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(54) **Title:** THE RPAP1 GENE PRODUCT IS EXPRESSED IN MULTIPLE STEM CELL NICHES, REGULATES STEM CELL DIFFERENTIATION AND REPRESSES IPS REPROGRAMMING

(57) **Abstract:** The RPAP1 gene product is expressed in multiple stem cell niches, regulates stem cell differentiation and represses iPS reprogramming. Thus, it can be used as a marker of pluripotent or multipotent cells, such as embryonic, or adult stem cells and induced pluripotent cells. Its suppression or inhibition can be used to increase the efficiency of the transformation of more differentiated cells, such as fibroblast, into induced pluripotent cells, as well as to increase the number of pluripotent cells in cultures of stem cells.



The RPAP1 gene product is expressed in multiple stem cell niches, regulates stem cell differentiation and represses iPS reprogramming.

5 **Technical Field**

The present invention relates to the field of Biotechnology. More particularly, the invention relates to a novel gene (RPAP1) which we identify as a marker of both embryonic and adult mammalian stem cells, and which we characterize as a regulator of differentiation. We also demonstrate that inhibition of RPAP1 function can improve the efficiency of reprogramming differentiated cells to give rise to induced Pluripotent Stem (iPS) cells. Then, the invention refers to a method for determining and/or maintaining or modifying the pluripotency of mammalian cells, that is based on assessing or modifying the levels and/or the activity of the rpap1 gene product.

15 **Background of the invention**

Multicellular species must correctly orchestrate complex transcriptional programs to guide the transition from self-renewal to cell specialization and development. Plantae and Animalia represent 2 major branches in the multicellular evolutionary tree, and although they diverged approximately 1,600 million years ago, the possibility remains that fundamental aspects of the organization of multicellular development may have been conserved (Meyerowitz, 2002).

In both plants and animals, a core network involving key transcription factors promotes and regulates pluripotency and indefinite self-renewal (Birnbaum and Sánchez-Alvarado, 2008). For example, in mammalian ES cells this involves Oct4, Sox2 and Nanog (Loh et al., 2006; Orkin et al 2008). In both Kingdoms during the initiation of differentiation a transcriptional switch occurs, characterized by the down-regulation of pluripotency-promoting factors and the induction of the transcriptional programs of lineage determination and cell specialization (Birnbaum and Sánchez-Alvarado, 2008). The regulation of RNA Pol II promoter-proximal pausing or release into productive elongation has been identified as a conserved mechanism to launch the rapid and coordinated transcriptional programs of early development (Min et al., 2011; Levine, 2011). Several factors have been identified which can promote elongation of

paused RNA Polymerase II (RNAPII) complexes (Shilatifard et al., 2003). However, during the initiation of differentiation, hundreds of developmental genes are switched from pausing to elongation, and the identification of the trigger for this selective transcriptional program remains unclear.

5 RPAP1 (RNAPII-associated protein) is a novel RNA Pol II-associated protein, whose human form (NCBI Reference sequence: NP_056355.2, and NM_015540.2 for the mRNA sequence) has 1393 amino acids and shows highly significant alignments over the full length of its sequence with proteins from *Mus musculus* (accession no. BAC65787.1 to the base of EMBL-EBI), *Rattus norvegicus* (accession no. XP_230480) and significant similarities with proteins of *Drosophila melanogaster* (accession no. NP_648573 and CG32104_PB) and with *Saccharomyces cerevisiae* Ydr527wp protein (accession no. NP_010816) (Jeronimo et al., 2004), a protein that is required for cell viability and was known to bind the Rpb10 subunit of RNAPII and to affect global gene expression. Therefore, it has been thought that the association of RPAP1 with RNAPII
10 might be physiologically relevant, but its exact role remained to be elucidated.
15

 Recently, in Spanish Patent Application ES201130812, some of the present inventors found that the plant gene that they called *IYO* or *MINIYO* is a molecular switch for initiating cell differentiation in plants, such as *Arabidopsis thaliana* plants. *IYO* corresponds to *Arabidopsis thaliana* gene At4g38440 described on TAIR, the
20 database of the Arabidopsis Information Resource (available at <http://www.arabidopsis.org/>), a gene with unknown function before the publication of the works disclosed in ES201130812, but with homologs in all eukaryotic kingdoms. The role of *IYO* in initiating differentiation is linked to their direct interaction with RNAPII, acting as a positive regulator of transcriptional elongation that is essential for
25 cells to initiate differentiation. *IYO* is expressed in embryos, meristems and organ primordial and not in mature tissues. Nuclear accumulation of *IYO* seems to function as a transcriptional switch for initiating differentiation in *Arabidopsis thaliana*. While *IYO* overexpression induces premature cell differentiation and leads to meristem termination phenotypes, a mutation (the hypomorphic allele *iy0-1*) of a single amino acid strictly
30 conserved in all the putative *IYO* orthologs from plants, lead to a delayed differentiation of every cell type and organ in *iy0-1* plants. Moreover, the decrease in the expression and/or the inhibition of activity of *IYO* leads to a delay or even to a block of the entry

into differentiation of in embryos and in shoot, floral and root meristems, leading to an increase in the size and number of meristems, as well as an increase in the size and number of the organs stemming from said meristems. Thus, transgenic plants where expression of *IYO* and/or its orthologs is reduced or abolished results in plants with a better yield as compared with wild type plants, due to their larger meristems and organs generated from them, and the presence of ectopic meristems that give rise to additional inflorescences, multiple flowers and a larger number of lateral roots and double embryo seeds.

As it was supposed that the beginning of differentiation in animals is associated to a transcriptional reordering that allows the activation of developing programs that are silenced in progenitor stem cells, and some evidences indicate that the silencing of such programs is due to the block of transcripcional elongation of developmental control genes, the findings about the activity of *IYO* as a molecular switch for initiating differentiation in plants through its role in promoting paused-RNA Poll II into elongation on developmental genes, open the possibility of finding a similar pro-differentiation factor for animal cell differentiation among the mammalian homologues of *IYO*, such as RPAP1, which is a protein that shows 24% sequence identity to the protein encode by the *IYO* gene and that, similarly to *IYO*, as its name indicates, is associated to RNA Polymerase II.

The finding of such a pro-differentiation factor could help to control and regulate the differentiation of pluripotent and stem cells and, even, might be useful to distinguish pluripotent cells from differentiated cells in tissue samples and cell cultures. Therefore, the present inventors have characterized the mammalian homologue of RPAP1 to investigate a putative role in mammalian stem cell self-renewal and differentiation.

SUMMARY OF THE INVENTION

The present invention is based on the findings observed by the authors about the function of the RPAP1 gene product in mammalian stem cells and mouse embryonic fibroblasts (MEFs), the role of the RPAP1 gene product in differentiation, and the impact of manipulating RPAP1 expression during application of the Yamanaka

reprogramming method to MEFs, (Takahashi and Yamanaka, 2006), (the method that allows to obtain “induced Pluripotent Stem Cells” or iPS cells, that is, to give to adult skin cells the properties of embryonic stem (ES) cells by a simple genetic manipulation: inducing over-expression of Oct4, Klf4, Sox2 and cMyc). These findings can be summarized as follows:

- The RPAP1 gene product is highly conserved across all multi-cellular animals, from Plantae to Mammalia.
- The RPAP1 gene product is selectively and highly expressed in mammalian pluripotent cell types: embryonic stem (ES) cells, embryonal carcinoma (P19 EC) cells and induced pluripotent stem (iPS) cells. It may therefore be used as a marker of pluripotency in cells.
- The RPAP1 gene product is selectively and highly expressed in mammalian adult tissue stem cell niches in adult progenitor cells. It may therefore be used as a marker of stem cell niches in adult tissues.
- Upon differentiation of pluripotent cells, the levels of expression of the RPAP1 gene product are rapidly down-regulated. It may therefore be used as a marker of the differentiation process.
- Following differentiation and down-regulation of its expression, the RPAP1 gene product retains the ability to be reactivated and up-regulated to high levels of expression upon reprogramming of differentiated cells, such as MEFs, to form iPS cells. It may therefore be used to monitor the reprogramming process during the formation of iPS cells.
- The expression of an shRNA against *Rpap1* in ES cells does not affect ES cell proliferation or survival, however, upon induction of differentiation, it has a negative impact, resulting in aberrant differentiation. Therefore the *Rpap1* gene product has an important role in guiding normal differentiation.
- The expression of a shRNA against *Rpap1* in MEF cells has a positive impact on the efficiency of iPS generation, increasing the ratio of successfully reprogrammed cells, indicating together that repression of the *Rpap1* gene product is rate limiting for reprogramming.

Taking these findings together, it can be concluded that the *Rpap1* gene product is expressed in multiple stem cell niches including Embryonic Stem cells and in adult tissues (testis, hair follicle and brain).

Thus, the present invention refers to a method for determining and/or
5 maintaining or modifying the pluripotency of a mammalian cell characterized in that the level of expression and/or the activity of the *Rpap1* gene is determined and/or modify, which comprises at least one of the following alternative steps:

- 10 a) decreasing the level of expression of the *Rpap1* gene and/or inhibiting the activity of the RPAP1 protein for increasing the efficiency of the transformation of adult cells into induced Pluripotent Stem cells (iPS);
- b) decreasing the level of expression of the *Rpap1* gene and/or inhibiting the activity of the RPAP1 protein in embryonic stem cells cultivated in vitro for preventing its differentiation;
- 15 c) assessing the expression of the *Rpap1* gene for identifying pluripotent cells as those cells where the expression of the *rpap1* gene product is elevated.

Thus, a first aspect of the present invention relates to the transient inhibition of the RPAP1 gene product function in differentiated cells such as mouse embryonic fibroblasts for enhancing iPS reprogramming, thereby providing a method for improving the efficiency of reprogramming by inhibiting the function of the *rpap1* gene
20 product. In a preferred embodiment of the invention, the inhibition of the activity of the protein is carried out using a chemical inhibitor of *Rpap1* to inhibit its function, or alternatively, using a shRNA or siRNA directed against the gene coding for *Rpap1* to decrease, preferably until repression is achieved, its expression levels. As the repression / inactivation of *Rpap1* seems to favour MET (the conversion of MEF cells into cells
25 undergoing Mesenchymal-Epithelial-Transition, the main barrier of the reprogramming process) process, the repression / inactivation of *Rpap1* or the corresponding protein, RPAP1, can be combined with any alternative way of inducing reprogramming, regardless of the factors used, that can be the traditional 4-factors Oct4, Sox2, Klf4 and c-myc, or can be also simply the three factors Oct4, Sox2, Klf4, any other combination
30 of factors known to induce iPS reprogramming selected not only among Nanog, LIN28, and the Oct-3/4, Sox, Klf or Myc families, but also combinations only with Klf4 or Klf4 together with myc-c or the combinations with chemical compounds that are under

research. In the examples of the present application assays are provided showing the applicability of the reprogramming induced by Klf4, optionally combined with:

- i) myc-c;
- ii) Oct 4 and Sox2;
- 5 iii) Oct-4, Sox2 and myc-c

The cells where reprogramming is induced can be embryo fibroblasts as in the examples of the present application (particularly, MEFs) or any other known suitable cell.

Another aspect of the invention is based on the elevated expression of the Rpap1
10 gene product in different pluripotent stem cells, such as embryonic stem cells, stem cells present in niches of adult tissues or cells lines derived from stem cells such as lines of embryonic carcinoma stem cells. That elevated expression is also found in induced pluripotent cells (iPS) and even cells beginning differentiation. Therefore, the invention can be defined as a method for identifying pluripotent cells as those cells where the
15 expression of the rpap1 gene product is elevated, in vivo or in vitro, in tissue samples or in cells in culture. The cells are preferably mammalian cells, provided that they are not human embryonic stem cells. In any case, the determination of RPAP1 protein is preferred over the determination of the corresponding mRNA.

Yet another aspect of the present invention is the advantage of inhibiting the
20 function of the Rpap1 gene product in relation to the maintenance of self-renewal. In particular, the RPAP1 gene product is required for correct embryonic stem cell differentiation. Therefore, reversible inhibition of the Rpap1 gene product provides a method of blocking ES cell differentiation, thereby enriching ES cell cultures for truly pluripotent ES cells. Thus, the method of modifying the expression or function of the
25 Rpap1 gene for acting on the differentiation status of a cell can be defined as a method where the level of expression of the *Rpap1* gene is decreased and/or inhibiting the activity of the RPAP1 protein in embryonic stem cells cultivated in vitro for preventing its differentiation. The options for decreasing the expression of the *Rpap1* gene and or inhibiting the activity of the corresponding protein are analogous to those described for
30 increasing the efficiency of the reprogramming of cells to iPS cells.

The invention will be explained in more detail by means of the Examples and Figures that appear below.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 corresponds to the characterization of the expression levels of the Rpap1 gene product in mammals: Rpap1 is selectively highly expressed in pluripotent stem cells compared to adult tissues, and it is down-regulated in association with differentiation by a post-transcriptional mechanism.

- Panel a shows fragments of photographs of Western blots corresponding to the areas where the bands of factors on the right (Rpap1, Gapdh, Actin) are expected to appear. The photographs confirm the expression of Rpap1 in pluripotent ES cells, iPS cells and P19 EC cells, whereas Rpap1 expression is extremely low or undetectable in adult whole tissue lysates.

- Panel b shows the Western-blot analysis of expression of RPAP1 protein levels in a range of mouse pluripotent cell lines and early passage MEF clones from 3 separate litters. The expression of Rpap1 is high in Nanog-positive pluripotent ES cells, iPS cells, early embryoid bodies (EBs) and P19 EC cells, whereas Rpap1 expression is extremely low in multiple MEF clones.

- Panel c shows the Western-blot analysis of expression of RPAP1 protein levels in a timecourse wt ES cells exposed to a differentiation-induction protocol (which is outlined in the lower schematic, where LIF: leukemia inhibitory factor). RPAP1 expression is clearly down-regulated during wt ES differentiation.

- Panel d shows the Western-blot analysis of expression of RPAP1 protein levels in a timecourse P19 EC cells exposed to a differentiation-induction protocol (which is outlined in the lower schematic, where LIF: leukemia inhibitory factor). RPAP1 expression is clearly down-regulated during P19 EC differentiation.

- Panel e shows the qRT-PCR analyses of Rpap1 mRNA levels during a timecourse of wt ES cells exposed to a differentiation-induction protocol (as in Panel 1c, above).

- Panel f shows Rpap1 sub-cellular localization in wt ES cells by immunofluorescence (IF) microscopy. The expression and localization of Nanog and Oct4 are also shown as controls indicating the ES cells are pluripotent.

- Panel g shows Rpap1 sub-cellular localisation in P19 EC cells by immunofluorescence (IF) microscopy. The expression and localization of p53 are also

shown as controls. Upon inhibition of nuclear-export using LeptomycinB, Rpap1 displays rapid nucleo-cytoplasmic shuttling within 3 hours. RPAP1 accumulates in the nucleus similar to the positive control p53 which has well-documented nucleo-cytoplasmic shuttling kinetics.

5

Fig. 2 refers to the characterization of Rpap1 expression and localisation in stem cell niches and proximal differentiation zones in vivo, both in embryonic and adult tissues

10 - Panel a shows Rpap1 is expressed in vivo in the mouse e3.5 blastocyst. Immunofluorescence (IF) and Immunohistochemistry (IHC) for Rpap1 in a e3.5 blastocyst. IHC for Rpap1 is also shown for ES cells and MEFs as positive and negative controls respectively.

15 - Panel b shows RPAP1 is expressed in vivo in the stem cell regions of teratomas. Immunohistochemistry (IHC) for RPAP1 in a day 30 teratoma. Magnified inset highlights an example of teratoma regions which have retained stemness and which stain positively for RPAP1.

20 - Panel c shows RPAP1 is expressed in vivo in anagen-phase hair follicles. Immunohistochemistry (IHC) for RPAP1 in a transverse section of skin containing anagen-phase hair follicles.

 - Panel d shows RPAP1 is expressed in vivo in the Brain Sub-Ventricular Zone (SVZ). Immunohistochemistry (IHC) for RPAP1 in a transverse section of Brain SVZ. Magnified insets with red arrows highlight the SVZ. Also highlighted: Hypothalamus (H), and Ventricles (V).

25 - Panel e shows RPAP1 is expressed in vivo in the Brain Cerebellum Purkinje Cell Layer. Immunohistochemistry (IHC) for RPAP1 in a longitudinal section of Brain Cerebellum. Magnified insets with red arrows highlight the Purkinje Cell Layer (PCL), Inner Granular Layer (IGL), and Molecular Layer (ML).

30 - Panel f shows RPAP1 is expressed in vivo in adult testis in the seminiferous tubule basal layer spermatogonial cells. Immunohistochemistry (IHC) for RPAP1 in a transverse section of adult testis seminiferous tubule. Magnified inset highlights the

selective staining of spermatogonial progenitor cells versus the sertoli cells or mature spermatozoa.

5 **Fig. 3** relates to the epigenetic status of *Ink4a/Arf* locus in iPS cells:

- Panel a shows shRNA knockdown of Rpap1 protein expression by Western blot analysis using 5 different lentiviral shRNA at timepoints of +3 days or +7 days of Puromycin selection after lentiviral infection. The shRPAP1#5 is the most efficient.

10 - Panel b shows shRNA knockdown of RPAP1 is efficient and persists throughout differentiation. RPAP1 mRNA levels during a timecourse of wt ES cells exposed to a differentiation-induction protocol (as in Fig. 1c , above).

- Panel c shows shRNA knockdown of RPAP1 does not effect proliferation of wt ES or P19 EC cells under control conditions. Following 3 days of selection for lentiviral knockdown of RPAP1, the cells were reseeded at 1.2×10^5 per dish and
15 counted every 3 days.

- Panel d shows shRNA knockdown of RPAP1 delays SSEA1 downregulation in wt ES cells. Wt ES cells were cultured in control conditions +/- LIF for 24 hours, followed by FACS measurement of the % of SSEA1+ cells in the population

20 - Panel e shows shRNA knockdown of RPAP1 delays morphological changes associated with differentiation and induces cell death in P19 EC cells exposed to Retinoic Acid. Cells were treated according to the schematic in Fig. 1d above.

