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(54) Title: PLURIPOTENT STEM CELLS

(57) Abstract: The invention provides processes for producing or propagating a formative stem (FS) cell line, from one or more precursor pluripotent stem cells, which process comprises: (a) providing a one or more precursor pluripotent stem cells; (b) culturing the precursor pluripotent stem cells in formative stem cell culture media. The formative stem cell culture media comprises limited amounts of activin and exogenous fibroblast growth factor, but includes a Wnt inhibitor, and typically a retinoic acid receptor inhibitor. The invention further provides related materials and methods for use in preparing and utilising FS cell lines.



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Pluripotent Stem Cells

Technical field

5 The present invention relates generally to methods and materials for use in deriving and propagating pluripotent stem cells in the “formative” state. The invention further provides such cells, and uses thereof.

Background to the invention

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The regulative capability of single cells to give rise to all primary embryonic lineages is termed “pluripotency”.

15

Mammalian embryos develop from pluripotent cells that are first set aside as “naïve” founders in the blastocyst, subsequently gain competence for multi-lineage induction, and finally acquire lineage-specific “priming” prior to commitment.

20

Various conditions have been reported that can confer long term expansion in vitro on pluripotent cell populations from rodent or primate embryos (Wu and Izpisua Belmonte, 2015).

25

The early naïve and late primed phases of pluripotency are reflected in vitro in propagation of two distinct types of mouse stem cell, embryonic stem (ES) cells and post-implantation epiblast-derived stem (EpiS) cells. Naïve pluripotent stem cells, such as mouse ES cells, are closely related to nascent epiblast in the pre-implantation ICM. They are considered to represent the earliest or most primitive form of pluripotency. Primed pluripotent stem cells, such as mouse EpiS cells, show features of gastrulating epiblast cells. They are considered to represent a late form of pluripotency poised for differentiation (Kojima et al., 2014; Wu et al., 2015a).

30

ES and EpiS cells are therefore considered to represent distinct naïve and primed stem cell states respectively (Nichols and Smith, 2009).

35

Naïve and primed pluripotent stem cells show global differences in gene expression, epigenetic features, metabolism, embryo colonisation potential, and various other properties. They have distinct culture requirements, for example, dual inhibition of MEK and GSK3 plus the cytokine leukaemia inhibitory factor (“2i+LIF”) for mouse ES cells (Wray et al., 2011; Ying et al., 2008) and high dose activin with fibroblast growth factor and optionally Wnt inhibition (“A_{hi}F+XAV”) for primed EpiS cells (Kurek et al., 2015; Sumi et al., 2013; Tsakiridis et al., 2014).

40

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ES cells are self-renewing pluripotent cell lines derived from pre-implantation embryos (Evans and Kaufman, 1981; Martin, 1981). They undergo continuous division in culture while retaining the capacity to enter into multi-lineage differentiation both in vitro and upon return to the embryo.

ES cells can be propagated at scale in a substantially homogeneous condition termed the naïve ground state in 2i+LIF medium. Naïve ES cells are thereby suspended in a specific time window of early development (Boroviak et al., 2014; Hackett and Surani, 2014).

5 Naïve cells such as ES cells can be differentiated into primed cells in culture while primed cells can be reverted to naïve status by genetic manipulation and/or culture perturbations.

10 It has commonly been held that when ES cells are released from ground state culture conditions, they enter directly into the primed phase of pluripotency in which transcriptional programs associated with different lineages are heterogeneously co-expressed along with pluripotency factors (Loh and Lim, 2011). This “precarious balance” model is drawn from observations of promiscuous and heterogeneous gene expression in ES cells maintained in foetal calf serum (Chambers et al., 2007; Hayashi et al., 2008; Torres-Padilla and Chambers, 2014). However, serum contains ill-defined factors that are
15 not physiological for early embryonic cells and may corrupt or destabilise cell identity.

An alternative proposition is that exit from naïve pluripotency, both in the embryo and in vitro, is succeeded by a “formative” phase during which direct competence for germline and somatic lineage specification is acquired (Kalkan and Smith, 2014; Smith, 2017).

20 In the embryo, definitive lineage specification factors are not observed until some 30 hours after implantation. A similar temporal segregation is seen at the onset of ground state ES cell differentiation in defined conditions (Kalkan et al., 2016; Kalkan and Smith, 2014). Transcription factor circuitry specifically associated with the naïve state (Dunn et al., 2014) is first rapidly extinguished, (Kalkan and Smith, 2014; Leeb et al., 2014), up-regulation of de novo methyltransferases (DNMTs) leads to widespread DNA methylation (Lee et al., 2014), the enhancer landscape is extensively remodelled (Buecker et al., 2014), and X chromosome inactivation is initiated in female cells. These changes proceed while general pluripotency factors Oct4, Sall4 and Sox2 persist, but prior to evidence of
25 lineage specification. During this period pluripotent cells become responsive to germline induction either by cytokines or by forced transcription factor expression (Hayashi et al., 2011; Nakaki et al., 2013). Notably, naïve ES cells cannot respond to such stimuli but must be differentiated for 24-48hrs into a post-implantation epiblast-like cell (EpiLC) state to acquire responsiveness.

35 Data consistent with the idea of a formative transition are presented in (Kalkan et al., 2016) and (Mulas et al., 2016). These two papers describe a transient pluripotent population at early stages of naïve ES cell differentiation with similarity to E4.75-5.75 epiblast and EpiLC.

40 The period of formative pluripotency in the mouse embryo is postulated to extend from the late pre-implantation epiblast at around E4.75 until regionalised patterning becomes evident in the egg cylinder from around E5.75. During this window epiblast cells remain unpatterned and without expression of exclusive lineage-specification factors. They have
45 completely down-regulated the naïve pluripotency transcription factor circuitry and can no

longer give rise directly to ES cells (Boroviak et al., 2014; Boroviak et al., 2015; Brook and Gardner, 1997).

5 Primed pluripotent EpiS cells have been derived by culture in medium supplemented with
Knockout serum replacement (KSR) or high levels of activin (10-20 ng/ml) together with
fibroblast growth factor (FGF, 10-12.5ng/ml) on a feeder cell layer (Brons et al., 2007;
Tesar et al., 2007) or without feeders (Guo et al., 2009). Importantly, however,
10 established EpiS cell lines are distinct from E5.5 epiblast. Notably they display global
transcriptome similarity to gastrula-stage primed epiblast at E7.0, from which they can
also be derived efficiently (Kojima et al., 2014; Osorno et al., 2012). Furthermore, EpiS
cells do not respond to primordial germ cell induction unlike E5.5 epiblast or EpiLC
(Hayashi et al., 2011; Murakami et al., 2016; Ohinata et al., 2009).

