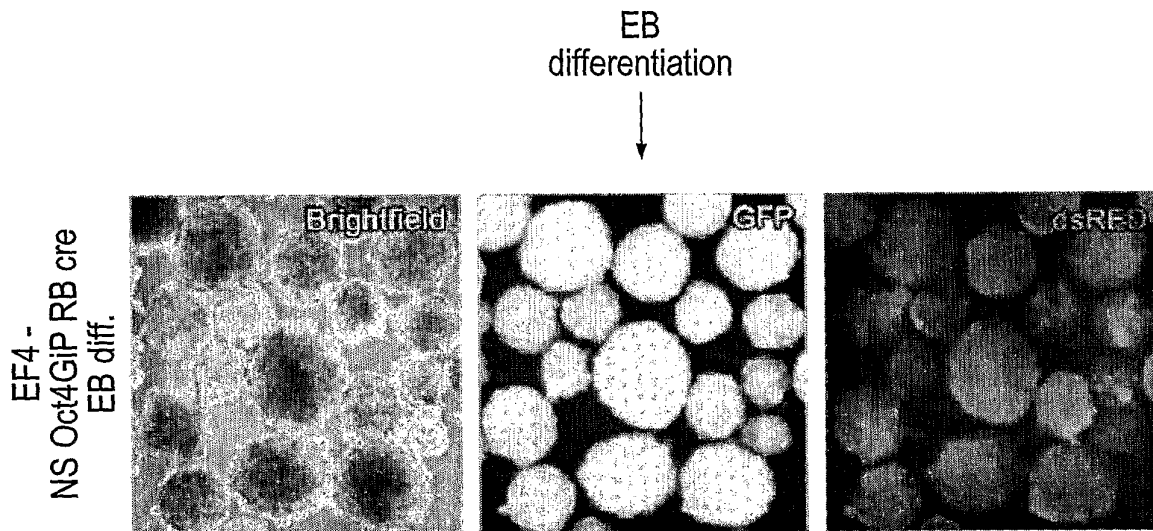
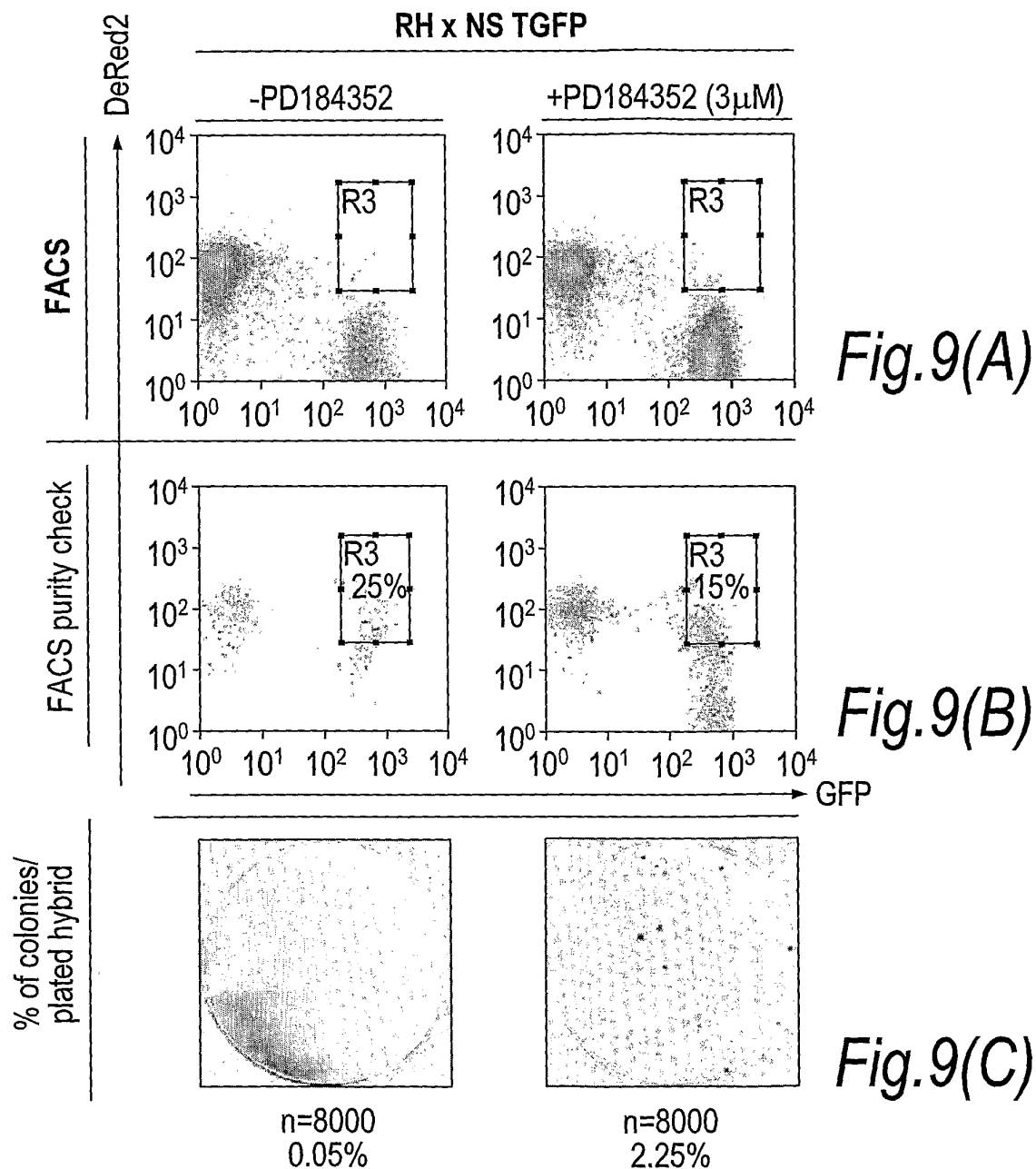