- Panel f shows bar chart indicating that shRNA knockdown of RPAP1 in P19 EC cells undergoing differentiation upon exposure to Retinoic Acid is associated with increased cell death. Cells were treated according to the schematic in Fig. 1d above,
25 followed by FACS analysis of the percentage of cells positive for the cell surface AnnexinV apoptosis indicator.

- Panel g shows that shRNA knockdown of RPAP1 results in delayed/aberrant development in Embryoid Bodies (EB's). Following 3 days of selection for lentiviral knockdown of RPAP1, wt ES cells were reseeded in a hanging drop differentiation
30 protocol and inspected at intervals for the percentage of EBs which developed beating cells in cardiac centres. Photographs show the differences in morphology of EBs formed from ES cells with shSCR or shRPAP1 at Day4 in suspension culture. Bar chart

on upper left shows the percentage of hanging drops which successfully developed an EB. Bar chart on lower left shows the percentage of EBs which successfully developed a focus of beating cells in a cardiac centre.

- Panel h shows the qRT-PCR analyses of mRNA levels of stemness markers in wt ES cells lentivirally-infected with shSCR or shRPAP1 under control culture conditions (left), or on Day 4 of the hanging-drop developmental assay (as in Panel 3g , above). Results are relative to GAPDH $\times 10^{-3}$.

- Panel i shows the qRT-PCR analyses of mRNA levels of differentiation markers in wt ES cells lentivirally-infected with shSCR or shRPAP1 under control culture conditions (left), or on Day 4 of the hanging-drop developmental assay (as in Panel 3g , above). Results are relative to GAPDH $\times 10^{-3}$.

- Panel j shows the qRT-PCR analyses of mRNA levels of cardiac differentiation markers in wt ES cells lentivirally-infected with shSCR or shRPAP1 under control culture conditions (left), or on Day 4 of the hanging-drop developmental assay (as in Panel 3g , above). Results are relative to GAPDH $\times 10^{-3}$.

Fig. 4 relates to the shRNA knockdown of RPAP1 and the increase in iPS colony formation efficiency.

- Panel a shows shRNA knockdown of RPAP1 increases iPS colony formation efficiency. Following lentiviral knockdown of RPAP1, 4F-MEFs were treated with doxycyclin to induce reprogramming. Plates were inspected daily for the appearance of iPS colonies. Results from 3 different MEF clones in 2 separate cell experiments are shown.

- Panel b shows shRNA knockdown of RPAP1 increases iPS colony formation efficiency. Following lentiviral knockdown of RPAP1, 4F-MEFs were treated with doxycyclin to induce reprogramming. Plates were stained for alkaline phosphatase to identify iPS colonies. A representative staining is shown. Graph shows results from 3 different MEF clones in 2 separate cell experiments are shown.

- Panel c shows shRNA knockdown of RPAP1 improves reprogramming efficiency. Shown are scans of alkaline phosphatase-stained MEF cultures at the indicated number of days after retroviral infection with combinations of the Yamanaka

reprogramming factors and either shSCR control or shRPAP1 lentiviral shRNA. Retroviral delivery of OCT4, SOX2, KLF4 or MYC.

- Panel d shows that shRNA knockdown of RPAP1 improves reprogramming efficiency by inducing the MET process. MEFs which were simultaneously retrovirally infected to over-express Klf4 and lentivirally infected with shRPAP1. Cell foci formed and on Day 30, clones were picked and expanded for 2 passages on feeders followed by 3 passages on gelatin before harvest for qRT-PCR analyses of mRNA levels of RPAP1, Klf4 and MET markers relative to Actin $\times 10^{-3}$.

DETAILED DESCRIPTION OF THE INVENTION

As set forth above, the present invention is based on the findings of the authors of the invention related to the influence of the level of expression of the Rpap1 gene product on the reciprocal processes of differentiation and reprogramming, discovered during the studies carried out by the authors of the invention in order to characterize the putative role of Rpap1 in stem cell differentiation and in reprogramming of fibroblasts towards iPS cells.

Similarly to its homologue in plants, *IYO* or *MINIYO*, RPAP1 seems to act as a pro-differentiation factor, that is required for normal differentiation of ES cells. In this cells RPAP1 is highly expressed, although it is not required for their survival under control self-renewal conditions. However, upon initiation of differentiation, RPAP1 appears to be required to promote normal down-regulation of stemness and the concomitant induction of the transcription programs of early development. The expression of RPAP1 is also high in the subset of multipotent stem cells present in adult tissues, but a rapid down-regulation linked to differentiation can be observed, the protein being practically undetectable in fully-differentiated regions. Surprisingly, loss of RPAP1 expression favours reprogramming of iPS cells, an increase in the number of iPS colonies being observed when reprogramming using retroviral overexpression of the four Yamanaka transcription factors (Oct4, Sox2, Klf4 and c-Myc) is provoked in cells where an shRNA directed against RPAP1 is expressed.

Thus, a method for determining and/or maintaining or modifying the pluripotency/multipotency of a mammalian cell characterized in that the level of

expression and/or the activity of the *Rpap1* gene is determined and/or modified, which comprises at least one of the following alternative steps:

- a) decreasing the level of expression of the *Rpap1* gene and/or inhibiting the activity of the RPAP1 protein for increasing the efficiency of the transformation of adult cells into induced Pluripotent Stem cells (iPS);
- b) decreasing the level of expression of the *Rpap1* gene and/or inhibiting the activity of the RPAP1 protein in embryonic stem cells cultivated in vitro for preventing its differentiation;
- c) assessing the expression of the *Rpap1* gene for identifying pluripotent/multipotent cells as those cells where the expression of the *Rpap1* gene product is elevated.

Then, generalizing, the invention can be defined as a method for identifying or acting on the status of pluripotency/multipotency of a cell by acting on the *Rpap1* gene as pro-differentiation marker, determining its gene product or acting on its functioning, either acting of its level or in its activity.

The main findings of the authors of the invention that support such applications are as follows:

- RPAP1 expression is tightly associated with pluripotency/multipotency, its expression being high in multiple stem cell niches including Embryonic Stem cells and in stem cell niches present in adult tissues (testis, hair follicle and brain). RPAP1 expression is also tightly associated with cells initiating differentiation. This conclusion is supported by the immunohistochemical analyses of RPAP1 protein expression in adult tissues using validated antibodies that are shown in Examples of the present application, where it can be seen that RPAP1 protein is highly expressed in pluripotent cell types (wild type embryonic ES cells, iPS cells and the embryonal carcinomal cell line P19) compared with a range of adult whole tissues (Example 1). Example 2, in turn, demonstrates that RPAP1 expression is a common feature of both embryonic stem cells and multiple adult stem cell niches, such as spermatogonia (testicular progenitor cells), anagen-phase hair follicle progenitor layers, the Sub-Ventricular Zone (SVZ) of the brain, and the Purkinje cell layer of the cerebellum, where a subset of cells selectively expressed the gene.

This finding has an important commercial potential, since adult stem cells are under constant research because they are thought to maintain the integrity of adult tissues by a process of cell replacement, a property that might make them useful for the healing and regeneration of damaged organs in vivo and, even for the generation of organs in vitro that could later transplanted to suitable recipients. However, the studies are being hindered because it is difficult to identify, isolate and study new adult stem cell niches until specific markers have been identified. As RPAP1 expression is highly localized in adult tissues in multiple adult stem cell niches (brain, testis, hair follicle), RPAP1 can be exploited by researchers as a marker for adult stem cells, thereby enhancing their identification and isolation for further investigation.

Thus, in one of its aspects, the present invention can be also defined as a method for the identification of pluripotent/multipotent cells in a sample, wherein the pluripotent/multipotent cells are those with elevated expression of the Rpap1 gene product. It can be also defined as the use of the Rpap1 gene product as marker for the identification of pluripotent/multipotent cells in a sample, with the same possible embodiments.

As used in the present application, the term "pluripotent/multipotent cell" is used as a synonym of stem cell, that is, a cell capable to self-renewal by mitotic divisions or to initiate differentiation to a more specialized type of cell. As such, it includes both adult stem cells present in adult tissues (generally considered as "multipotent") as well as embryonic stem cells (that can differentiate into any of the three germ layers, endoderm, mesoderm or ectoderm, and can give rise to any fetal or adult cell type, and for that reason usually referred to as "pluripotent") and even embryonic carcinoma cells (such as the murine line P19, which are pluripotent cells than can be induced to differentiate into neuronal and glial cells in the presence of retinoic acid, whereas aggregates of P19 cells differentiate into cardiac and skeletal muscle in the presence of dimethyl sulfoxide (DMSO); in the presence of both retinoic acid and DMSO cells develop as if exposed to retinoic acid only) and cells belonging to other embryonic cell lines. The term is intended to cover progenitor cells, that are cells that have the potencial to give rise to a limited number of lineages or kinds of differentiated cells. Preferably, the cells will be mammalian cells provided that, in the case of embryonic stem cells, the cell is not a cell from a human embryo. The term

"pluripotent/multipotent cell", as used in the present application, also covers iPS cells (induced pluripotent cells), which are cells with the potencial of embryonic stem cells but artificially derived from a non-pluripotent cell - typically an adult somatic cell - by inducing a "forced" expression of specific genes. Induced pluripotent stem cells are similar to natural pluripotent stem cells, such as embryonic stem (ES) cells, in many aspects, such as the expression of certain stem cell genes and proteins, chromatin methylation patterns, doubling time, embryoid body formation, teratoma formation, viable chimera formation, and potency and differentiability, but the full extent of their relation to natural pluripotent stem cells is still being assessed.

As used in the present application, the term "elevated" must be understood as, at least, 10x-fold higher levels of RPAP1 in pluripotent/multipotent cells than in reference value corresponding to differentiated cells or tissues. The reference value will depend on the sample where the presence of pluripotent/multipotent cells is assayed and the technique used to determine the expression of the *Rpap1* gene product, as will be obvious for one skilled in the art.

Preferably, the *Rpap1* gene product whose level is determined is the RPAP1 protein, not the mRNA, because the assays described in Example 1 of the present application indicate that it is the RPAP1 protein, and not its mRNA, what is strongly down-regulated at an early time-point during cell differentiation.

When the gene product is the RPAP1 protein and its level is determined by any technique that makes use of antibodies linked to compound that emits any kind of signal (as can be the Western blot assays or immunofluorescent assays of tissue samples or cultures of cells), the reference value can be the value obtained from cells known to not be pluripotent/multipotent. That reference value can be obtained previously to the performance of the assays, as an average reference value obtained from previous assays carried out under the same or very similar conditions, or it can be the value obtained from control cells included in the sample and assayed simultaneously with the putative pluripotent/multipotent cells. The value can also be that obtained from cells previously identified as non pluripotent cells because of the expression or lack of expression of another marker.

As can be seen in Example 1, for instance in Fig. 1, *Rpap1* expression is extremely low or undetectable in adult whole tissue lysates, when the level is assessed,

as in the assay of Fig. 1, by Western blot. Then, the reference value can be assumed to be zero when the assessment is done visually. Preferably, the relation between the intensity of the signals obtained with the cell under assays and the value corresponding to the adult tissue or cell known to be differentiated is calculated. Also preferably, the cell will be identified as a pluripotent/multipotent cell when its level of Rpap1 expression is over 100x fold higher than that of the cell, tissue or value used as control or reference, as it happens in Example 1, particularly when the expression is compared to the level of the protein in a sample of brain, heart, lung, liver, spleen, kidney, small intestine, muscle (from arm), fat tissue (dorsal, white), or skin (preferably, tail skin in the case of mouse samples).

-The RPAP1 gene product is required for correct mouse embryonic stem cell differentiation. The present inventors used lentiviral shRNA to knockdown RPAP1 expression in embryonic stem cells (ES cells), finding that ES cells in which RPAP1 expression has been knocked down, display delayed and/or aberrant differentiation ability. For example, during the differentiation of ES cells into Embryoid Bodies (EB's) with cardiac centres, the knockdown of RPAP1 expression results in: (i) delayed and aberrant development of cardiac centres; (ii) delayed and aberrant silencing of stemness markers; (iii) delayed and aberrant induction of differentiation markers. However, when ES cells of P19 EC cells were cultured under "self-renewal" conditions (conditions not favouring differentiation, as explained below in the "Methods" section of the Examples), no effect on the survival, morphology or proliferation was observed. These data suggest that RPAP1 is required for normal differentiation of ES cells, but it is not required for their survival under control self-renewal conditions. This property can be exploited for research scientists to improve the maintenance and analysis of embryonic stem cells and iPS cells in vitro.

Thus, in a second aspect, the invention can also be defined as a method for blocking embryonic stem (ES) cell differentiation and/or enriching ES cells in in vitro cultures of ES cells, wherein the Rpap1 gene product is inhibited. The inhibition can be achieved either but decreasing / blocking the expression of the gene (including in this option the interference in the expression of the gene by small interfering or silencing RNAs) or by inhibiting the activity of the RPAP1 protein.

In this sense, the use of chemical inhibitors of RPAP1, that could be easily eliminated from the culture medium when differentiation is desired, would be a preferable alternative. The knockdown of RPAP1 by the use of silencing RNAs is a possible alternative, wherein siRNAs or shRNAs targeted against said gene are provided to the cultured cells. The shRNA of SEQ ID NO:1 is a possible example. As in the Examples of the present application (see Example 4), the shRNA can be synthesized from a lentiviral vector; in that case, for this particular application, placing the synthesis of the shRNA under an inducible promoter, easy to silence when the presence of the shRNA (and, therefore, the cessation of RPAP1 knockdown) is no longer desired, could be a good option.

This aspect of the invention has an important commercial potential. It is known that a major difficulty during the in vitro culture of embryonic stem cells is that some cells lose the ability to self-renew and enter into differentiation. This results in a continuous sub-population of differentiated cells whose presence contaminates and hinders the analysis of stem cell self-renewal. Chemical inhibition of RPAP1 function may improve the efficiency of culturing embryonic stem cells in vitro. During RPAP1 inhibition, embryonic stem cells which attempt to differentiate will die, enriching the population for undifferentiated cells and thereby favouring the analysis of stem cell self-renewal. This property could be exploited by research scientists to improve the maintenance and analysis of embryonic stem cells and iPS cells in vitro.

- Suppression of RPAP1 function in mouse embryonic fibroblasts enhances iPS reprogramming. Mouse Embryonic Fibroblasts (MEFs) can be induced to reprogram to form Induced Pluripotent Stem cells (iPS cells) by overexpression of 4 transcription factors (Oct4, Sox2, Klf4 and c-MYC). The present inventors have observed that lentiviral knockdown of RPAP1 enhances 4-Factor iPS reprogramming ~7x fold. Lentiviral knockdown of RPAP1 enhances 3-Factor iPS reprogramming (Oct4, Sox2, Klf4) ~3x fold.

Moreover, the results set forth in the Examples of the present application indicate that knockdown of RPAP1 may enhance MEF reprogramming by promoting the Mesenchymal-Epithelial-Transition (MET) process of epithelialisation, which is one of the earliest barriers during the reprogramming towards iPS cells.

Then, the present invention, in a third aspect, can be also defined as a method for increasing the efficiency of the reprogramming process of a differentiated cell to an iPS cell wherein RPAP1 is knocked-down and/or inhibited. As previously commented, the inhibition can be understood as the inhibition of the activity of RPAP1 protein, as well as the decrease or blocking of the expression of the Rpap1 gene by any other means. RPAP1 can be knocked down by the presence of a shRNA targeted to the expression of its corresponding gene, as in the case of Example 4, wherein the shRNA of SEQ ID NO:1 is used. A possibility of the presence of the shRNA is its expression from an expression vector after having transformed the cell with it, lentiviral vectors, as in the case of Example 4, being a common possibility.

This aspect of the invention has a very important industrial application, because the reprogramming of adult cells to Induced Pluripotent Stem cells (iPS cells) represents a very recent biomedical innovation possessing significant clinical potential as an unlimited source of patient-specific stem cells for therapeutic tissue repair and replacement. However, the reprogramming of adult cells to iPS cells faces 3 major difficulties: (i) the process is highly inefficient; (ii) the process is poorly understood; (iii) the process involves the random insertion of viral genes into the genome which is potentially oncogenic; the same risk arises from the use of some of the reprogramming factors used, such as c-myc, which is known proto-oncogene implicated in cancer. For that reason, many works have focused on finding alternative ways of inducing the reprogramming process, finding, for instance, that c-myc is unnecessary for the generation iPS cells, reason why c-myc is currently absent from many protocols, the use of Oct4, Sox2 and Klf4 (3-Factor iPS reprogramming) without c-myc being a possible alternative.

Current research in the field is focused on replacing virally-induced reprogramming with chemical treatment regimes to indirectly induce the reprogramming process. Understanding the reprogramming process is a very important point for achieving that purpose.

Embryo fibroblasts have been one of the cells preferred for carrying out the process. In the particular case of the reprogramming of MEFs to form iPS, one of the earliest barriers described during the reprogramming process involves what is called a Mesenchymal-Epithelial-Transition (MET). Approximately 48-96 hours after inducing

4F-reprogramming, the MET process can be observed as MEFs undergo a morphological transition: target MEFs lose their typical fibroblastic morphology and slow proliferation, instead becoming smaller, spherical, and rapidly proliferative, eventually forming loose clusters or foci of cells. Through a selective process which
5 remains to be elucidated, a subset of these cell foci subsequently continue to proliferate to form iPS colonies.

The assays shown in Example 4 of the present application not only demonstrate that the repression of RPAP1 expression significantly enhances the efficiency of the whole process of reprogramming to iPS cells, but also that RPAP-1 knockdown in
10 combination with 2-factor (c-myc+Klf4) or 1-factor (Klf4) reprogramming results in a morphological transition in the MEF population that closely resembles the normal process observed using 4F reprogramming, specifically the formation of loosely aggregated and rapidly proliferating cell foci. The present inventors have concluded that RPAP1 knockdown promoted the MET process and that clones obtained, for instance,
15 with the combination of Klf4 and an appropriate shRNA represent partially reprogrammed cells in a metastable but intermediate state in the process towards iPS cells. Knockdown or inhibition of RPAP1, in combination with particular factors, such as Klf4 (optionally together with c-myc), can be a good way of obtaining such intermediate cells.