Disclosure of the invention

15 The present inventors have investigated whether pluripotency progression may be
paused at the intermediate “formative” phase described above. They hypothesised that
global regulatory remodelling during the formative transition instates the molecular
machinery for multi-lineage commitment and subsequent differentiation. Notably, the
20 abrupt elimination of naïve transcription factors which control the ES cell state means
they no longer play a role. The formative remodelling of pluripotency is proposed to
generate a group of cells that are uniformly equipped to respond to patterning and lineage
specification cues.

25 Therefore formative pluripotency may be manifest in profound transcriptional and
epigenetic resetting relative to naïve pluripotency, lack of lineage-affiliated transcriptional
activity characteristic of primed pluripotency, and different requirements for propagation
compared to earlier naïve or later primed pluripotency.

30 The disclosure herein provides methods for generating a discrete pluripotent cell
population that is molecularly and functionally distinguishable from previous
heterogeneous pluripotent cultures and from defined naïve ES cells or primed EpiS cells.
It is believed that these culture conditions described herein sustain a stable pluripotent
stem cell state corresponding to the formative phase of pluripotency in the embryo.

35 The invention thus provides cells which possess the expected core features of formative
pluripotency, but which can be maintained as continuous stem cell cultures. These cells
and lines are termed formative stem (FS) cell lines herein.

40 Provided herein are processes for producing FS cell lines from precursor pluripotent stem
cells, and processes for propagating FS cell lines. This is achieved by suppressing
signals for lineage specification whilst sustaining proliferation via autocrine FGF
stimulation. In preferred embodiments, the inventors have utilised developmentally
appropriate minimal activation of the nodal/activin pathway combined with autocrine
45 stimulation of the FGF/Erk pathway and inhibition of the Wnt pathway. Preferred media
further include a pan-retinoic acid receptor inverse agonist, (RARI) and a γ -secretase

inhibitor. Also provided are related methods and materials relating to the novel media and the formative stem cell lines of the invention and to their generation from more mature cell types by resetting or reprogramming.

5 The ability to capture or derive and propagate stable and homogenous cell lines in the formative pluripotent state is unexpected because it was previously considered that the natural formative phase is transient and may not represent a stable attractor state, since formative cells quickly become primed in mouse embryos and there is no precedent for pausing development at this stage in rodents or primates.

10

Without wishing to be bound by theory, it is surmised that the formative pluripotent state is sustained by autocrine low level activation of nodal/activin/TGF- β signalling and of the FGF/Erk MAP kinase pathway and that progression to primed pluripotency is blocked by Wnt inhibition and RAR inhibition. Low level exogenous activin (or other TGF β superfamily member) is normally required, potentially together with autocrine nodal. However, receptor stimulation may be dispensable in the presence of a γ -secretase inhibitor.

15

20

Irrespective of the precise mechanism, the inventors have shown that FS cell lines can be provided from different species, including from human cells, and different cell types, including embryonic naïve pluripotent stem cells, and other pluripotent cell types, as well as pluripotent cell lines and reprogrammed somatic cells.

25

“Formative pluripotent cells” as defined herein lack expression of naïve pluripotency factors or gastrulation markers. At the whole transcriptome level they cluster apart from naïve pluripotent cells and primed pluripotent cells. Functionally they display robust multi-lineage differentiation in vitro and unlike either ES or EpiS cells they respond directly to germ cell induction. Furthermore, mouse formative stem cells show widespread contribution to chimaeras after blastocyst injection.

30

Further characteristics of the FS cells are described hereinafter, and in Table 4 hereinafter.

35

The provision of stable FS cells provides a useful contribution to the art.

40

For example FS cells are believed to retain imprinted gene status. Current human naïve pluripotent stem cells do not stably retain imprints. Thus one advantageous embodiment of the invention for obtaining a fully competent human pluripotent stem cell involves transient derivation or resetting to naïve status then conversion to formative status before erasure of imprints occurs in the naïve state. FS cells may therefore be a superior or alternative source material for chimaera formation or directed differentiation compared with other forms of pluripotent stem cell. Formative pluripotent stem cells may be a desirable intermediate stage for population expansion prior to directed differentiation of human naïve pluripotent stem cells.

45

Further utilities of the FS cells are described hereinafter.

While multiple conditions have previously been described for propagating rodent and primate pluripotent stem cells, these primarily result in heterogeneous populations. The notable exception is the 2i+LIF culture system for mouse naïve ES cells (Ying et al., 2008)) and the recently reported conditions for human naïve pluripotent stem cells (Guo et al., submitted; Guo et al., 2016; Takashima et al., 2014; Theunissen et al., 2014)

Other conditions, such as FGF with KSR or high concentrations of activin (or TGF β), yield cell populations that are exclusively or predominantly primed (Brons et al., 2007; Tesar et al., 2007; Wu et al., 2015a), or are mixed and metastable, such as mouse ES cells in serum (Enver et al., 2009; Filipczyk et al., 2015; Fishell, 1995; Marks et al., 2012). Such cultures may in principle contain minority sub-populations of uncharacterised cells that have features of formative pluripotency, but those cells have not been identified in any previous study, and the mixtures are sub-optimal for initiating multi-lineage differentiation or chimaera formation, and cannot reliably be standardised.

In contrast the present specification provides conditions for deriving and propagating cells which sustain substantially homogeneous cell populations that: (i) are genetically and phenotypically stable; (ii) exhibit consistent characteristics and behaviour between cell lines; (iii) display robust multi-lineage differentiation; (iv) are distinct from previously well-characterised naïve and primed pluripotent stem cell cultures.

These and other aspects of the invention will now be described in more detail:

Production of FS cell lines using FS cell culture media

In one aspect the invention provides a process for producing a formative stem (FS) cell line, from one or more precursor pluripotent cells, which process comprises:

- (a) providing one or more precursor pluripotent cells;
- (b) culturing the precursor pluripotent cells in formative stem cell culture media.

“Formative stem cell culture media” is explained in more detail herein. Formative stem cell culture media allows proliferation via autocrine or limited exogenous FGF stimulation while suppressing signals for lineage specification.