Fig.8(D)



Fig.8(E)

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PD184352 treatment	+ (3 μ M)	-
Hybrid pair	RH-NSTGFP	RH-NSTGFP
n FACS Sorted cells	8000	8000
FACS Purity	15%	20%
n true double positive cells	1200	1600
n hybrid colonies	27	1
% of Colonies/ Plated hybrids	2.25%	0.05%

Fig.9(D)

RH-NS TGFP hybrid colonies

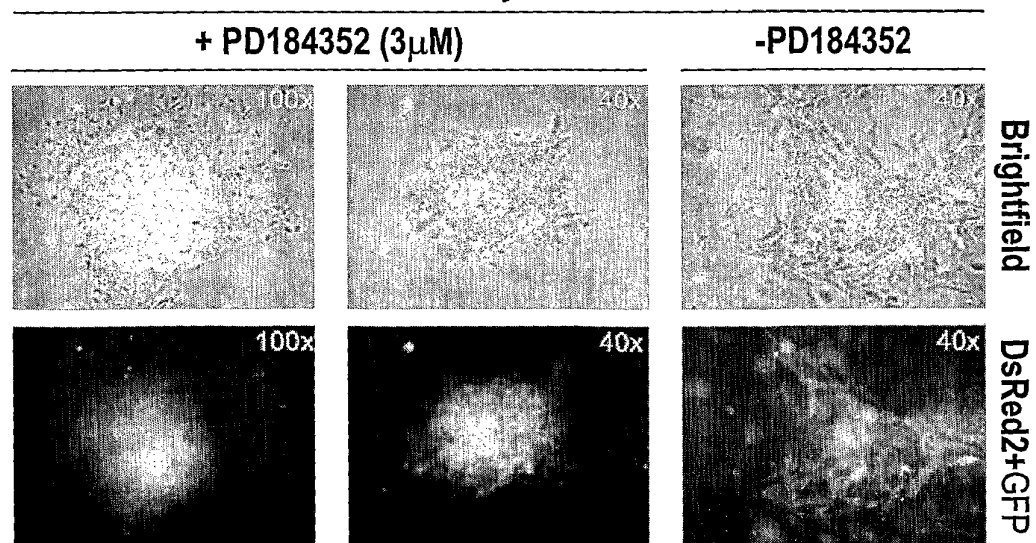


Fig.9(E)