20 All these findings provide a novel avenue of research into the mechanism of cellular reprogramming. Also, chemical inhibition of RPAP1 function is predicted to enhance iPS reprogramming, and the combination of chemical inhibitors with RPAP1 knockdown could be used to achieve full chemically-induced reprogramming.

25 As can be seen, the present findings are consistent with the previous findings related to the plant homologue, *iyu* or *miniyo*, but with a surprising and unexpected application related to the possibility of increasing the efficiency of the reprogramming process to iPS.

30 The inventions will be explained in more detail with the aid of the examples below.

EXAMPLES

Methods

The examples set forth in the present section were carried out by the use and
5 application of the following products and techniques:

- Culture conditions

Primary mouse embryo fibroblasts (MEFs, passage 2) of the indicated genotypes
(wild-type (wt) or 4-Factor OSKM) were obtained from pure inbred C57BL6
10 background mice, as described previously (Palmero et al. 2001). MEFs were cultured
in standard DMEM medium with 10% FBS (Gibco). Murine ES (mES) cells and
murine iPS (miPS) cells were cultured in DMEM (high glucose) supplemented with
serum replacement (KSR, 15%, Invitrogen 10828028), LIF 1000 u/ml (ESGRO™,
Millipore ESG1107), non-essential amino acids (Invitrogen 11140035), glutamax
15 (Invitrogen 35050061) and beta-mercaptoethanol (Invitrogen 31350) (hereinafter
referred as "complete KSR medium": these conditions are considered to be "self-
renewal" conditions (not favouring differentiation) in the present application.

Mouse ES cells were derived at the Transgenic Mice Unit of the Spanish
National Cancer Research Center (commonly abbreviated as CNIO, from the name in
20 Spanish: Centro Nacional de Investigaciones Oncológicas) from blastocysts following
standard procedures in the field.

- Generation of mouse iPS cells from MEFs

Reprogramming of primary (passage 2-4) mouse embryo fibroblasts was
25 performed following a previous protocol (Li et al., 2009). Briefly, retroviral
supernatants were produced in HEK-293T cells (5×10^6 cells per 100-mm-diameter dish)
transfected with the ecotropic packaging plasmid pCL-Eco (4 µg) together with either
one of the following retroviral constructs (4 µg), pMXs-Klf4, pMXs-Sox2, pMXs-Oct4,
or pMXs-cMyc (obtained from Addgene and previously described (Takahashi and
30 Yamanaka, 2006)): the backbone is pMXs plasmid in all cases (information about the
structure available through
<https://www.lablife.org/ct?vectorid=5987&f=v&a=showvecinfo>) and the expression of

the coding sequences of the reprogramming factors are driven by the MMLV LTR promoter).

-RPAP1 Lentivirus

5 Briefly, lentiviral supernatants were produced in HEK-293T cells (5×10^6 cells per 100-mm-diameter dish) transfected with 3 plasmids:

(i) the ecotropic lentiviral packaging plasmid pMD2.G (0.3 μ g)(from: Addgene; http://www.lablife.org/p?a=products_show&id=15937; containing the VsVg gene;).

(ii) the lentiviral packaging plasmid pCMV-dR8.91 (3.0 μ g); (from: Addgene; http://www.lablife.org/p?a=vdb_view&id=g2%2e7G67wbCOYgVJMGj8Kyw5FTUgEr8%2d).

(iii) plus either one of the following 6 lentiviral shRNA constructs (3.0 μ g) expressing mouse shRNAs against RPAP1 (shRPAP1#1-5, respectively), and the corresponding control (Scramble, Scr) vector (shSCR) were obtained from Open Biosystems (Open Biosystems: Catalog Number: RMM4534-NM_177294; Product Description: TRC Mission Library Mouse pLKO.1 lentiviral target gene shRNA set (5 clones); <https://www.openbiosystems.com/Query/?i=0&q=RPAP1>). From these 5 clones we identified that the best knockdown of RPAP1 expression was achieved using clone TRCN0000173186, hereafter "clone shRPAP1#5":

20 *shRPAP1#5 shRNA clone and vector backbone details:*

Target gene Pubmed reference: NM_177294

"Clone ID": TRCN0000173186

"Clone Name": NM_177294.3-3838s1c1

25 "Target Taxon": mouse

"Target Gene": 68925

"Target Gene Symbol": Rpap1

Vector: pLKO.1

30

"Match Position on target mRNA": +3838

"Match Region": Coding DNA Sequence

"Match %": 100%

"SDR Match %": 100%

"Target Sequence": CCAAGTCTTGCCAAACCTCAA (SEQ ID NO:2)

"Forward Oligo Sequence":

5 CCGGCCAAGTCTTGCCAAACCTCAACTCGAGTTGAGGTTTGGCAAGACTTG
GTTTTTG (SEQ ID NO:3)

"Reverse Oligo Sequence":

10 AATTCAAAAACCAAGTCTTGCCAAACCTCAACTCGAGTTGAGGTTTGGCAA
GACTTGG (SEQ ID NO:4)

Hairpin sequence for TRCN0000173186 (SEQ ID NO:1), (wherein nucleotides underlined and in bold represent the target sequence, the nucleotides in italics correspond to the loop, underlined nucleotides are the reverse target sequence:

15 CCGG**CCAAGUCUUGCCAAACCUCAA**
CUC
GAG
GUUUUUGGUUCAGAACGGUUUAGAGUU
20

Vector transfections were performed using Fugene-6 transfection reagent (Roche) according to the manufacturer's protocol. Two days later, viral supernatants (10 ml) were collected serially during the subsequent 48 hours, at 12-hour intervals, each time adding fresh medium to the cells (10 ml). The recipient MEFs had been seeded the previous day (1.5×10^5 cells per well in a 6-well plate) and received 1.0 ml of each of the corresponding retroviral and lentiviral supernatants as indicated in each Figure. This procedure was repeated every 12 hours for 2 days (a total of 4 additions). After infection was completed, media was replaced by complete KSR medium (see above).
30 Cultures were maintained in the absence of drug selection with daily medium changes (Li et al., 2009).

Colonies with ES-like morphology were counted as they became visible and were subsequently scored by Alkaline Phosphatase staining according to manufacturer's protocol (AP detection kit, Chemicon International). Colonies were picked after 2
35 weeks and expanded on feeder fibroblasts using standard procedures.

- Differentiation with retinoic acid

Differentiation with retinoic acid (RA) was performed essentially as described (Savatier, 1996), and as outlined in the schematics of Fig. 1c (wt ES cell) and Fig. 1d (P19 EC cells). Cultures were grown to near confluence in complete KSR medium with LIF (day 0) and, then, were trypsinized and seeded at lower density in the absence of LIF for one day (day 1). During the following two days (days 2 and 3), RA was added at a concentration of 10^{-6} M.

-FACS

FACS was performed essentially as in Li et al 2009. Briefly, for SSEA1 analysis, cells were collected by scraping and pipetting to unicellularize, before resuspension in 500 uLs 1xPBS and incubation with anti-SSEA1 antibody conjugated to allophycocyanin (R+D Systems, #FAB2155A) for 15 mins at room temperature. For AnnexinV analysis of apoptosis, the cells were collected by trypsinization before resuspension in 1xbinding buffer and incubation with anti-AnnexinV antibody conjugated to FITC (BD Pharmingen, # 556570).

-Hanging-Drop Differentiation

This was performed essentially according to Marikawa et al., 2009. ES cells were transferred to Differentiation medium (that is DMEM (high glucose) supplemented with serum 15%, non-essential amino acids, glutamax and beta-mercaptoethanol; hereinafter referred as "differentiation medium"), and suspended in hanging drop culture at a cell density of 5000 cells/20 uLs. ES cells were allowed to form spherical aggregates known as Embryoid Bodies (EBs) for 48 hours in the hanging drops before transfer to suspension culture in low-adherence petri-dishes. In suspension culture, fresh Differentiation medium was added every 3 days, and the EBs were inspected daily for the development of beating cells in cardiac centres.

- Quantitative real-time PCR

Total RNAs from cells were extracted using Qiagen RNeasy columns and samples were treated with DNaseI on-column (Qiagen # 74104), before reverse transcription using random priming and Superscript Reverse Transcriptase (Life Technologies), according to the manufacturer's protocols. Quantitative real-time PCR

was performed using an ABI PRISM 7700 (Applied Biosystems), using DNA Master SYBR Green I mix (Applied Biosystems). Calculation for the values were made using the $\Delta\Delta C_t$ method, as previously described (Yuan, 2006).

5 The primers used were:

GAPDH-F 5'-TTCACCACCATGGAGAAGGC-3' (SEQ ID NO:5)

GAPDH-R 5'-CCCTTTTGGCTCCACCCT-3' (SEQ ID NO:6)

β actin-F: 5'-GGCACCACACCTTCTACAATG-3' (SEQ ID NO:7)

10 β actin-R: 5'-GTGGTGGTGAAGCTGTAGCC-3' (SEQ ID NO:8)

Endo-Oct4-F: 5'-TCTTTCCACCAGGCCCCCGGCTC-3' (SEQ ID NO:9)

Endo-Oct4-R: 5'-TGCGGGCGGACATGGGGAGATCC-3' (SEQ ID NO:10)

15 Endo-Sox2-F: 5'-TAGAGCTAGACTCCGGGCGATGA-3' (SEQ ID NO:11)

Endo-Sox2-R: 5'-TTGCCTTAAACAAGACCACGAAA-3' (SEQ ID NO:12)

Endo-Klf4-F: 5'-GCGAACTCACACAGGCGAGAAACC-3' (SEQ ID NO:13)

Endo-Klf4-R: 5'-TCGCTTCCTCTTCCTCCGACACA-3' (SEQ ID NO:14)

20

Nanog-F: 5'-CAGGTGTTTGAGGGTAGCTC-3' (SEQ ID NO:15)

Nanog-R: 5'-CGGTTCATCATGGTACAGTC-3' (SEQ ID NO:16)

RPAP1-F: 5'-CACCTTCTCTGCCTGGGCC-3' (SEQ ID NO:17)

25 RPAP1-R: 5'-TAGCAGCTGCGGATGCTGGG-3' (SEQ ID NO:18)

E-Ras-F: 5'-CCCTCATCAGACTGCTACTCCTGG-3' (SEQ ID NO:19)

E-Ras-R: 5'-CACGCAGAGCCCGGTGAATATCCT-3' (SEQ ID NO:20)

30 E-cadherin-F: 5'-TTTTCGGAAGACTCCCGATTCA-3' (SEQ ID NO:21)

E-cadherin-R: 5'-AGCTTGTGGAGCTTTAGATGC-3' (SEQ ID NO:22)

N-cadherin-F: 5'-CTGATAGCCCGGTTTCACTTG3' (SEQ ID NO:23)

N-cadherin-R: 5'-CAGGCTTTGATCCCTCTGGA3' (SEQ ID NO:24)

Snail-F: 5'-cacacgtgccttgtgtct3' (SEQ ID NO:25)

5 Snail-R: 5'-ggtcagcaaaagcacggtt3' (SEQ ID NO:26)

Slug-F: 5'-tggtcaagaaacatttcaacgcc3' (SEQ ID NO:27)

Slug-R: 5'-ggtgaggatctctggttttgta3' (SEQ ID NO:28)

10 HoxA1-F: 5'-ACTTCAGTGC GCCTTATGGC3' (SEQ ID NO:29)

HoxA1-R: 5'-ATGGGAGTCGAGAGGTTTCCA3' (SEQ ID NO:30)

HoxB1-F: 5'-CCCTTCCAACTCAGTTCAGTGCCT3' (SEQ ID NO:31)

HoxB1-R: 5'-TTGGTGGCGATTGGGCTCACACTC3' (SEQ ID NO:32)

15

Myo-D1-F: 5'-CCACTCCGGGACATAGACTTG3' (SEQ ID NO:33)

Myo-D1-R: 5'-AAAAGCGCAGGTCTGGTGAG3' (SEQ ID NO:34)

Fgf5-F: 5'-TGTACTGCAGAGTGGGCATC3' (SEQ ID NO:35)

20 Fgf5-R: 5'-ACAATCCCCTGAGACACAGC3' (SEQ ID NO:36)

Cyp26a1-F: 5'-CCCGTGATCGCTGAGGAAG3' (SEQ ID NO:37)

Cyp26a1-R: 5'-GGGCACGTCAATGGGAAGAG3' (SEQ ID NO:38)

25 Cardiac alpha-actinin-F: 5'-CTGGTATTGCCGATCGTATG3' (SEQ ID NO:39)

Cardiac alpha-actinin-R: 5'-CTTGCTGATCCACATTTGCT3' (SEQ ID NO:40)

A.N.P. Atrial Natriuretic Peptide-F: 5'-ACTAGGCTGCAACAGCTTCC3' (SEQ ID NO:41)

30 A.N.P. Atrial Natriuretic Peptide-R: 5'-TGACACACCACAAGGGCTTA3' (SEQ ID NO:42)

Pax3-F: 5'-TTTCACCTCAGGTAATGGGACT3' (SEQ ID NO:43)

Pax3-R: 5'-GAACGTCCAAGGCTTACTTTGT3' (SEQ ID NO:44)

Zeb1-F: 5'-GCTGGCAAGACAACGTGAAAG3' (SEQ ID NO:45)

5 Zeb1-R: 5' GCCTCAGGATAAATGACGGC3' (SEQ ID NO:46)

Zeb2-F: 5'-CAGGCTCGGAGACAGATGAAG3' (SEQ ID NO:47)

Zeb2-R: 5'-CTTGCAGAATCTCGCCACTG3' (SEQ ID NO:48)

10 Twist1-F: 5'-GGACAAGCTGAGCAAGATTCA3' (SEQ ID NO:49)

Twist1-R: 5'-CGGAGAAGGCGTAGCTGAG3' (SEQ ID NO:50)

P16/Ink4a-F: 5'-CGTACCCCGATTTCAGGTGAT-3' (SEQ ID NO:51)

P16/Ink4a-R: 5'-TTGAGCAGAAGAGCTGCTACGT-3' (SEQ ID NO:52)

15

- Western blot

Cell extracts from in vitro cultured cells or from tissues obtained from mice (generally 3-4 months old) were prepared using RIPA buffer (Sigma-Aldrich, #R0278), resolved on NuPAGE 4-12% gradient Bis-Tris gels, transferred to nitrocellulose and
20 hybridized using Antibodies against RPAP1 (1:1000; Proteintech # 15138-1-AP), Nanog (1:5000; Chemicon/Millipore #AB5731), GAPDH (1:10000; Sigma #G8795-200); beta-Actin (1:20000; Sigma #A5441).

- Immunohistochemistry

25 Tissue was fixed in formalin at 4°C, embedded in paraffin wax, and sectioned at a thickness of 5 um. Sections were stained with hematoxylin and eosin for pathological examination or processed for immunohistochemical analysis with antibodies against mouse RPAP1 (Proteintech # 15138-1-AP; CosmoBio MK14030910).

30 - Immunofluorescence

ES cells were grown on chamber slides using the same protocols as for the rest of the experiments. At day 3 and 8, cells were fixed with 3.7% paraformaldehyde for 10

minutes at room temperature, washed with PBS and permeabilized with PBS containing 0.02% Tween-20 for 20 minutes. Cells were blocked in PBS with 50%BSA for 1 h and incubated with antibodies against mouse RPAP1 (Proteintech # 15138-1-AP; CosmoBio MK14030910; 1; 1:200 in PBS-4%BSA) for 2 h, washed with PBS and further
5 incubated with secondary anti-rabbit antibodies conjugated with Alexa-488 or Alexa-647 (1:500 in PBS-4%BSA).

Results

10 Example 1: Characterization of *Rpap1* expression levels and localisation during development.

In order to assess the level of expression of *Rpap1* gene in adult differentiated tissues with regard to pluripotent/multipotent cells, cell extracts from C57BL6 adult mice of 3-4 months of age were prepared from brain, heart, lung, liver, spleen, kidney, small intestine, arm muscle, dorsal white fat tissue and tail skin. The extracts were used
15 to perform Western blot analyses, comparing the RPAP1 protein levels with those of the following pluripotent cell types: murine C57BL/6 ES cells (wt ES cells in Fig. 1a), iPS cells derived from primary mouse embryo fibroblasts (obtained as described above) and the murine embryonic carcinoma (EC) cell line P19 (purchased from Sigma Aldrich, #95102107). Assays were also carried out to compare the level of RPAP1 in pluripotent
20 cells with the level in mouse embryo fibroblasts (MEFs).

RPAP1 protein was highly expressed in pluripotent cell types, with over 100xfold expression compared to a range of adult whole tissues (Fig. 1a). Furthermore, RPAP1 protein expression was also significantly lower in MEFs compared to all pluripotent cell types examined (Fig. 1b), suggesting that RPAP1 expression is down-
25 regulated during early development.

Indeed, in vitro differentiation assays using wt ES cells and P19 EC cells confirmed that RPAP1 protein, but not mRNA, is strongly down-regulated at an early time-point during cell differentiation (Fig. 1c-e). These results also suggest that RPAP1 down-regulation occurs most-likely by a post-transcriptional mechanism.

30 Conversely, RPAP1 protein expression was up-regulated during reprogramming of MEFs towards induced Pluripotent stem cells (iPS; Fig. 1a, b).

Immunofluorescence indicated further evidence of post-transcriptional regulation, related to the subcellular localization: RPAP1 was largely cytoplasmic in control wt ES (Fig. 1f) and P19 EC stem cells (Fig. 1g), but displayed rapid nucleo-cytoplasmic shuttling kinetics (within 3 hours) upon inhibition of nuclear-export using
5 LeptomycinB (Fig. 1g): RPAP1 accumulates in the nucleus similar to the positive control p53, which has well-documented nucleo-cytoplasmic shuttling kinetics. That result is consistent with similar results that were observed by sub-cellular fractionation (data not shown), with the results previously obtained with the plant homologue *iyv*, and the existence of multiple nuclear localization and export sequences in the N-
10 terminal region which are highly conserved.