Specifically FS cell culture medium provides developmentally appropriate moderate stimulation of the FGF/Erk pathway by autocrine ligands, FGF4 and FGF5, optionally supplemented with exogenous FGF (typically less than 10ng/ml e.g. 0.5-5ng/ml, or 0.5 to 3 ng/ml), together with low level activation of the nodal/activin/TGF- β pathway using less than 5ng/ml exogenous activin A (e.g. 1-4ng/ml, e.g. 1.5-3 ng/ml) or nodal or TGF- β family member.

Lineage specification is blocked by inhibition of the Wnt pathway. This can be achieved by using a tankyrase inhibitor such as XAV939 (e.g. range 0.5-10 μ M, for example 2 μ M) or IWR1 (e.g. 0.5-10 μ M, for example 2.5 μ M) or a porcupine inactivator such as IWP2 (e.g. 0.5-10 μ M, for example 2 μ M).

As explained herein, the FS cell culture medium preferably includes an inhibitor of the retinoic acid receptor (RAR) e.g. a selective inverse agonist such as BMS 493 (0.1-1.0 μ M).

5

A preferred FS cell culture medium comprises:

(i) a low concentration of activin (or other TGF β superfamily member);

(ii) low or no exogenous fibroblast growth factor;

(iii) tankyrase inhibitor or other Wnt inhibitor;

10 (iv) absence of serum or serum substitutes such as KSR, and preferably

(v) a retinoic acid receptor inhibitor.

Canonical Wnt pathway inhibition has previously been reported to reduce mesoendodermal gene expression and shift the regional specification of EpiS cells and human PSC, but not to alter their primed identity or confer ability to colonise blastocyst chimaeras (Tsakiridis et al., 2014; Wu et al., 2015b).

15

Primed pluripotent stem cells, both mouse and human, are highly dependent on exogenous stimulation of the nodal/activin/TGF β signalling and FGF pathways (Brons et al., 2007; Tesar et al., 2007; Thomson et al., 1998; Vallier et al., 2005) through ligands provided by feeder cells, serum or KSR, or as growth factor supplements. For example a medium typically used for mouse and human primed cells is supplemented with an activator of the Erk/MAP kinase pathway (typically FGF at 4-12.5ng/ml) and also of the activin/nodal/TGF- β pathway (typically activin at 10-20ng/ml, or TGF- β at 2ng/ml) which may also be provided by MEF feeders (the concentration of activin A secreted by feeders is 6.2-15.0 ng/ml (Kojima et al., 2014) and feeders also secrete TGF- β s) and/or KSR or serum.

20

25

As shown in the Examples, the inventors have demonstrated pluripotent cells such as early post-implantation mouse epiblast (E5.5) cells can be converted into stem cell lines that retain formative properties by culture on a fibronectin substrate in defined medium and can be expanded long term (>2 passages) in this medium while retaining diploid karyotype. For brevity this medium, based on 'low activin plus XAV' may be termed "A_{lo} X" herein.

30

35

Thus a process of the invention may comprise use of a formative stem cell culture medium which comprises:

(i) activin at a concentrations of 1 - 4ng/ml, more preferably 1.5 - 3 ng/ml;

40 (ii) less than 10ng/ml of exogenous FGF; more preferably less than 4 or 5ng/ml e.g. 0.5 to 4 ng/ml of exogenous FGF;

(iii) a wnt inhibitor to suppress Wnt signalling (e.g. XAV939, e.g. at 2 μ M, or IWP2 at 2 μ M).

In one embodiment the formative stem cell culture media comprises no exogenous stimulator of the FGF pathways.

45

Preferably the formative stem cell culture media comprises a Wnt inhibitor which is a tankyrase inhibitor e.g. XAV939, optionally at 0.5-10 μM e.g. 2 μM .

5 The inventors have observed that mouse formative pluripotent stem cells cultured in A₁₀X display sporadic neural differentiation, particularly during early passages. Furthermore, retinoic acid (10^{-7}M) induced differentiation of FS cells. It is desirable to suppress this endogenous retinoid signalling which can be achieved by addition of a retinoic acid receptor (RAR) inhibitor. As shown in the Examples herein, addition of RAR inhibitor stabilises formative pluripotent stem cells in the presence of very low concentrations of
10 activin.

One preferred inhibitor is a pan-retinoic acid receptor inverse agonist such as BMS 493 applied at a concentration range of 0.1-1.0 μM e.g. 0.5 μM . Other RAR inhibitors may include LG100815 (Stavridis et al., 2010), AGN 193109, AGN194310 .
15

Notably, inhibitors of the related pan-RXR receptors, such as UVI 3003 (0.1-1.0 μM) were not effective in conditions where BMS493 was effective. For example BMS493 could block neural differentiation of FS cells cultured in A₁₀X with addition of 1×10^{-7} M Retinoic acid, whereas UVI3003 could not.
20

The inventors further observed that addition of a γ -secretase inhibitor (" γSi ") further increased the homogeneity and stability of FS cell cultures by eliminating residual neural specification.

25 Thus a preferred culture condition for FS cells comprises low activin, Wnt inhibition, retinoic acid receptor inhibition and gamma-secretase inhibition. For brevity this medium, may be termed "A₁₀X+RARi+ γSi " herein, and may optionally inclusion of a low to moderate concentration of FGF (e.g. < 5, 4, 3, 2, or 1ng/ml).

30 Thus in one embodiment the formative stem cell culture media further comprises RARi e.g. BMS 493, optionally at 0.1-1.0 μM .

Thus in one embodiment the formative stem cell culture media further comprises a γ -secretase inhibitor e.g. CAS 209984-56-5 at 1.7nM-1.0 μM e.g. 1.7nM to 0.1 μM , e.g.
35 0.1 μM . In another embodiment the γ -secretase inhibitor may be CAS 209986-17-4 or other commercially available agent.

The formative stem cell culture media may be based on N2B27 medium (Ying and Smith, 2003) or other media known in the art designed for serum free culture of pluripotent stem cells, such as E6 or SF-O3. The formative stem cell culture media may comprise insulin, selenium, transferrin, anti-oxidants and lipid supplements.
40

The FS media may also be characterised by an absence of serum, KSR, BMP or Wnt. These components have all been previously used in various combinations with other
45 components in medium cocktails for culturing ill-defined or heterogeneous pluripotent stem cells. At concentrations normally used in cell culture, such as 10% serum or 20%

KSR, they each induce upregulation of somatic genes and/or overt differentiation of FS cells.

The inventors observed that cultures differentiated or died upon inhibition of PI3 kinase (Ly294002, 25 μ M), Src (WH-4-023, 1 μ M), JNK (SP-600125, 10 μ M), MEK (PD0325901, 1 μ M) or FGF receptor (PD173074, 0.1 μ M). Furthermore inhibition of GSK3 with CHIR99021 at 1 μ M induced expression of Brachyury, although this effect was not evident at 0.5 μ M. The FS media may be characterised by an absence of any one or more of these, although may optionally contain a low level of GSK3 inhibitor (e.g. up to around 0.5 μ M).

Thus the formative stem cell culture media may optionally not comprise any one or more of: serum; KSR; BMP; Wnt, retinoic acid, MEF feeders. Furthermore, the formative stem cell medium may optionally not comprise a PI3 kinase inhibitor; a Src inhibitor; a JNK inhibitor; a GSK3 inhibitor, a MEK inhibitor or an FGF receptor inhibitor at concentrations such as those indicated that are standardly used for achieving inhibition in cell culture.

Culture conditions

Suitable culture conditions in which the novel FS media of the invention may be utilised may be as described herein. In the Examples below, and without limitation, E5.5 epiblasts were obtained by dissection and plated individually on fibronectin-coated 4-well plates in N2B27 medium. Cultures were propagated in 7% CO₂ and 5% O₂. After several days, explants were treated with Accutase for 5-10 seconds at room temp and wash buffer was gently added to detach and fragment the outgrowths into small clumps. Cells were collected by centrifugation and seeded into fresh 4-well plates. Subsequently, expanding cultures were treated with Accutase briefly (30-60 seconds, at room temp) for dissociation into cell clusters by gentle trituration. Established cultures were routinely passaged every 2-3 days and plated onto new fibronectin coated dishes at a split ratio of 1/10-1/15. Medium was changed every other day.

In one embodiment, a culturing process of the invention comprise one or more of: culturing on fibronectin coated plates; propagation in 7% CO₂ and 5% O₂; passaging every 2 to 3 days with a split ratio of 1/10-1/15; change of medium every other day. It is preferable to avoid dissociation to single cells during passaging. Use of ROCK inhibitor is optional.

It will be readily appreciated by those skilled in the art that the exact conditions for pluripotent cell culture may be varied without departing from the invention.

Sources of FS cells

FS cells as described herein may be derived from a number of different sources.

For example FS stem cells can be derived most directly from embryonic epiblast tissue at the formative phase of development (early post-implantation up to mid-gastrulation in

most mammals, including primates), by explant or dissociation into formative culture conditions.

5 FS cells can be derived from naïve pluripotent cells either taken directly from embryos, or established as naïve embryonic stem cells, or generated as induced pluripotent stem cells by somatic cell reprogramming (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007) coupled with naïve resetting. Naïve cells are transitioned to the formative phase of pluripotency and then 'captured' by culture in formative stem cell conditions. Transitioning from naïve to formative phases will typically entail withdrawal of
10 naïve pluripotency maintenance conditions (e.g. "2iLIF" for mouse; or titrated 2iLIF plus the PKC inhibitor Gö6983, "t2iLIF+Gö" (Takashima et al., 2014) for human) and transfer to formative pluripotency culture conditions as described herein, either immediately or after a short period with no factor addition.

15 FS cells can be derived from primed pluripotent stem cells by resetting to a naïve state using methods known in the art (see e.g. published patent application WO2016/027099), then transitioning to the formative phase as described above. The period in the naïve state culture conditions may be transient but should be sufficient to achieve epigenetic erasure.

20 'Formative-like' stem cells can be isolated from primed pluripotent stem cells by resetting or selection/adaptation directly in formative culture conditions. However, since this is expected to occur without comprehensive erasure of epigenetic restrictions or abnormalities, the resulting cells may retain impediments to multi-lineage differentiation.

25 FS cells can also be derived from somatic cells via known factor-based reprogramming methods, reviewed by (Takahashi and Yamanaka, 2015), coupled directly with resetting to a naïve state, then treating as described above.

30 As explained below, FS cells can be provided from desired mammalian or avian species. Preferred mammals include rodent (e.g. mouse), porcine mammals, lagomorph, artiodactyla, or primate (e.g. human). The invention finds utility for, without limitation, mammals which are companion animals, model animals or livestock. Preferred avian species include poultry such as chickens.

35 *Use of cells at a formative phase of development*

In one embodiment the processes of the invention utilise precursor pluripotent cells which are already in a formative phase of development e.g. obtained by explant or dissociation from early post-implantation embryonic epiblast tissue.

40 Those skilled in the art are generally familiar with the stages of pluripotency lineage in different mammalian embryos, from naïve to primed phases. Thus sources of cells at the formative phase of development suitable for culture by the methods of the invention may be identified according to the known embryonic development progression of the species
45 of interest, and the disclosure herein.

For example, where the FS cells are rodent (e.g. mouse) these may be epiblast cells between E4.75 and E6, preferably E5.0- 5.5 – see e.g. Kalkan, T. and Smith, A. (2014).

5 In other mammals the formative phase may last for several days. For example, in the non-human primate Cynomolgus, cells with expected features of formative pluripotency are evident at the first stage examined after implantation (E13) and persist during gastrulation up to at least E17 (Nakamura et al., 2016).

10 In some species, however, such as the rabbit and pig, axis determination and gastrulation commence before implantation. Accordingly formative epiblast in these species is sourced from the late blastocyst stage prior to or co-incident with formation of the primitive streak.

15 In the case of human, in utero post-implantation formative epiblast cannot be sourced directly and the starting material may for example be a pre-implantation inner cell mass (ICM) explant culture that is matured in vitro to the formative embryonic disc stage (Deglincerti et al., 2016; O'Leary et al., 2012).

20 The embryology of others species can be found in the literature – see e.g. https://embryology.med.unsw.edu.au/embryology/index.php/Main_Page which describes or curates literature on the embryology of many animal models e.g. Bat, Cow, Chicken, Dog, Kangaroo, Mouse, Opossum, Pig, Platypus, and Rat etc.

Use of naïve cells

25

As explained above, in one embodiment the precursor pluripotent cells are naïve cells. These may be derived, for example, from pre-implantation embryonic epiblast tissue e.g. ICM explant/dissociation.