Example 2: Characterization of Rpap1 expression levels and localisation in vivo in adult tissues

ES cells are isolated from the Inner Cell Mass (ICM) of the blastocyst at e3.5 of
15 development.

We confirmed that RPAP1 was also expressed in vivo in the e3.5 blastocyst, both in the emerging ICM and trophoblast (Fig. 2a), by immunofluorescence and immunohistochemistry.

Moreover, RPAP1 expression was localized exclusively in stem cell regions and
20 proximal differentiation-zones in vivo in day 30 teratomas, but not in the fully-differentiated regions (Fig. 2b, which shows teratoma regions which have retained stemness and which stain positively for RPAP1).

We further investigated the low levels of RPAP1 which were detectable in adult whole tissue lysates (Fig. 1a), carrying out immunohistochemical assays with sections of
25 the same tissues used to obtain the lysates of Example 1. Notably, RPAP1 was selectively expressed in a small subset of cells which coincided with previously reported adult stem cell niches, for example in skin anagen phase hair follicles (Fig. 2c), in the brain Sub-Ventricular Zone (SVZ; Fig. 2d), in the brain cerebellum Purkinje cell layer (Fig. 2e), and in the testis seminiferous tubules in the basal layer spermatogonial
30 progenitor cells (Fig. 2f). Interestingly, RPAP1 expression was exclusive to follicles in anagen phase, staining positive in all differentiating progenitor cell layers before down-regulation as the follicles exited anagen (Fig. 2c). An analogous gradient of RPAP1

expression was also observed in the basal layers of the seminiferous tubule, where the onset of spermatogonial differentiation/maturation and migration towards the centre of the lumen coincided with loss of RPAP1 expression (Fig. 2f).

5 Taken together, the results revealed that RPAP1 expression is tightly associated with pluripotent/multipotent cells types, both in vitro in embryonic cell lines and in vivo in normal embryonic development and in adult progenitor cell niches. Moreover, a gradient of RPAP1 expression exists: high expression in pluripotent/multipotent cells, with rapid down-regulation linked to differentiation.

10 Thus, these features could be exploited by researchers as a marker for adult stem cells, thereby enhancing their identification and isolation for further investigation.

Example 3: Rpap1 is required for normal differentiation.

We employed a lentiviral shRNA system to efficiently and stably knockdown RPAP1 expression in wt ES cells under control conditions or during differentiation
15 (Fig. 3a, b), carrying out assays with cells transfected with a lentivirus expressing the shRNA of SEQ ID NO:1, targeted against RPAP1 (assays labelled "shRPAP1" in the Figures), or expressing a scrambled shRNA (assays labelled "shSCR" in the Figures) as control.

20 Despite the high RPAP1 expression levels in stem cells (Fig. 1, 2), we observed no effect on the survival, morphology or proliferation of wt ES cells or P19 EC cells when they were cultured under control "self-renewal" conditions (Fig. 3c and data not shown). However, loss of RPAP1 expression during differentiation was associated with a moderate delay in the down-regulation of the surface stemness marker SSEA1 (Fig. 3d), delayed and aberrant morphology changes (Fig. 3e), and with a significant increase
25 in apoptosis levels (Fig. 3f).

To further explore the possibility that RPAP1 might be required for normal differentiation, the effect of RPAP1 knockdown was investigated in the context of standard Embryoid Body (EB) development assays. Loss of RPAP1 expression in wt ES cells had no effect on the number of EBs which formed (data not shown), however,
30 RPAP1-knockdown was associated with an increase in the number of malformed (non-spherical) EBs (Fig. 3g). In particular, RPAP1-knockdown was associated with both a delay, and an overall decrease, in the percentage of EBs which successfully developed

beating cells in cardiac centres (Fig. 3g). The data suggested that RPAP1 was required for normal differentiation of ES cells.

In agreement, qRT-PCR analyses indicated that down-regulation of a number of stemness markers was delayed in RPAP1-knockdown-ES cells undergoing
5 differentiation in the EB developmental assays (Fig. 3h). Moreover, loss of RPAP1 expression was associated with aberrant induction of several differentiation markers (Fig. 3i), including cardiac developmental markers (Fig. 3j) which was notable given the observed defects in cardiac centre development (Fig. 3g, above).

Taken together, the results indicate that RPAP1 is highly expressed in ES cells
10 but it is not required for their survival under control self-renewal conditions. However, upon initiation of differentiation, RPAP1 appears to be required to promote normal down-regulation of stemness and concomitant induction of the transcriptional programs of early development.

We hypothesize that RPAP1 exerts these effects by shuttling from the cytoplasm
15 into the nucleus where it interacts with RNA Pol II to promote a shift in the transcriptome, repressing pluripotent self-renewal and promoting lineage specification and differentiation, similarly to the behaviour of its plant homologue, *ityo* or *miniyo*.

Thus, inhibition of Rpap1 in ES cells may improve the efficiency of culturing embryonic stem cells in vitro. During RPAP1 inhibition, embryonic stem cells which
20 attempt to differentiate will die, enriching the population for undifferentiated cells and thereby favouring the analysis of stem cell self-renewal. As commented in the "Detailed Description of the invention", this property could be exploited by research scientists to improve the maintenance and analysis of embryonic stem cells and iPS cells in vitro.

25

Example 4: Rpap1 inhibition promotes iPS reprogramming efficiency

The reversibility of differentiation has been elegantly demonstrated in the reprogramming of MEFs to form iPS cells using retroviral overexpression of the four Yamanaka transcription factors: Oct4, Sox2, Klf4 and c-Myc (OSKM).

30 Given our findings linking RPAP1 with promoting normal differentiation, we also explored a possible role for RPAP1 in reprogramming using doxycyclin-inducible 4-Factor (4F) MEFs previously derived in our laboratory. Lentiviral knockdown of

RPAP1 resulted in approximately 6-7x fold increase in the formation of alkaline-phosphatase positive iPS colonies (Fig. 4a, b). Therefore, although MEFs express a low level of RPAP1 (Fig. 1b), the presence of RPAP1 appeared to be a barrier to reprogramming. Similarly, use of three retroviral factors encoding Oct4, Sox2 and Klf4 results in reprogramming and in this setting lentiviral knockdown of RPAP1 resulted in approximately 3x-fold increase in the formation of alkaline phosphatase positive iPS colonies.

We hypothesized that RPAP1 may act as a pro-differentiation factor, such that loss of RPAP1 expression favours reprogramming to iPS cells.

To further explore the mechanism by which RPAP1-knockdown might promote MEF reprogramming to iPS, we investigated the expression of MET markers during MEF reprogramming to iPS cells. Indeed, we observed that RPAP1-knockdown in combination with 2-Factor (2F) reprogramming (c-Myc+Klf4+shRPAP1), or 1-Factor (1F) reprogramming (Klf4+shRPAP1), resulted in a morphological transition in the target MEF population which closely resembled the normal process observed using 4F reprogramming, specifically the formation of loosely aggregated and rapidly proliferating cell foci which stained positively for the stemness marker alkaline phosphatase (Fig. 4c). These changes were not observed in control MEF cultures (Klf4+shSCR).

In the Klf4+shRPAP1 cultures several of these cell foci were picked, expanded, and observed to proliferate indefinitely, with silencing of Klf4 overexpression and lentiviral shRNA knockdown of RPAP1 (Fig. 4d). In addition, the expression of the cell cycle inhibitor p16, and the differentiation markers Pax3, cardiac alpha-actinin and ANP were abolished in these clones (Fig. 4d), consistent with their increased proliferation and morphological similarity to stem cells.

In particular, qRT-PCR analyses of MET markers indicated up-regulation of E-cadherin, and concomitant down-regulation of N-cadherin, Snail1, Slug1, Twist1, Zeb1, and Zeb2 (Fig. 4d and data not shown), consistent with a MET process.

Since these phenotypical changes were only observed with Klf4-overexpression plus RPAP1-knockdown, and not by Klf4-overexpression alone, we conclude that RPAP1-knockdown promotes the MET process and that these clones represent partially re-programmed cells in an intermediate but metastable state between the starting

population of differentiated MEFs and iPS cells. Moreover, since the MET process represents one of the earliest barriers to MEF reprogramming, we hypothesize that RPAP1-knockdown improves 4F-reprogramming by significantly promoting the MET process.

- 5 Thus, inhibition of Rpap1 by any other inhibitors different from shRNA, such as specific chemical inhibitors, may also promote the reprogramming process.

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CLAIMS

1. A method for determining and/or maintaining or modifying the pluripotency of an animal cell characterized in that the level of expression and/or the activity of the *Rpap1* gene is determined and/or modified, which method comprises at least one of the following alternative steps:

- a) decreasing the level of expression of the *Rpap1* gene and/or inhibiting the activity of the RPAP1 protein for increasing the efficiency of the transformation of adult cells into induced Pluripotent Stem cells (iPS);
- 10 b) decreasing the level of expression of the *Rpap1* gene and/or inhibiting the activity of the RPAP1 protein in embryonic stem cells cultivated in vitro for preventing its differentiation;
- c) assessing the expression of the *Rpap1* gene for identifying pluripotent/multipotent cells as those cells where the expression of the *rpap1* gene product is elevated.

2. The method according to claim 1, wherein the level of expression of the *Rpap1* gene is decreased and/or the activity of the RPAP1 protein is inhibited in an adult cell for increasing the efficiency of the transformation of adult cells into induced Pluripotent Stem cells (iPS).

3. The method according to claim 2, wherein the expression of the *Rpap1* gene is decreased by the use of a shRNA directed against the *Rpap1* gene.

4. The method according to claim 3, wherein the shRNA is represented by SEQ ID NO:1.

5. The method according to any one of claims 2 to 4, wherein reprogramming is induced by Klf4, optionally combined with any other factors, such as:

- i) myc-c;
- ii) Oct 4 and Sox2;
- iii) Oct-4, Sox2 and myc-c

6. The method according to any one of claims 2 to 5, wherein the cells are MEF cells.

7. The method according to claim 1, wherein the expression of the Rpap1 gene is assessed for identifying pluripotent/multipotent cells as those cells where the expression of the Rpap1 gene product is elevated, that is carried out in vivo, in vitro, in tissue samples taken from an animal or in cells in culture.

8. The method according to claim 7, wherein the pluripotent/multipotent cells are embryonic stem cells, adult stem cells, induced pluripotent stem cells (iPS), cells belonging to a cell line derived from stem cells or cells beginning differentiation, provided that they are not human embryonic stem cells.

9. The method according to claim 8, wherein the cells are mammalian cells.

10. The method according to any one of claims 7 to 9, wherein the RPAP1 protein is determined.

11. A method for the identification of pluripotent/multipotent in a sample, wherein the pluripotent/multipotent cells are those with elevated expression of the Rpap1 gene product, .

12. The method according to claim 11, wherein the pluripotent/multipotent cells are adult stem cells, embryonic stem cells, embryonic carcinoma cells, cells belonging to embryonic cells lines, or induced pluripotent stem cells, provided that the embryonic stem cells are not human.

13. The method according to claim 11 or 12, wherein the cells are mammalian cells, provided that they are not human embryonic stem cells.

14. The method according to any one of claims 11 to 13, wherein the Rpap1 gene product whose level is assessed is the RPAP1 protein.

15. The method according to claim 14, wherein the pluripotent/multipotent cells are those whose level of the RPAP1 protein is at least 10x fold higher than that of a sample of a whole adult tissue.

5

16. A method for blocking embryonic stem (ES) cell differentiation and/or enriching ES cells in in vitro cultures, wherein the Rpap1 gene product is inhibited, either but decreasing / blocking its expression or by inhibiting the activity of the RPAP1 protein.

10

17. The method according to claim 16, wherein a siRNA or shRNA targeted against Rpap1 is provided to the cells in culture.

18. The method according to claim 17, wherein the shRNA is that of SEQ ID
15 NO:1.

19. A method for increasing the efficiency of the reprogramming process of a differentiated cell to an iPS cell wherein RPAP1 is knocked-down and/or inhibited.

20. The method according to claim 19, wherein the cells are embryo fibroblasts.

21. The method according to claim 20, wherein the cells are mouse embryo fibroblasts (MEFs).

22. The method according to any one of claims 19 to 21, wherein RPAP1 is knocked-down by the presence of a targeted shRNA.

23. The method according to claim 22, wherein the shRNA is that of SEQ ID
NO:1.

30

24. The method according to claim 22 or 23, wherein the shRNA is expressed from an expression vector.

25. The method according to claim 24, wherein the shRNA is expressed from a lentiviral vector.

5 26. The method according to any one of claims 19 to 25, wherein the differentiated cell is fully reprogrammed to an iPS cell.

27. The method according to claim 26, wherein Oct4, Sox2, Klf4 and, optionally or additionally, c-Myc are used as reprogramming factors.

10

28. The method according to any one of claims 19 to 25, wherein Klf4 and, optionally, together with c-Myc, are used as reprogramming factors.