30 Alternatively the naïve cells maybe from a naïve stem cell line. In this case, the cell line may first be cultured in known naïve pluripotency maintenance conditions, but these conditions are withdrawn and replaced, directly or indirectly with formative stem cell culture conditions.

35 The inventors have shown that naïve mouse ES cells could be used to consistently establish FS cell lines by withdrawal of naïve pluripotency maintenance conditions and transfer to formative pluripotency culture conditions, either immediately or after a short period with no factor addition (24-48 hours). For example ES cells could be transferred directly to FS cell culture conditions e.g. by passaging in A₁₀X every 2-3 days at the ratio
40 of 1/5-1/10 for the first several passages. This conversion process was most efficient with the addition of RARi.

45 In one embodiment human ICM explants may be transferred directly into FS cell conditions. In another embodiment naïve epiblast cells could be initially expanded in t2iLGö followed by transfer to FS cell conditions.

In relation to human stem cell lines, the naïve stem cell line is first cultured in naïve pluripotency maintenance conditions t2iLIFGö (Takashima et al., 2014) or an alternative naïve culture formulation such as 5iL/A/F or 4iL/A (Theunissen et al., 2016; Theunissen et al., 2014). They may then be transferred either directly to formative stem cell culture media such as A₁₀X or indirectly after 2-8 days in a basal culture medium. Human FS cells may be passaged for every 4-6 days in 1/10-20.

Use of 'primed' cells

In another embodiment, the source pluripotent stem cells may be primed pluripotent stem cells, such as rodent EpiS cells, which are transferred directly or indirectly to formative stem cell culture media, for example by passaging in media having reduced FGF and activin and gradual transition to A₁₀X+RARi. This process of adaptation or selection is accompanied by high cell death and differentiation but can nevertheless still be used to obtain FS cells if desired.

Examples of primed cells include mouse EpiS cells and conventional human ES cells (Thomson et al., 1998) or iPSC (Takahashi et al., 2007; Yu et al., 2007).

Human primed cells, for example cultured on matrigel in E8 medium, may also be converted to FS cells by transfer into A₁₀X with/without RARi and continuous passaging. Without wishing to be bound by theory, this suggests that such human primed cultures may, in some circumstances, include a proportion of formative-like cells. Conversion from primed cells without resetting to naïve status may not include any epigenetic erasure or correction, however, and is therefore less preferred in the context of the present invention.

In another embodiment naïve cells are provided as the source for FS cells by resetting primed pluripotent cells.

The primed pluripotent cells may be, for example, from embryonic tissue or from a cultured cell line.

For example the invention may utilise rodent (e.g. mouse) post-implantation embryonic epiblast tissue during gastrulation, E6 or E7, or mouse EpiS cells.

To obtain human FS cells, primed human pluripotent stem cells may be used after first resetting to naïve status by methods known in the art (Takashima et al., 2014; Theunissen et al., 2014), for example HDAC inhibition or transgene or mRNA reprogramming.

For example, published patent application WO2016/027099 describes a resetting medium which comprises a HDAC inhibitor, a MEK inhibitor, and optionally a STAT3 activator, and optionally one or more further inhibitors e.g. a PKC inhibitor, a GSK3 inhibitor, or a Wnt inhibitor.

Conventional primed human pluripotent stem cells can be reset to naïve state by transgenes or mRNAs (Nanog, KLF2, Klf4, Nr5a1/2) or following a chemical resetting protocol using HDAC inhibitors such as valproic acid or sodium butyrate followed by culture in t2iLGö. After 7 to 9 days resetting, naïve-like cells expressing naïve makers, KLF17, KLF4, TFCP2L1, DPPA3 emerge, which maintain pluripotent surface markers, Tra1-60, Tra1-81, but have down-regulated SSEA4, a marker for conventional human ES cells. Reset naïve cells can be isolated based on expression of EOS-GFP reporter or simply enriched by passaging. A stable naïve culture can be established by 3-5 passages in t2iLGö with ROCK inhibitor. The stable reset naïve culture can be used to establish human FS cultures as described herein.

For example, to capture human FS cells, human naïve cells are plated in t2iLGö on geltrex or laminin. After 24 hours FS culture medium is applied. The dome shaped naïve colonies acquire a flattened morphology after 4-5 days culture in FS medium. If present, EOS-GFP reporter or other naïve reporter expression is down regulated. The culture is passaged by dissociation in the presence of Rock inhibitor and replated. After 2-3 more passages, cells uniformly exhibit an epithelial morphology with lack of naïve marker expression, and low or undetectable lineage marker expression, and can then be propagated continuously as FS cell lines.

Alternatively, naïve cells can be isolated at early stages during resetting. For example, following 7-21 days resetting naïve cells can be distinguished by expression of naïve markers using a reporter line such as *KLF4*:GFP, or isolated by expression of surface antigens, for example via a combination of SSEA4 negative and Tra1-60/Tra1-81 positive, or expression of novel naïve surface markers, such as HAVCR1 (also called TIM1, TIMD1). The purified resetting cells can be replated to naïve medium briefly before transfer to FS medium, or may be plated directly in FS cell medium. This protocol can avoid loss of imprints associated with extended propagation of naïve stem cells.

Following these protocols, impaired lineage-specific differentiation of primed human pluripotent stem cells may be restored. For example, Shes6 human ES cells show poor neural lineage potential, measured for example by up-regulation of SOX1 and PAX6 in neural induction protocols. Induction of these neural markers is substantially increased following naïve resetting and conversion to FS cells.

Use of somatic cells

In another embodiment naïve cells are provided by reprogramming somatic cells to naïve induced pluripotent stem cells (iPSCs). Reprogramming can be done by methods known in the art. Human iPSCs may be derived from different cell types. For example, the cells can be produced from Fibroblasts, keratinocytes, adipose cells, bone marrow stromal cells or urinary epithelial cells. Human iPSCs may be derived from diploid cells which may be a 'wild-type' or non-transformed cell. In other embodiments an iPSC is derived from a transformed (tumour) cell. Cells may be obtained from an individual by standard techniques, for example by biopsy for skin cells. Cells may preferably be obtained from an adult. Methods for generating iPSCs are known in the art, for example as described

in: Takahashi et al Nature 2007; Yu et al, Science 2007, reviewed in (Takahashi and Yamanaka, 2015). Stable iPSCs may be reset to naïve status then converted to FS cells as above.. Alternatively, during early stages of reprogramming, for example after around 10 days of reprogramming with Yamanaka factors (Takahashi et al., 2007), cultures may be switched to naïve resetting medium with HDAC inhibitor, Erk inhibitor, and optionally PKC inhibitor and activation of Stat3 signalling for 5- 10 days. The dome-shaped naïve colonies expressing naïve markers including KLF17, KLF4 or DNMT3L can be further expanded in t2iGo medium or may be switched immediately to FS medium for conversion to formative stem cells.