29. Use of RPAP1 as a marker for the identification of pluripotent/multipotent
15 cells in a sample.

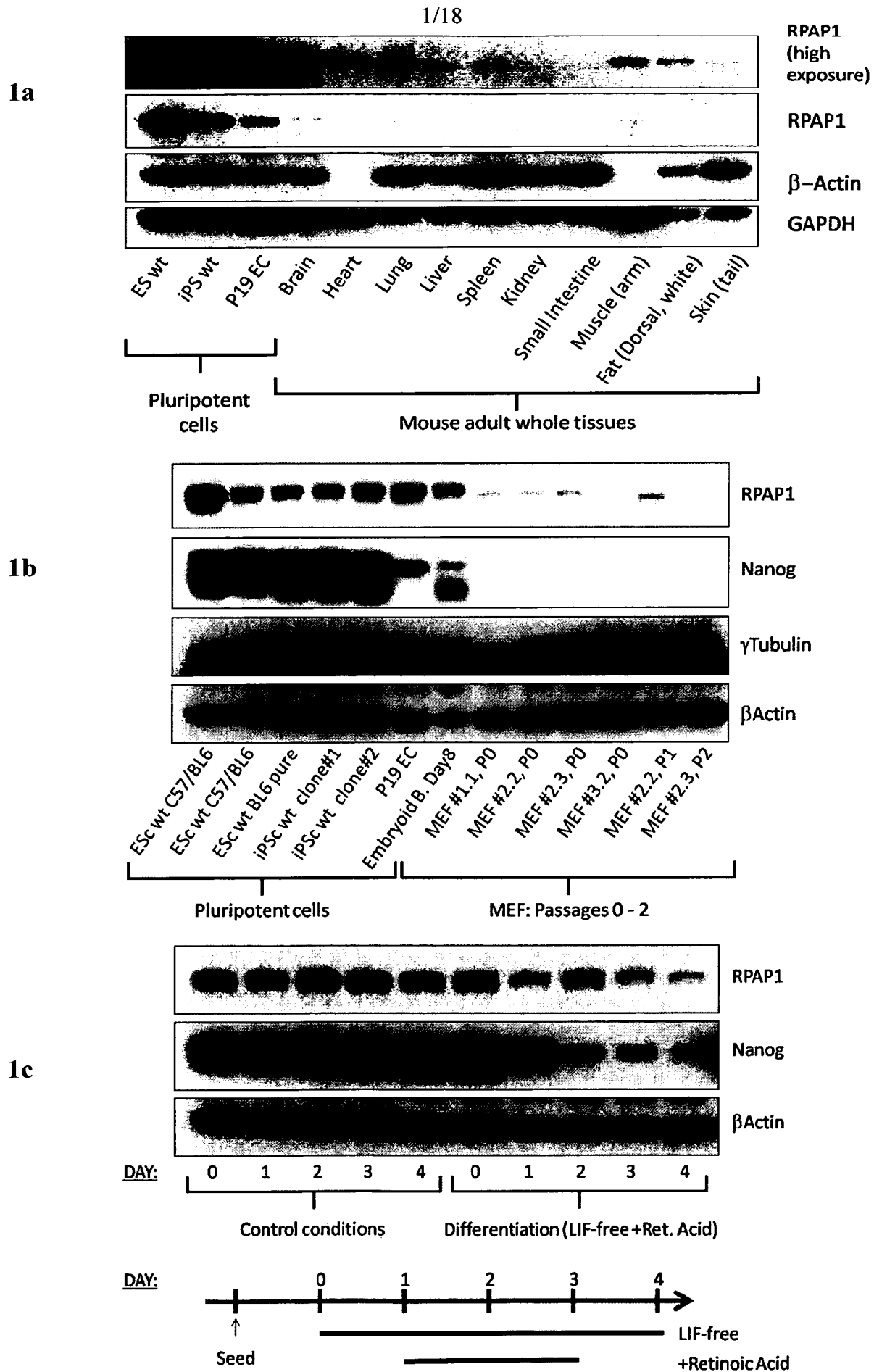


FIG. 1

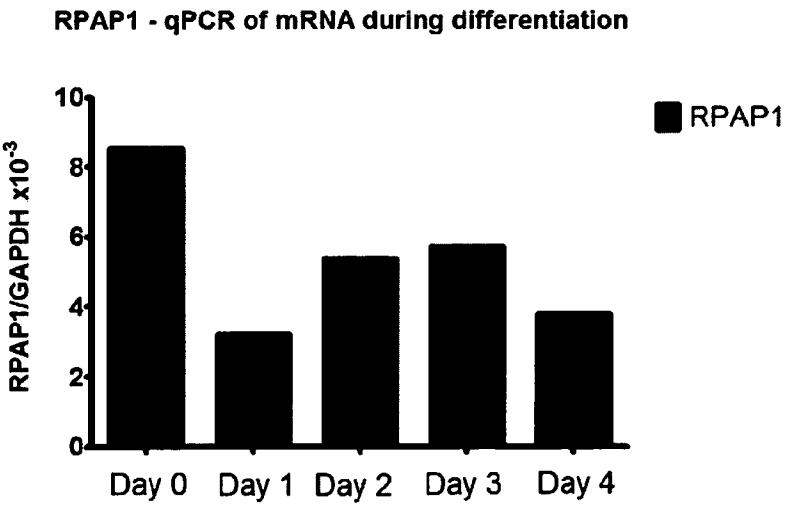
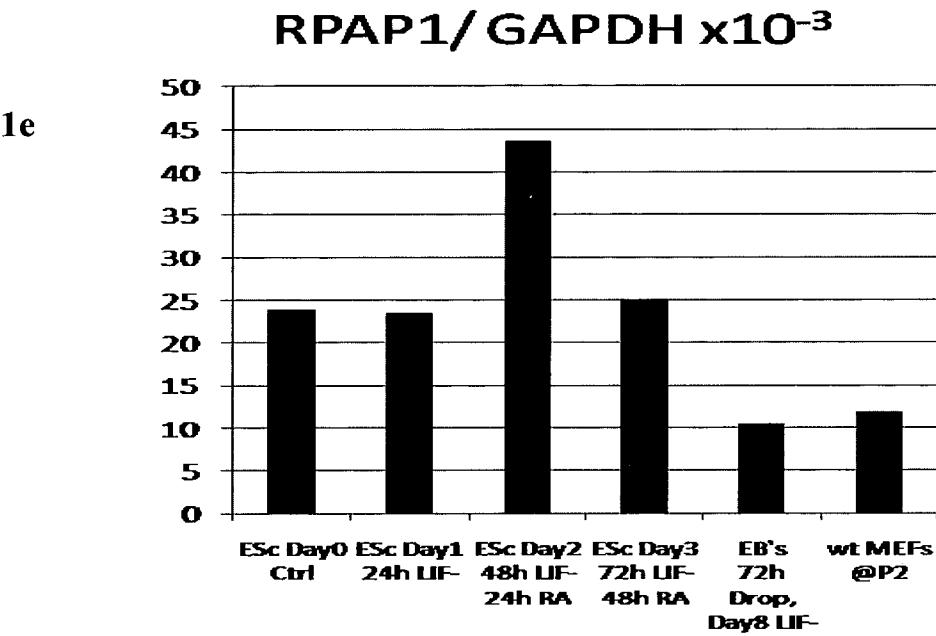
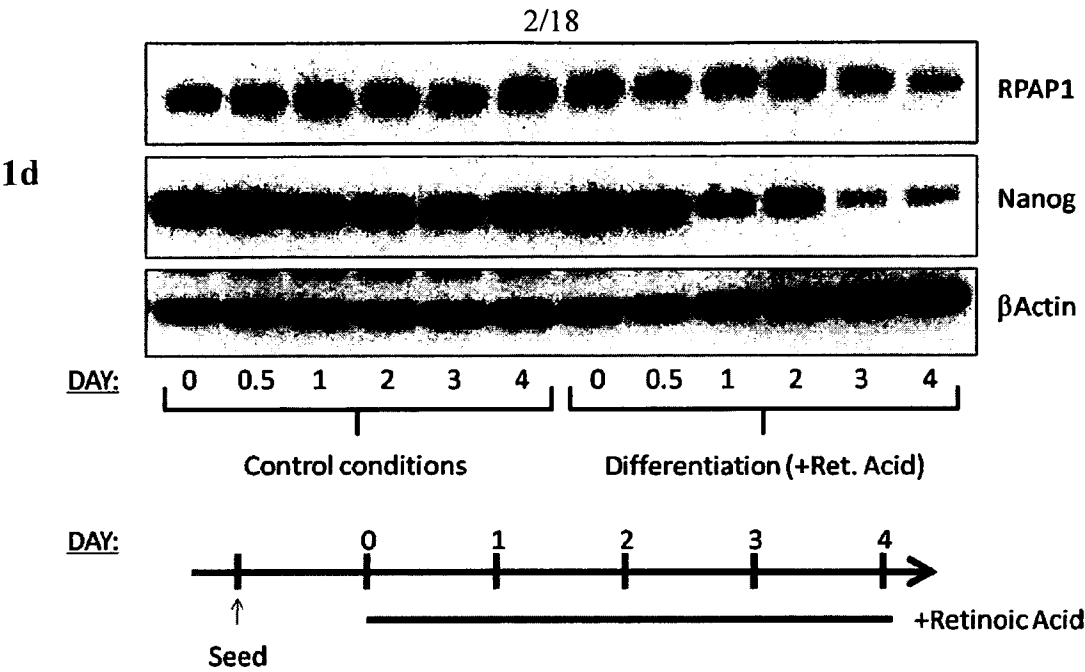
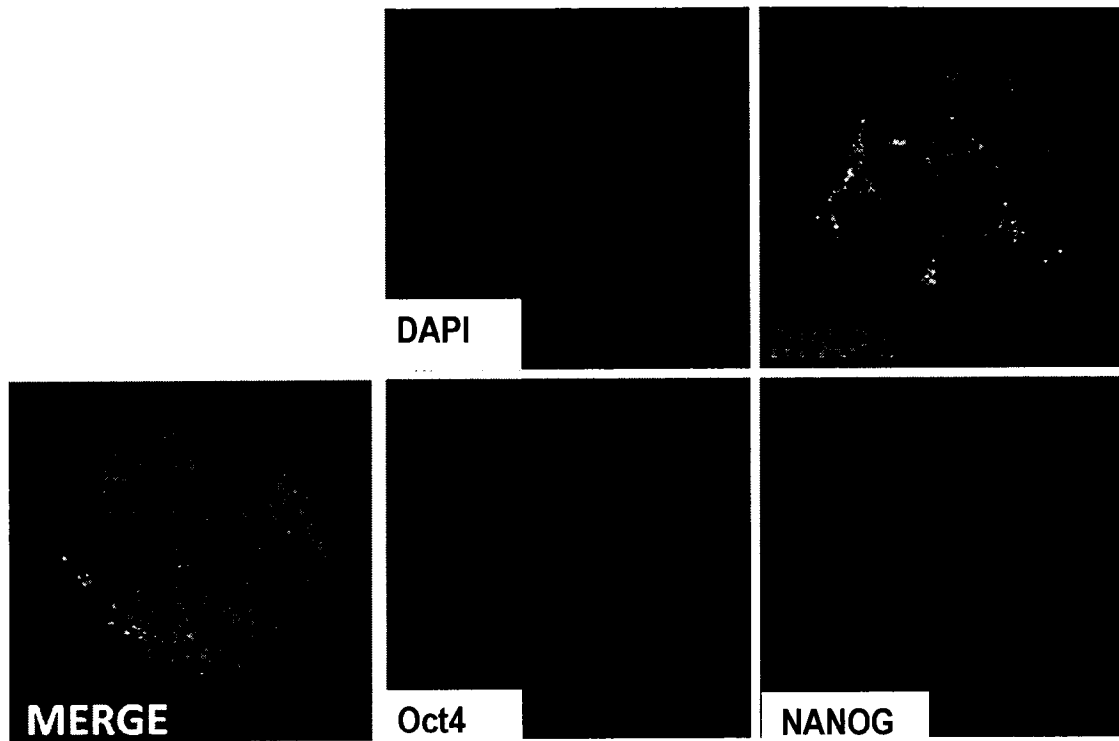


FIG. 1
SUBSTITUTE SHEET (RULE 26)

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1f



1g

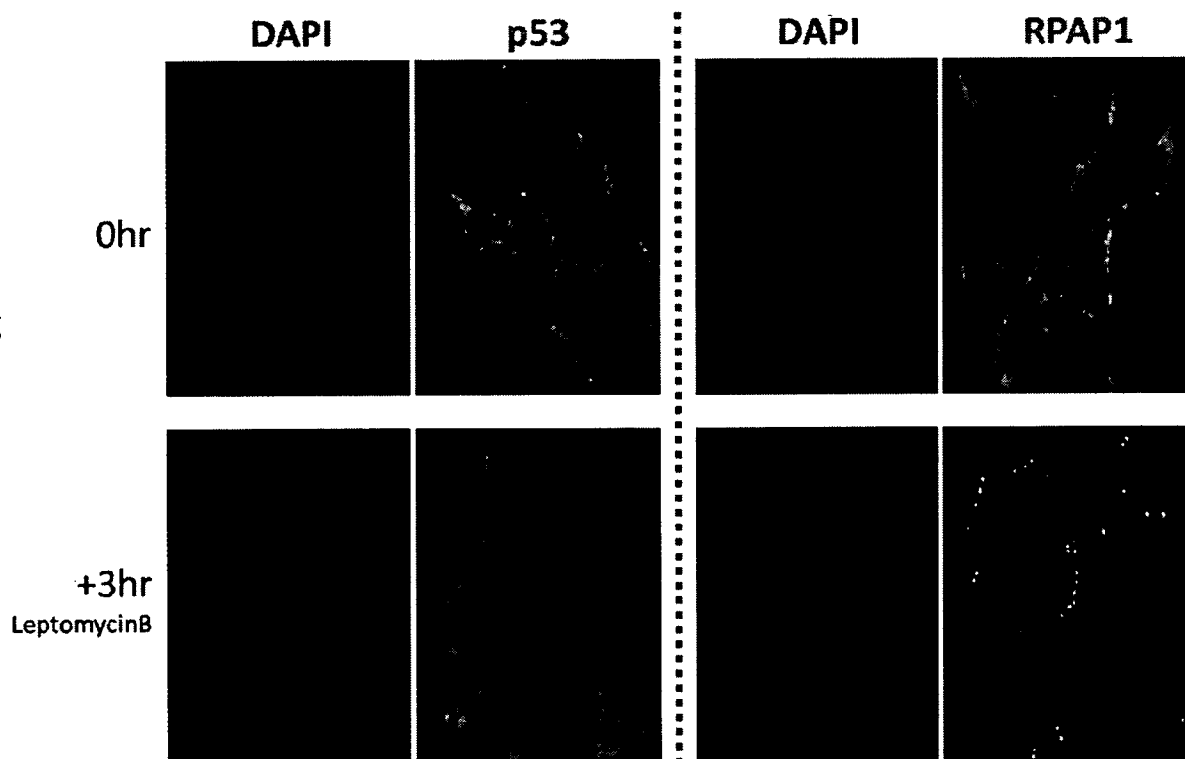
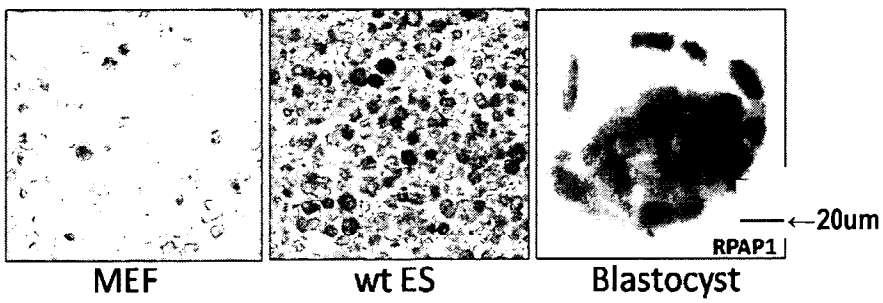
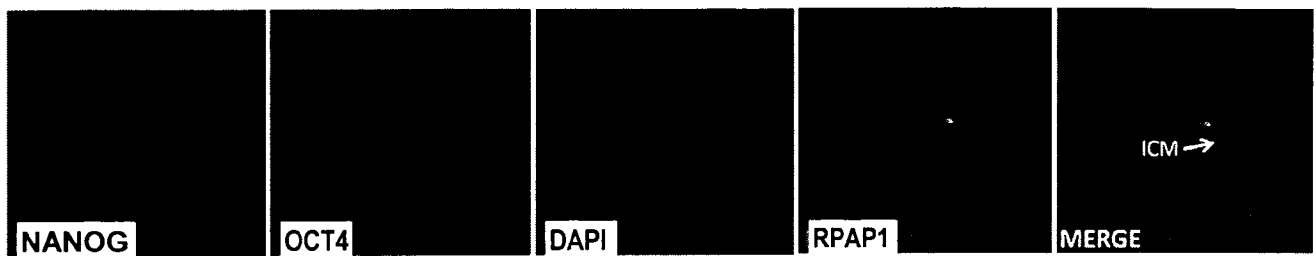


FIG. 1

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2a



2b

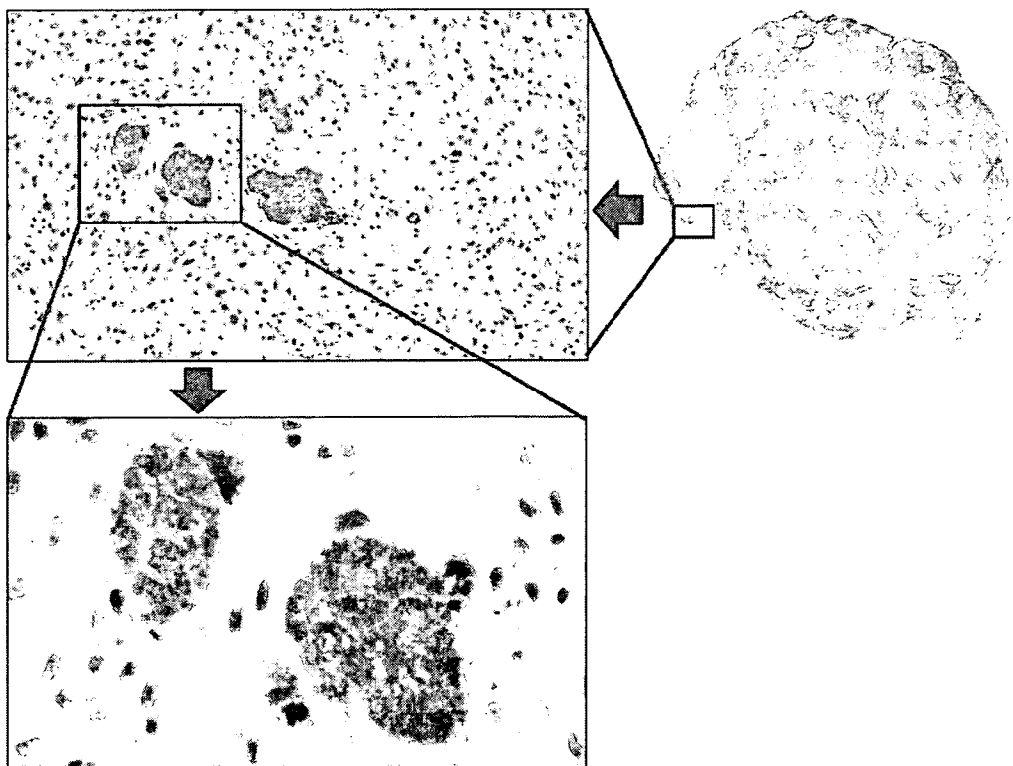
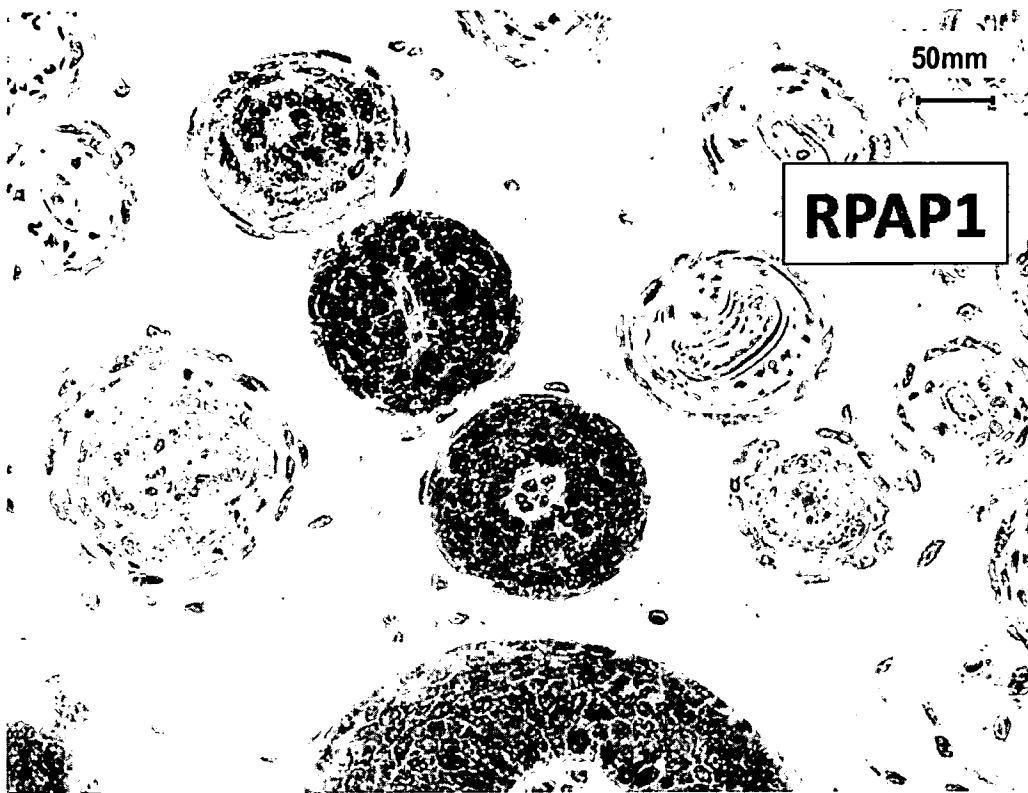


FIG. 2

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2c



2d

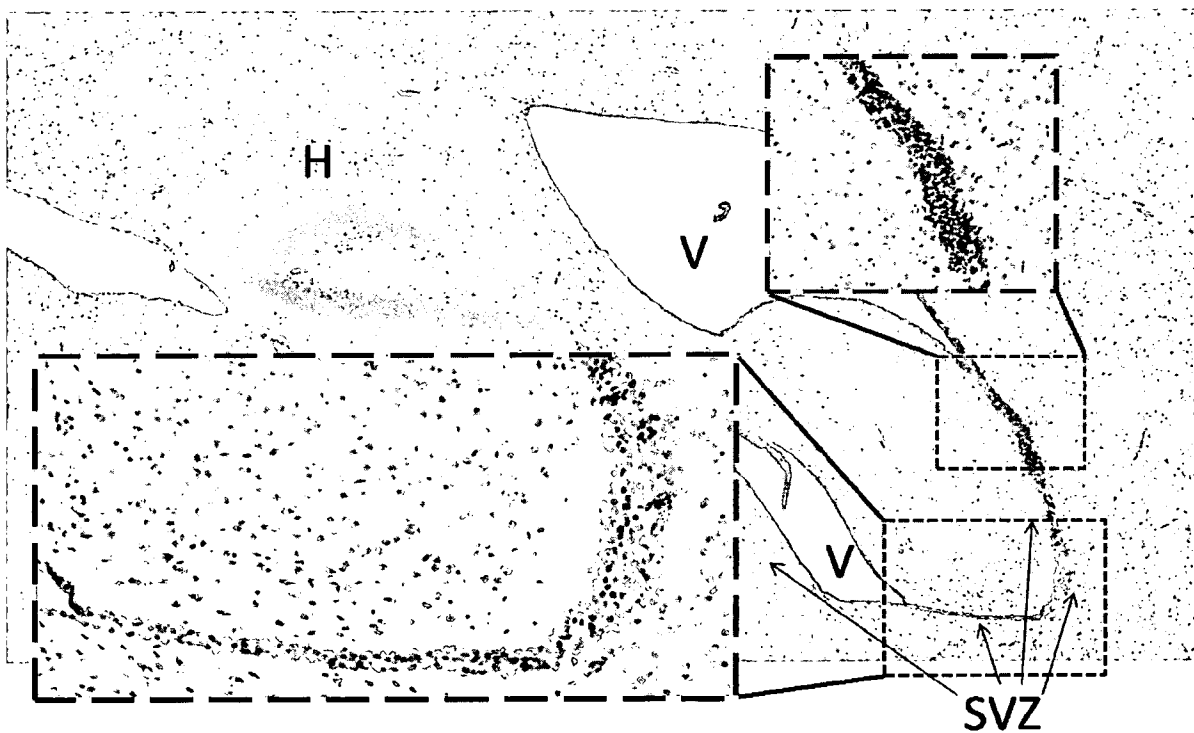
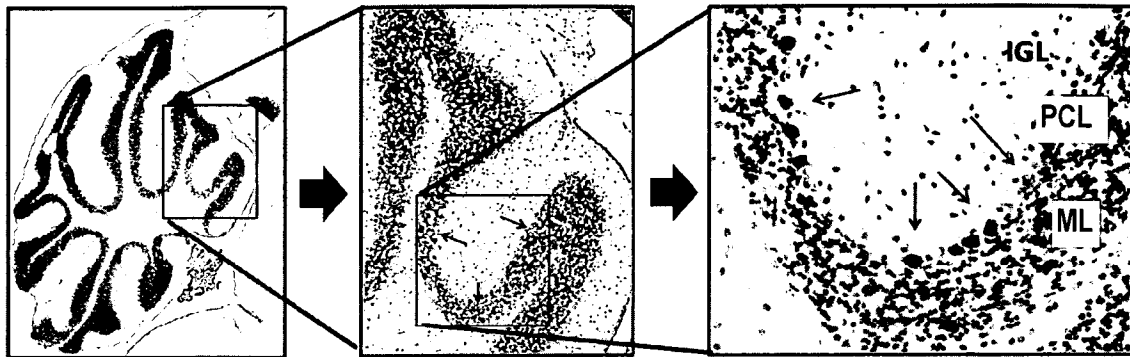


Fig. 2

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2e



2f

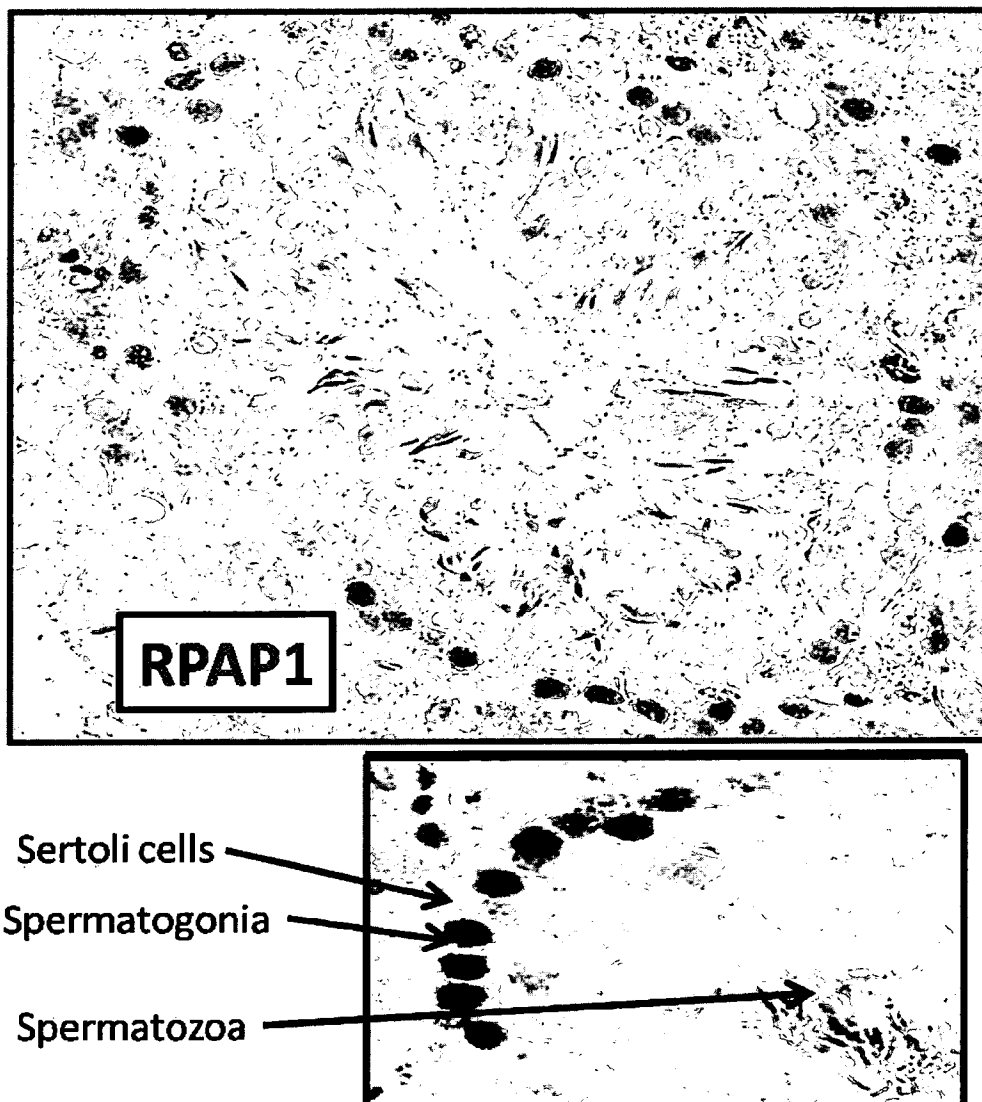
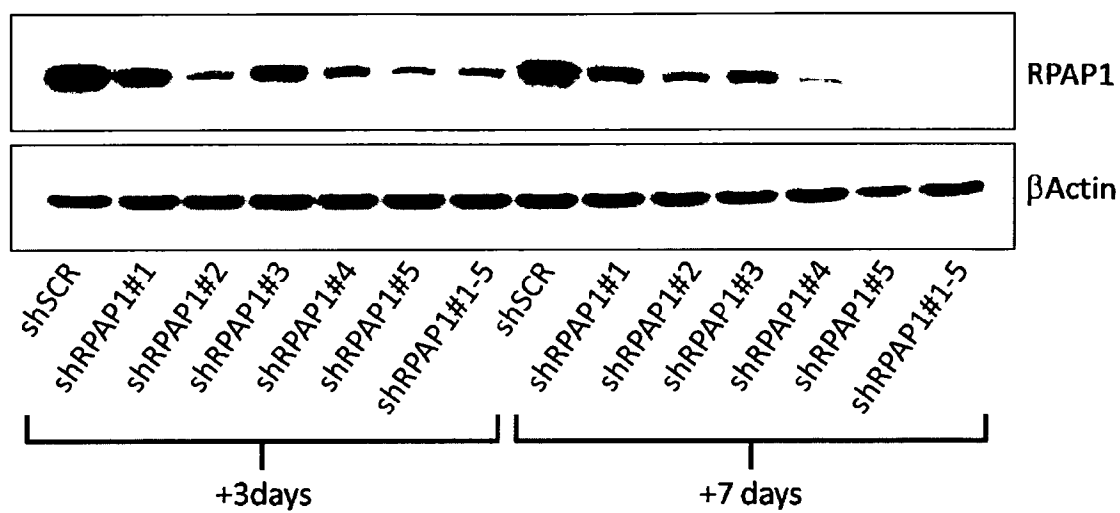


Fig. 2

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3a



3b

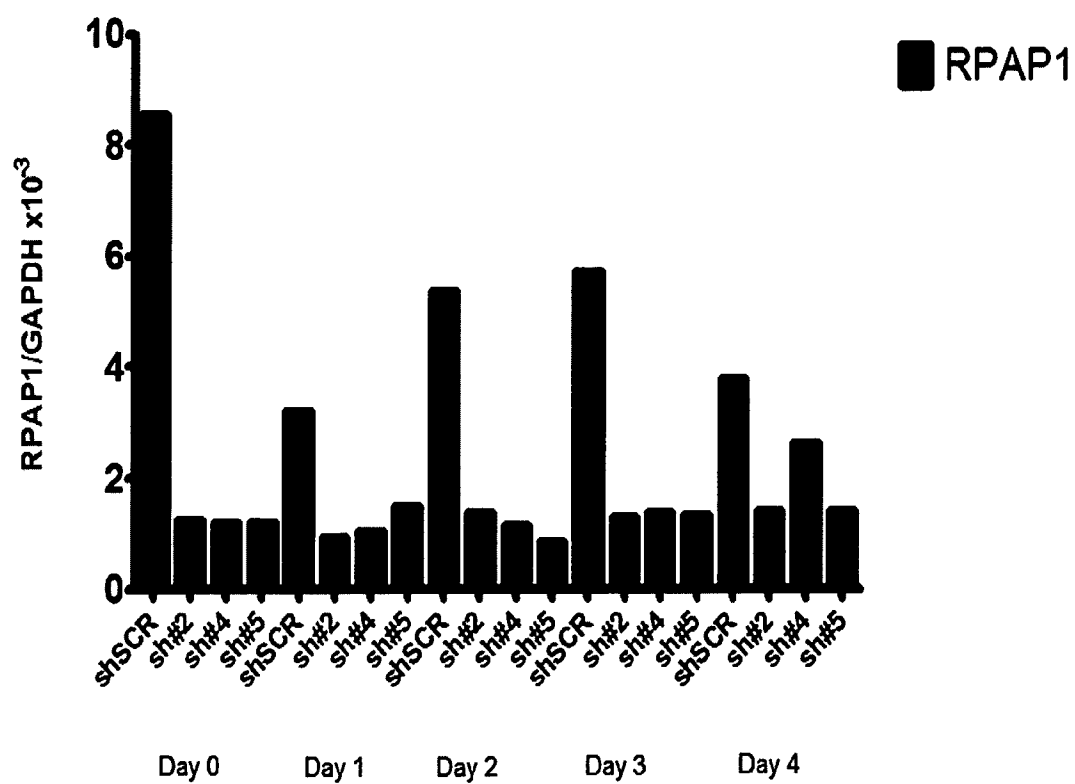
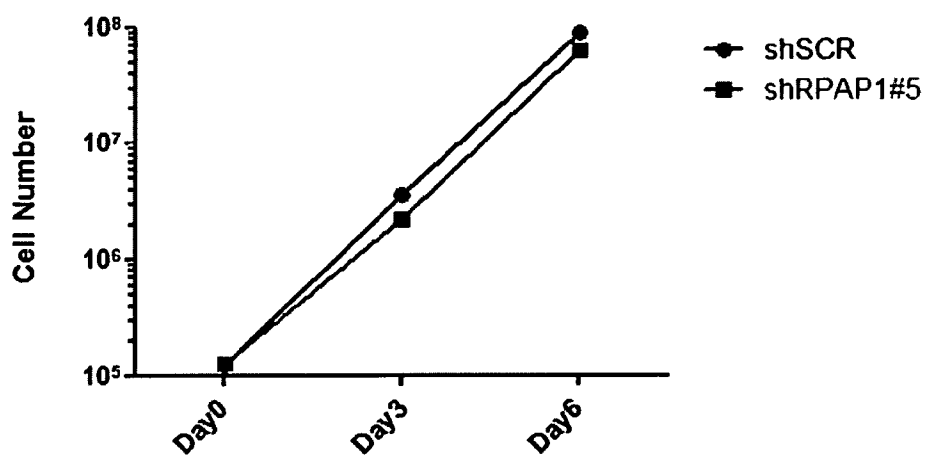
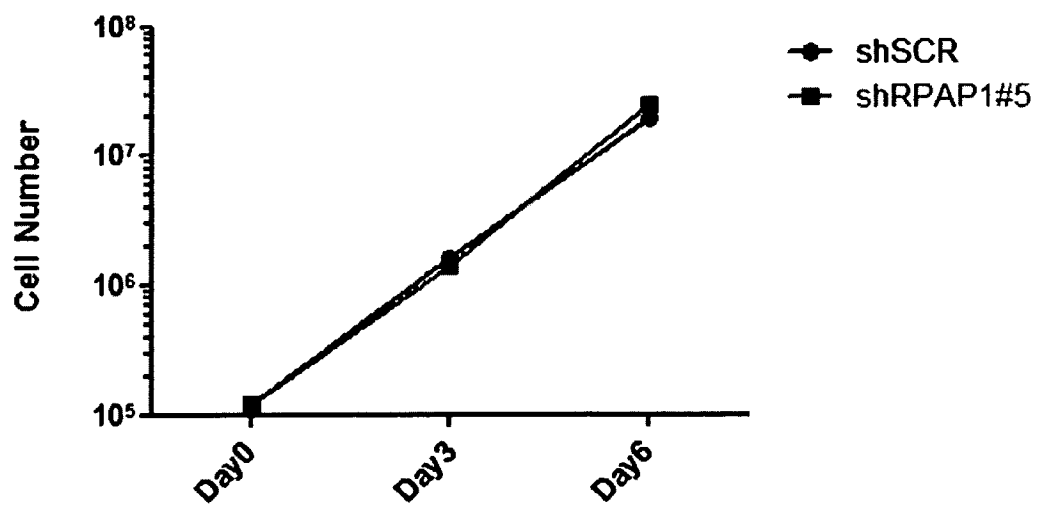


Fig. 3

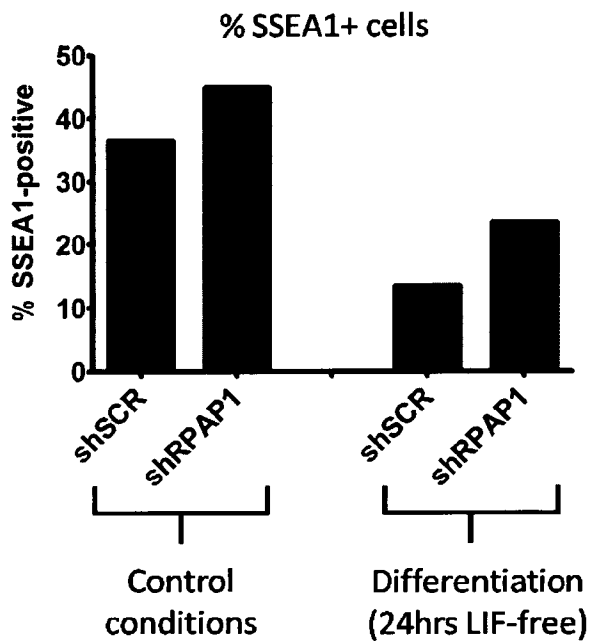
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3c

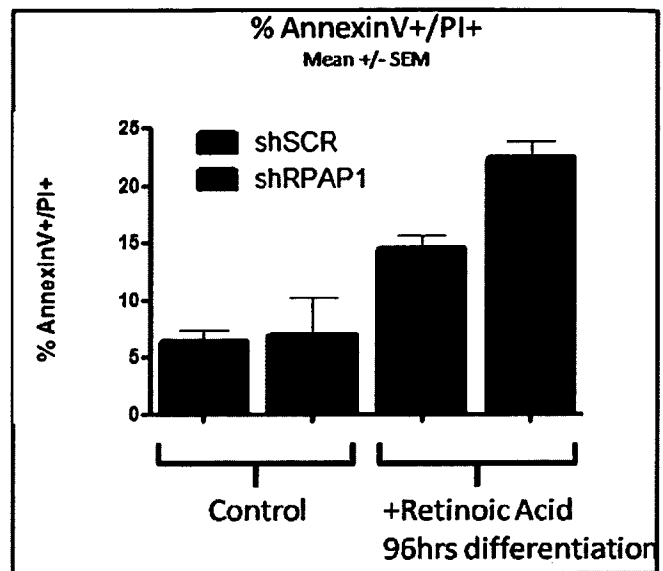
Proliferation Curve: E14 mESc shSCR vs shRPAP1**Proliferation Curve: P19EC shSCR vs shRPAP1****Fig. 3**