In one strategy for obtaining a fully competent human pluripotent stem cell, the precursor cell is transiently reset to naïve status, then converted to formative status before erasure of imprints occurs in the naïve state. It is believed a relatively brief period in naïve pluripotency conditions should be sufficient for major epigenome remodelling whilst preserving imprints. Reprogramming somatic cells to naïve status then rapidly converting to formative pluripotent stem cells is therefore an attractive option for obtaining robust and unbiased cultures with imprints maintained.

Characteristics of FS cells

As described above, the FS cells described herein have phenotypic properties quite different from previously characterised ES or EpiS cell lines or iPSC.

For example mouse FS cells can colonise multiple lineages of the developing mouse embryo following blastocyst injection, albeit at lower frequency than ES cells. It is notable that EpiS cells rarely make any significant contribution to blastocyst chimaeras unless they have been genetically manipulated.

In the presence of RARi and gamma secretase inhibitor, mouse FS cells can be propagated in the presence of the activin receptor (ALK5) inhibitor A-8301 (0.5-1.0µM) in contrast to EpiSC or primed human pluripotent stem cells.

Unlike naïve pluripotent stem cells, mouse FS cells cannot be propagated if FGF/Erk signalling is inhibited, for example in the presence of the Fgfr inhibitor PD-173074 or the Mek inhibitor PD-0325901, confirming reliance on autocrine FGF. FS cell cultures can expand and maintain undifferentiated morphology in the presence of exogenous FGF (0.1, 0.5, 1.0, 2.5, 5.0 and 12.5 ng/ml) although higher concentrations may induce some specification markers.

Mouse FS cells maintain expression of Oct4 but differ from ES cells by an absence of naïve transcription factors such as Esrrb, Tfcp2l1, Klf4 and Klf2. FS cells differ from EpiS cells by an absence of early lineage markers such as Tbra, FoxA2, Mixl1 and Sox1. Markers enriched in the FS cell state appear to include Dppa2, Dppa4 and Wdr86. FS express substantially lower levels of neural, mesoderm and endoderm lineage-affiliated transcripts than EpiS cells, even when EpiS cells are cultured in the presence of XAV939.

Human FS cells can be prepared as described above.

In the experiments described herein, human FS cells grew robustly with homogeneous morphology and a doubling time of 24-30 hours on plates coated with laminin and fibronectin. They displayed a down-regulation of naïve markers (KLF4, KLF17, TFCP2L1) and retention of the core pluripotency factor OCT4. They showed little or no expression of lineage priming markers (TBRA, FOXA2) typically detected in conventional human pluripotent stem cell cultures. They exhibited gain of early post-implantation epiblast marker OCT6 (*POU3F1*).

OCT6 is not normally expressed in conventional human pluripotent stem cells and thus provides a potential discriminating marker of the formative state.

Human FS cells can be differentiated efficiently into neural, mesodermal and endodermal lineages using protocols effective on primed hPSC. Methods of directing differentiation of pluripotent cells are known in the art, and described in the Examples hereinafter, which describe the use of inductive culture systems. For example, neural differentiation can be induced by dual SMAD inhibition using 500 nM LDN193189 and 1 μ M A 83-01 (Chambers et al., 2009). Lateral plate mesoderm may be generated by treatment with CHIR99021 (6 μ M), BMP-4 (40ng/ml), activin A (30ng/ml) for 1 day, and then with BMP-4 (30ng/ml), XAV939 (10 μ M) and A8301 (1 μ M) for 2 days (Loh et al., 2016). Endoderm differentiation is induced by exposure to 100 ng/ml Activin A, 100 nM PI-103, 3 μ M CHIR99021, 10 ng/ml FGF2, 3 ng/ml BMP4 for one day, followed by 2 days in 100ng/ml Activin A, 100nM PI-103, 20ng/ml FGF2, 250nM LDN193189 (Loh et al., 2014).

Human FS cells can also respond to germ cell inductive cytokines (Irie et al., 2015). Following germ cell induction with BMP2, SCF, LIF, EGF and KSR, human FS cells showed expression of surface markers such as TNAP and CD38 between days 4 and 7. Immuno-staining for SOX17, BLIMP1 and OCT4 confirmed PGCLC formation

Thus in embodiments of the invention, mouse formative stem cells are characterised by one or more (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) of the following phenotypes:

- (i) lack expression of naïve transcription factors such as *Esrrb*, *Tfcp2l1*, *Klf4* and *Klf2*, or early lineage markers such as *Tbra*, *FoxA2* and *Sox1*;
- (ii) display robust multi-lineage differentiation in vitro;
- (iii) respond directly to germ cell induction;
- (iv) widespread contribution to all lineages in chimaeras after blastocyst injection.
- (v) can be propagated in the presence of the activin receptor (ALK5) inhibitor A-8301 in the presence of RARi and gamma secretase inhibitor;
- (vi) maintain expression of Oct4 in all cells
- (vii) display near-absence of lineage-affiliated gene expression;
- (viii) do not survive if FGF/Erk signalling is inhibited with either the MEK inhibitor PD0325901 (1 μ M) or the FGF receptor inhibitor PD173074 (0.1 μ M);
- (ix) can expand and maintain undifferentiated morphology in the presence of exogenous FGF (0.1, 0.5, 1.0, 2.5, 5.0 ng/ml);
- (x) proliferate as flat monolayer colonies of epithelial-like cells;

- (xi) show enriched expression of markers: Dppa2, Dppa4 and Wdr86;
- (xii) differentiate if exposed to serum or KSR, BMP, or retinoic acid;
- (xiii) show evidence of initiation of X chromosome inactivation in female cells, for example foci of H3K27me3.