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3d



3f



3e

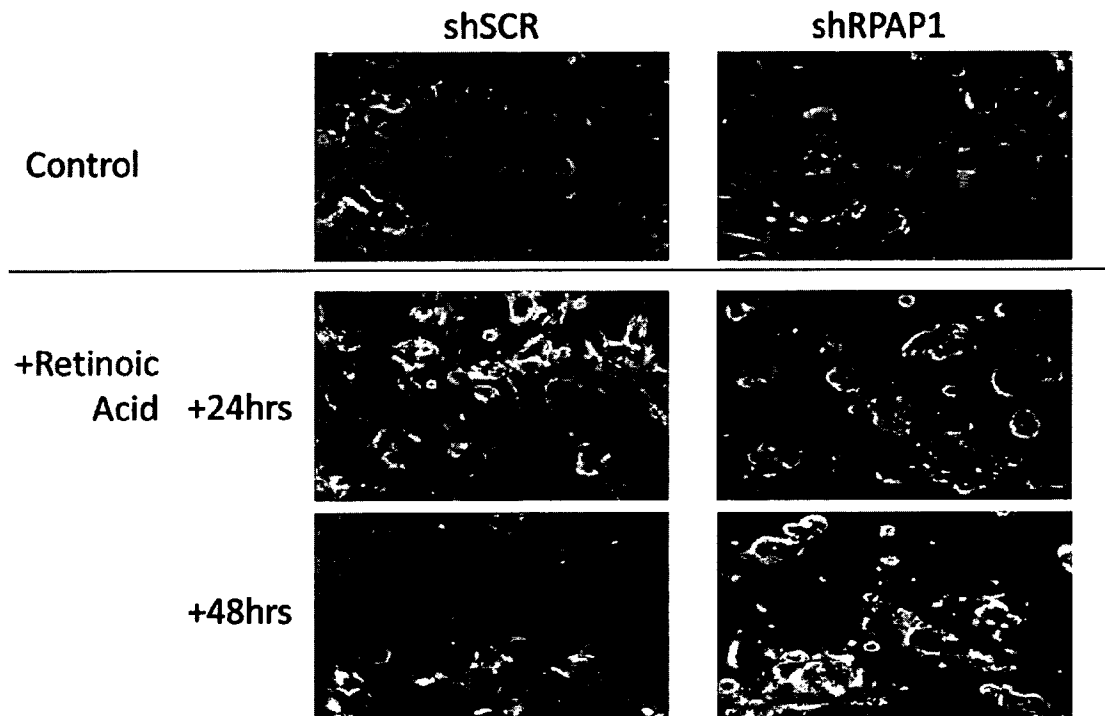


Fig. 3

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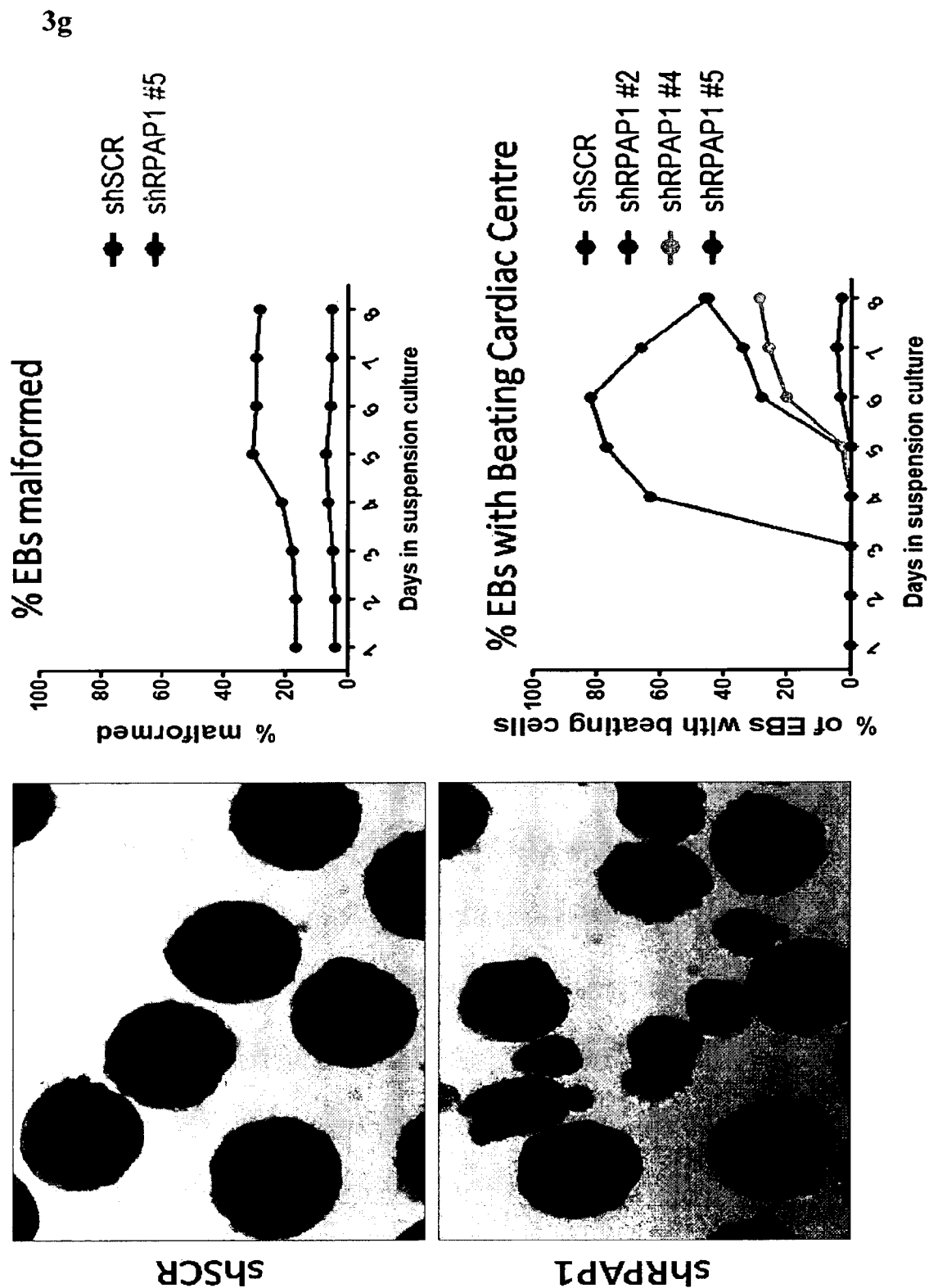


Fig. 3

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3h

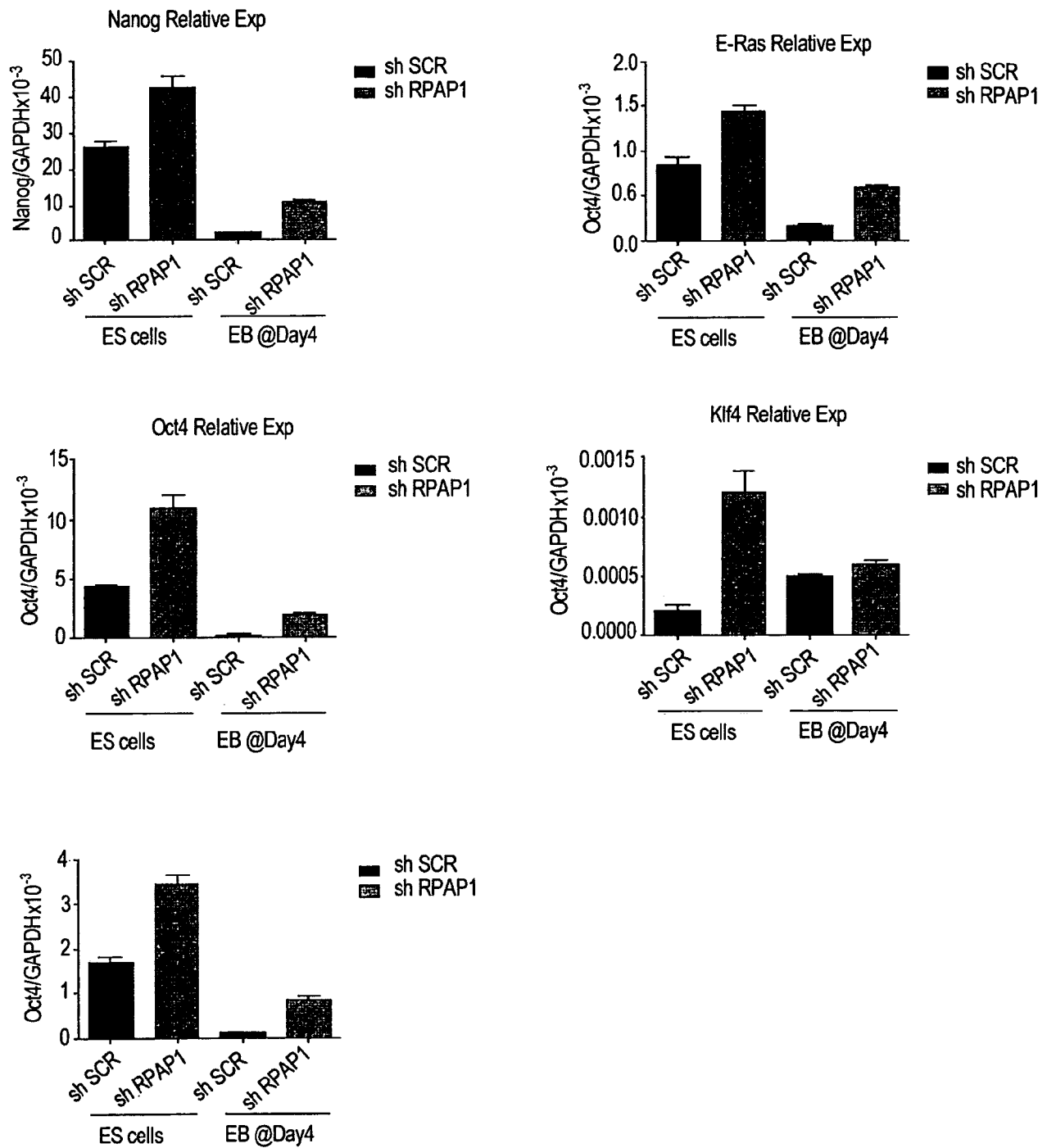


Fig. 3

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3i

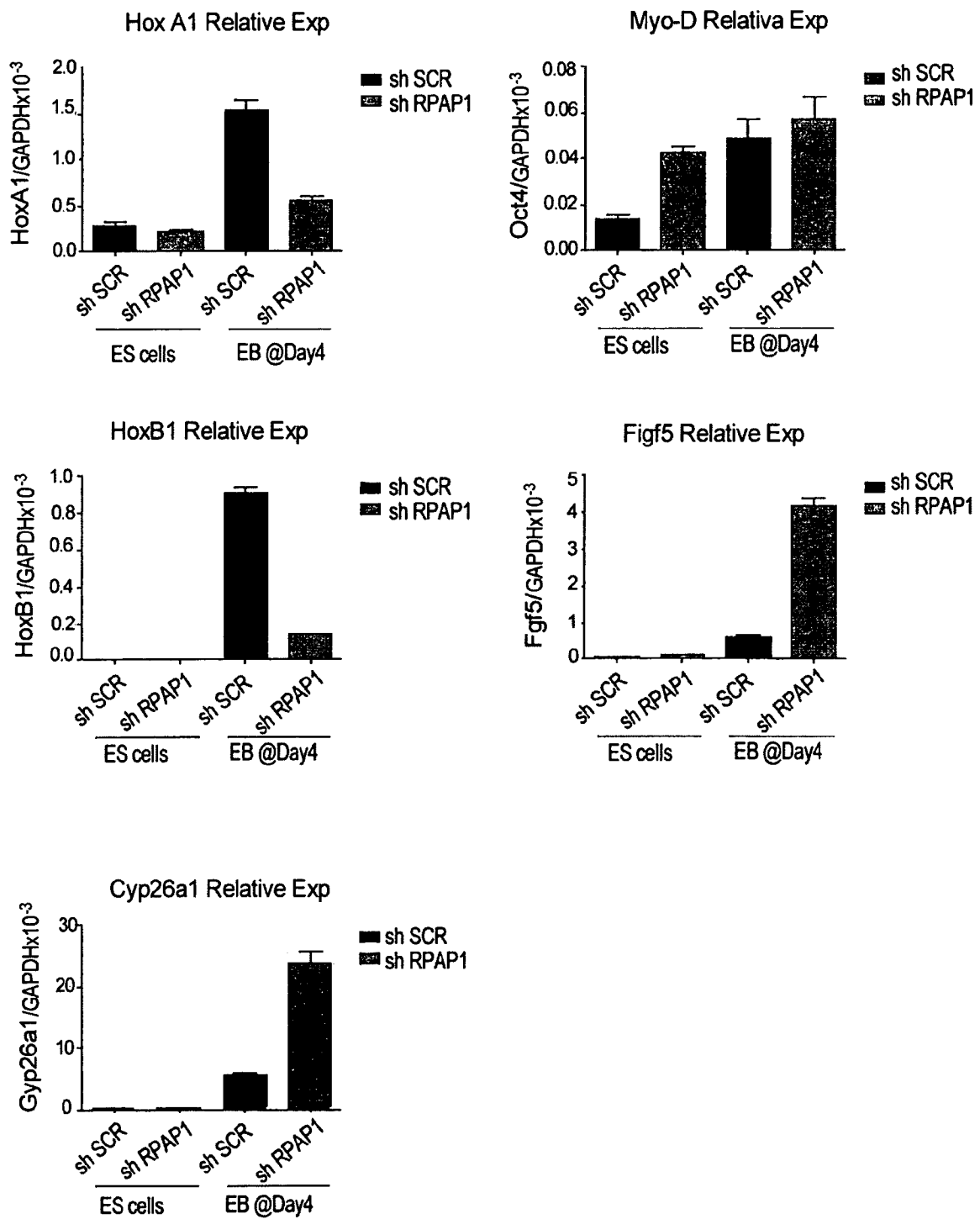


Fig. 3

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3j

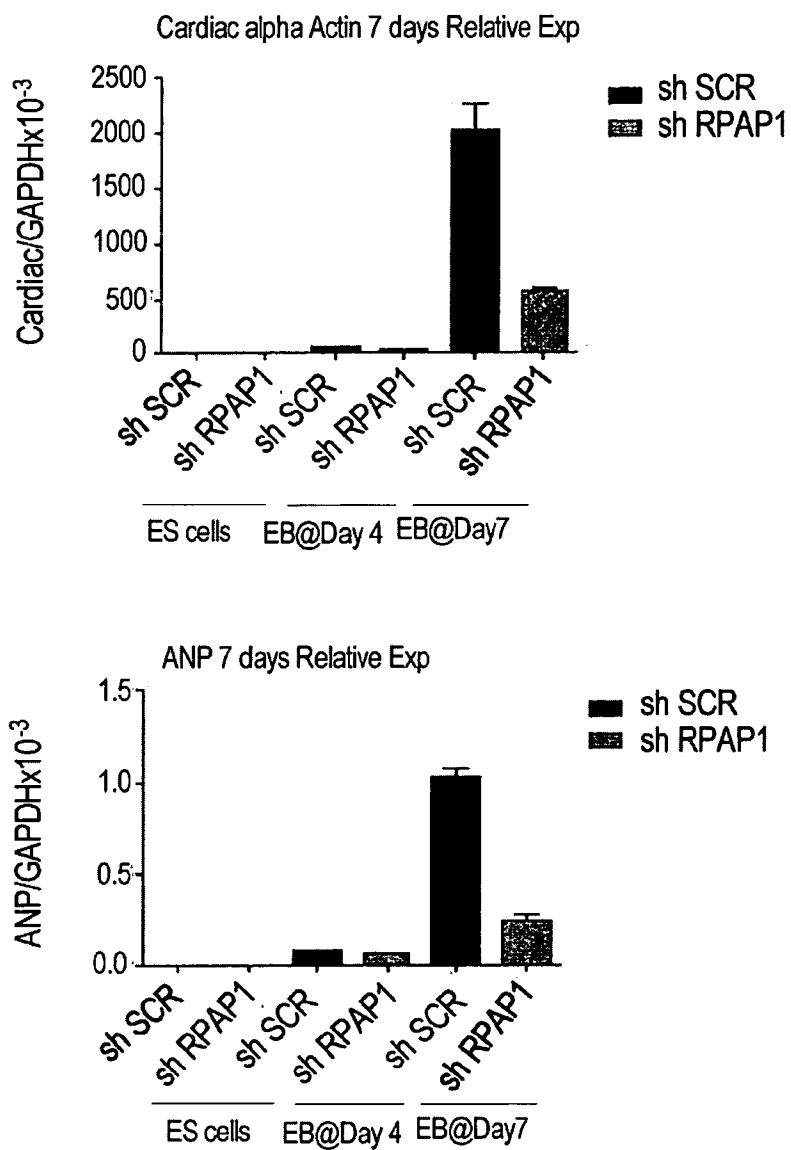
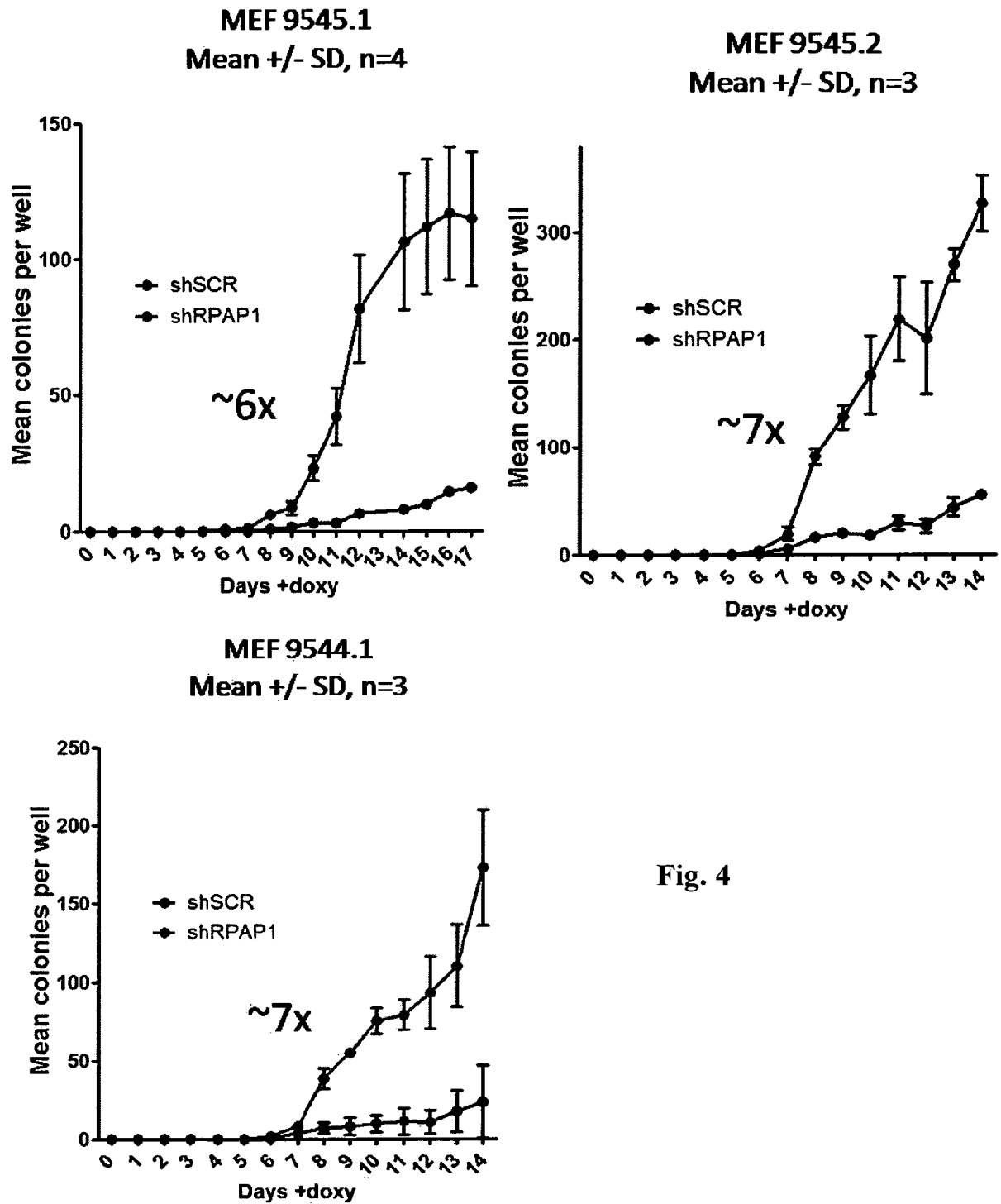


Fig. 3

4a

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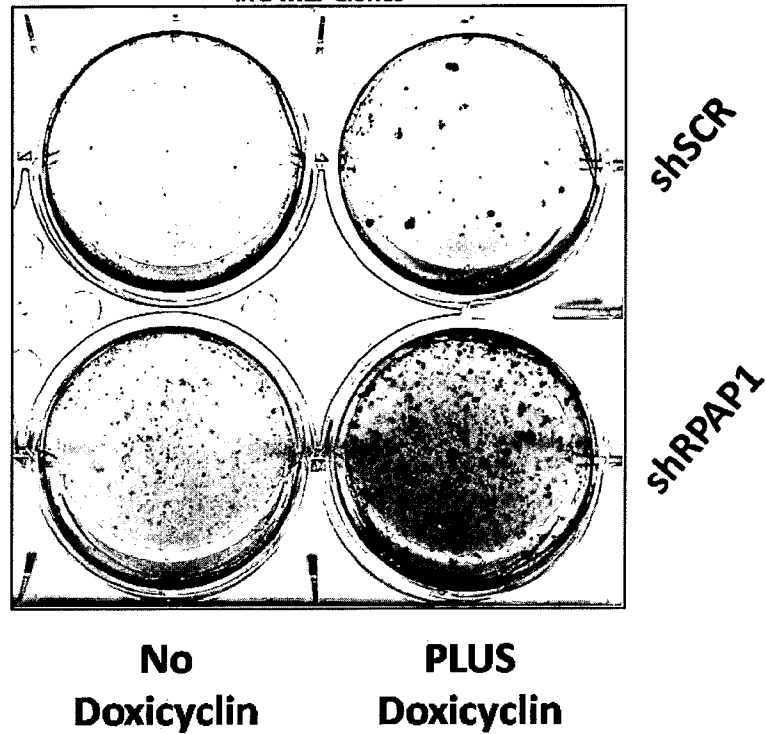


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4b

Alkaline Phosphatase Assay Colonies at Day14

Representative of reprogramming
in 3 MEF clones



Alkaline Phosphatase +Colonies

Day14 n=3 MEF clones, 3 wells each

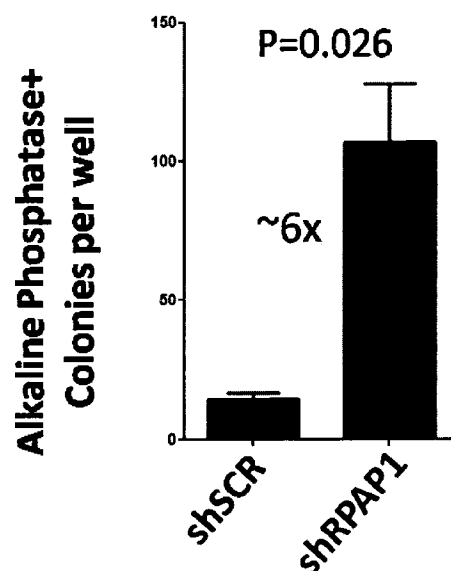


Fig. 4

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4c

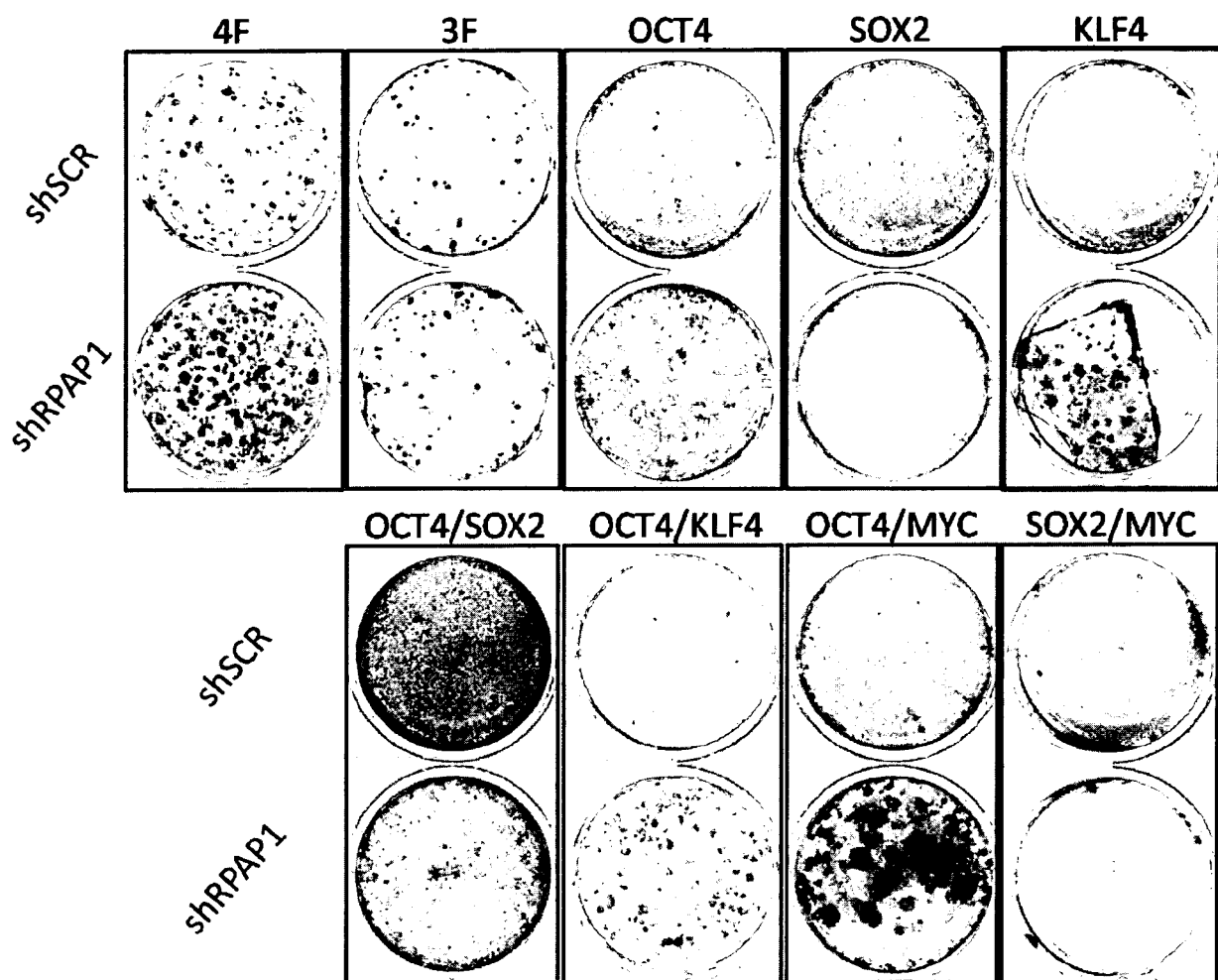


Fig. 4

4d

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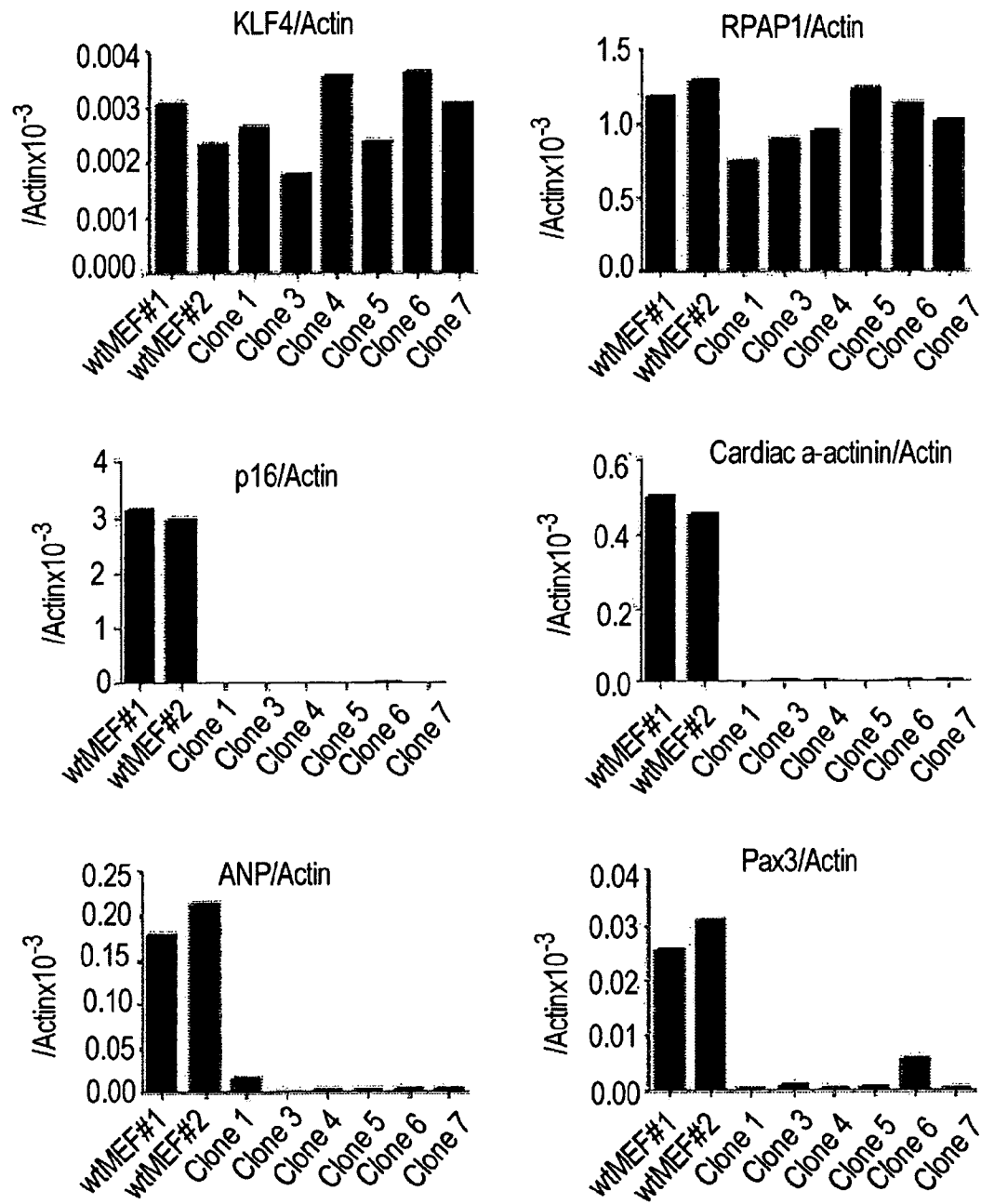


Fig. 4

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4d (cont.)

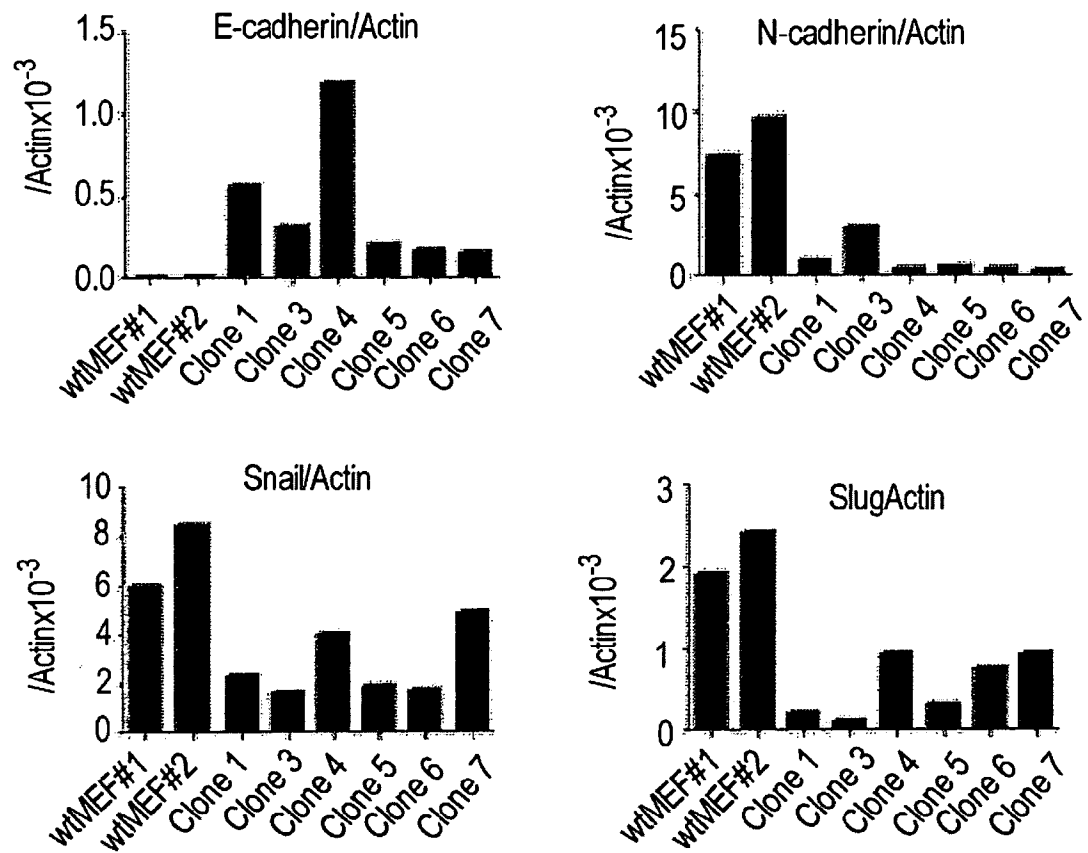


Fig. 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/059312

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/0735 C12N5/074
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

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

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

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NA-YU CHIA ET AL: "A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity", NATURE, vol. 468, no. 7321, 11 November 2010 (2010-11-11), pages 316-320, XP055031299, ISSN: 0028-0836, DOI: 10.1038/nature09531 cited in the application the whole document -/--	1



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

7 July 2012

Date of mailing of the international search report

30/07/2012

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Rutz, Berthold

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/059312

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	-& Na-Yu Chia et al.: "excerpt from Supplementary Table 1 to "A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity", Nature, vol. 468, 11 November 2010 (2010-11-11), page 2PP, XP002679217, Retrieved from the Internet: URL:http://www.nature.com/nature/journal/v468/n7321/extref/nature09531-s2.xls [retrieved on 2012-07-03] cited in the application Total pages of complete document are 1244	1
A	----- LEE T L ET AL: "Application of transcriptional and biological network analyses in mouse germ-cell transcriptomes", GENOMICS, vol. 88, no. 1, 1 July 2006 (2006-07-01), pages 18-33, XP024929414, ACADEMIC PRESS, SAN DIEGO, US ISSN: 0888-7543, DOI: 10.1016/J.YGENO.2006.03.008 [retrieved on 2006-07-01] the whole document	1-29
A	----- MARIA DE MIGUEL ET AL: "Pluripotent Stem Cells: Origin, Maintenance and Induction", STEM CELL REVIEWS AND REPORTS, vol. 6, no. 4, 1 December 2010 (2010-12-01), pages 633-649, XP055031661, ISSN: 1550-8943, DOI: 10.1007/s12015-010-9170-1 the whole document	1-29
A,P	----- MAITE SANMARTÍN ET AL: "A Molecular Switch for Initiating Cell Differentiation in Arabidopsis", CURRENT BIOLOGY, vol. 21, no. 12, 27 May 2011 (2011-05-27), pages 999-1008, XP055031340, ISSN: 0960-9822, DOI: 10.1016/j.cub.2011.04.041 cited in the application the whole document	1-29