5

In embodiments of the invention, human formative stem cells are characterised by one or more (e.g. 1, 2, 3, 4, 5, or more) of the following phenotypes:

- (i) down-regulation of naïve markers KLF4, KLF17, TFCP2L1.
- (ii) retention of core pluripotency factor OCT4;
- 10 (iii) little or no expression of lineage priming markers TBRA and FOXA2.
- (iv) gain of early post-implantation epiblast marker OCT6 (*POU3F1*).
- (v) capable of differentiation into neural, mesodermal and endodermal lineages;
- (vi) responsive to germ cell induction signal by direct treatment with the inductive cytokine cocktail.

15

Methods of propagating FS cells

In a further aspect of the invention there is provided a process for propagating an FS cell line, which process comprises:

- 20 (A) providing one or more FS cells;
- (B) culturing the formative stem cells in a formative stem cell culture media, as described above.

25

The FS cell may optionally be one obtained or derived as described herein. In one embodiment the FS cells are obtained by explant or dissociation from early post-implantation embryonic epiblast tissue or ICM culture, for example as described herein. In other embodiments the FS cell is derived from a different pluripotent stem cell type or somatic cells, for example as described herein.

30

The FS cell culture media and/or conditions may optionally be as described hereinbefore.

35

In preferred embodiments the processes of the invention maintains a substantially homogeneous population of FS cells in continuous proliferative culture for at least 30 days. More than 90% of cells, typically 99% of cells, express OCT4 protein as detected by immunostaining. Less than 5% of cells, typically less than 1% of cells, are immunopositive for Brachyury, SOX1, FOXA2, SOX17.

40

As explained above the substantially homogeneous cell populations of FS cells (i) are genetically and phenotypically stable; (ii) exhibit consistent characteristics and behaviour between cell lines; (iii) display robust multi-lineage differentiation; (iv) are distinct from previously well-characterised naïve and primed pluripotent stem cell cultures.

Products of the invention

45

In one aspect of the invention there is provided a formative stem cell line obtained or obtainable by the processes described herein.

5 Stem cell *lines* are products of human endeavour, and do not occur in nature. Indeed the combination of FS cell characteristics together with continuous long-term propagation, self-renewal, described herein do not correspond to cell behaviour observed in the organism.

10 The cell line may be “isolated” in the sense of homogeneous and relatively free of other types of cell. The cell line may be present in a suitable artificial culture container e.g. dish or well of glass or plastics material, and in suitable artificial medium as described herein, such as one containing additives such as XAV and RAR α that do not exist in the organism.

Utilities of the invention

15 The FS cells described herein have many utilities and potential advantages over existing pluripotent stem cell sources.

20 For example human naïve stem cells are unstable in all current culture conditions and even when semi-stable human naïve stem cells are established, they show loss of imprinting (Pastor et al., 2016)) which compromises their differentiation competence and utility.

25 Most naïve stem cells reported in the literature are for mice and rats. It appears that rodents may exhibit unusual stability of the naïve stem cell state, which may be intrinsically less stable in other species (Martello and Smith, 2014; Takashima et al., 2014)) and consequently more prone to culture stress, genetic instability and loss of imprinting.

30 The FS cell state provides an important alternative stable pluripotent state for species not limited to humans, biomedical model species such as rodents, and livestock. FS cells can be prepared by passing cells rapidly through the erasure process in the naïve state and then almost immediately converting them to a population of “clean” but also stable FS cells. Since FS cells have chimaera-forming potential with germline colonisation they provide a route to transgenic modification and genome engineering in non-human species.

35 Thus it will be understood that the methods and uses described herein also apply to other primates and non-human mammalian cells, and the features of the methods, uses and reset cells as described herein apply to non-human mammalian cells *mutatis mutandis* and to avian cells. Put another way, it will be understood (unless context demands otherwise) that where the term “human” is recited herein, it can be replaced with “mammalian” or any of the following: primate; non-human mammalian non-human primate; pig; sheep; cat; dog; goat; cow; camel; horse; llama; alpaca etc. In one embodiment the non-human mammalian cell is not a rodent cell. In another embodiment
45 the cell may be avian, for example chicken.

Human and non-human pluripotent stems cells have a number of general utilities including: differentiation to create cell culture models of human development and disease that can be applied in drug discovery and development, and in teratogenicity and toxicology testing; source of tissue stem cells and more mature cells for applications in clinical cell therapy; analysis of the relative contributions of genetics and epigenetics to developmental disorders, genetic disease and quantitative traits to facilitate advances in diagnostics, prognostics and patient treatment; generation of tissues and organs for transplantation either by bioengineering in vitro or by lineage/organ specific contribution to human-animal chimaeras.

Reset, reprogrammed, or embryo-derived formative state pluripotent stem cells from non-human primates and other mammals can be used for precision genome engineering to enhance or modify germline genetic constitution of animals. Germline modification is achieved by genome engineering or genome editing and clonal selection of ground state cells in culture, followed by production of chimaeras, breeding and screening for transmission of the modified genotype. Desired genetic alterations include single or multiple gene deletion, point mutation, or substitution. Chromosome-scale genome modifications/substitutions are also possible. Applications include: disease models; behavioural models; host compatibility for xenotransplantation and organ substitution; pharmaceutical, antibody and vaccine production; livestock improvement; breeding stock preservation and improvement. Non-human primate formative state cells may also be used in pre-clinical testing and evaluation of cell therapies.

Uses of the FS cells of the invention in any of these contexts forms further aspects of the invention.

Uses of FS cells to create primed cells

In one aspect the invention provides a process for producing a primed pluripotent cell from an FS cell by withdrawal from FS cell culture medium and transfer to primed cell medium such as E8 for human primed cells or A_{hi}F with or without XAV for mouse EpiS cells.

The primed pluripotent cell will be distinguished from the FS cell by virtue of characteristic phenotypes discussed herein.

In one aspect the invention provides a primed pluripotent stem cell obtained or obtainable by this process. The primed pluripotent stem cell may be isolated, or present in artificial context, as described above.

Uses of FS cells to create germ cells

FS cells can be induced to form primordial germ cell-like cells (PGCLC) by exposure to inductive cytokines. An example regime is described in the Examples below. In mouse neither naïve ES cells nor primed EpiSC respond to this induction regime. In the Examples below FS cells were cultured with hBMP2, mSCF, hLIF, and Egf for 4 days in

the presence of Rock inhibitor and 15% KSR in GMEM. PGCLC were detected by expression of the surface marker genes (CD61 and SSEA1 for mouse and Tissue Non-specific Alkaline Phosphatase (TNAP) and CD38 for human) by FACS, or by fixation and triple immuno-staining for Oct3/4, Stella and Blimp1 for mouse and OCT4, SOX17 and BLIMP1 for human.

In one aspect the invention provides a process for producing a PGCLC from an FS cell by cytokine induction.

The PGCLC will be distinguished from the FS cell by virtue of characteristic phenotypes discussed herein.

In one aspect the invention provides a PGCLC obtained or obtainable by this process. The PGCLC may be isolated, or present in artificial context, as described above.

Uses of FS cells to create somatic cells

Multi-lineage differentiation of FS cells may be carried out by use of differentiating protocols or conditions known in the art for differentiating pluripotent cells. An example method comprises aggregated of cells in suspension culture and withdrawal of FS cell culture medium. Aggregates may be maintained in serum-free medium or in the presence of KSR or serum. After several days aggregates may be transferred to adherent dishes for outgrowth of differentiating cells

As shown in the Examples below, mouse FS cells could be differentiated and efficiently into the three germ layers *in vitro*. For mesendoderm differentiation, FS cells could be differentiated using serum free medium in the presence of activin and 3 μ M Chiron (CHIR99021). For neural differentiation, FS cells could be treated in N2B27 optionally with Tgfb receptor inhibitor A83-01, similarly to primed pluripotent stem cells.

In one aspect the invention provides a process for producing somatic lineage differentiated cells from an FS cell line, which process comprises exposing said formative FS cell line to one or more differentiating agents or conditions appropriate to that lineage.

For example where the somatic lineage is mesendoderm, the FS stem cell line may be exposed to serum free medium (SF-03) or N2B27 in the presence of 10 ng/ml activin a and 3 μ M Chiron.

For example where the somatic lineage is neural, the FS cell line may be exposed to N2B27 optionally with Tgfb receptor inhibitor A83-01.

The differentiated cell will be distinguished from the FS cell by virtue of characteristic phenotypes discussed herein.

In one aspect the invention provides a differentiated cell obtained or obtainable by this process. The differentiated cell may be isolated, or present in artificial context, as described above.

5 *Uses of FS cells in chimaera formation*

In one aspect the invention provides a process for producing a chimaeric organism, which process comprises introducing a FS cell of the invention into a host embryo at pre-implantation or early post-implantation stages.

10

FS cell culture media

In one aspect the invention provides a formative stem cell culture media as described herein. A preferred medium comprises:

15

- (i) activin at a concentrations of 1 - 4ng/ml, more preferably 1.5 - 3 ng/ml;
- (ii) less than 10ng/ml of exogenous FGF; more preferably less than 4ng/ml of exogenous FGF;
- (iii) a Wnt inhibitor.

20

Preferred media are $A_{10}X$, $A_{10}X+RARi$ and $A_{10}X+RARi+\gamma Si$.

In one aspect the invention provides use of a formative stem cell culture medium of the invention to derive, produce or propagate an FS stem cell line.

25

Other aspects and embodiments

30

Human embryonic stem cells for use in the invention may be obtained using methods which do not require destruction of the embryo. For example, embryonic stem cells may be obtained from the human embryo by biopsy. Methods for obtaining embryonic stem cells from the embryo without destruction of the embryo were disclosed for example in Klimanskaya, I., Chung, Y., Becker, S., Lu, S.J., and Lanza, R., Human embryonic stem cell lines derived from single blastomeres, (2006). Nature 444, 481–485.

35

In some embodiments of the present invention, the methods and uses do not involve destruction of human embryos. In some embodiments, the methods do not involve or use cells obtained by methods requiring destruction of human embryos.

40

In some embodiments the methods and uses of the invention do not involve use of a human embryo for industrial/commercial purposes. In some embodiments, the methods do not involve cells obtained by methods requiring use of a human embryo for industrial/commercial purposes.

45

In some embodiments, the cell is not a human embryonic stem cell.

Any sub-titles herein are included for convenience only, and are not to be construed as limiting the disclosure in any way.

5 The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments of the invention will occur to those skilled in the art in the light of these.

10 The disclosure of all references cited herein, inasmuch as it may be used by those skilled in the art to carry out the invention, is hereby specifically incorporated herein by cross-reference.

Figures

Figure 1. Mouse formative pluripotent stem cells

- 15 (a) Bright field image of mouse FS cells ($A_{10}X+RARi$) in culture.
- (b) Relative gene expression compared to conventional EpiS cells ($A_{hi}F$) measured by RT-qPCR for ES cells (green), FS cells ($A_{10}X$) (Blue) and EpiS cells $A_{hi}F \times$ (red). Error bar represents S.D. from triplicate assays.
- 20 (c) RT-qPCR showing reduced expression of neural lineage marker genes in FS cells exchanged to, or derived in the presence of, RAR inhibitor.
- (d) RT-qPCR for Nodal/Activin target gene expression in the presence of A83-01 in $A_{10}X+RARi+gSi$ compared to $A_{10}X+RARi$.
- (e) Bright field image of ES cells transferred to $A_{10}X+RARi$ for 3 days.
- 25 (f) Immunostaining for T, Sox1 and Oct3/4 in EpiS (AFX) and FS ($A_{10}X+RARi$) cells.
- (g) Principal component analysis (PCA) of whole transcriptome RNA-seq data obtained from different pluripotent stem cell cultures.
- (h) Time course RT-qPCR analysis during lineage induction. FS cells ($A_{10}X$) were subjected to neural or mesendoderm induction conditions for 4 days or 3 days
- 30 respectively. Error bars represent S.D. from triplicate assays.
- (i) Immunostaining for H3K27me3 shows intense nuclear foci in female FS cells.
- (j) Immunostaining of day 4 PGCLC directly differentiated from FS cells by treatment with inductive cytokines. Merged image includes nuclear staining with DAPI (in white).
- (k) FS cell chimeras dissected at E9.5. FS cells were stably transfected with a constitutive mKO reporter and injected into mouse blastocysts. 19/48 recovered embryos showed FS
- 35 cell derived contributions detected by mKO fluorescence. Eight chimaeras with broad tissue contribution are shown.
- (l) FS cells ($A_{10}X+RARi$, top panel) rapidly differentiate in the presence of either KSR (20%, middle panel) or FCS (10%, bottom panel). Images were taken after 2 days
- 40 treatment.
- (m) qRT-PCR and FACS analysis during mesendoderm differentiation. FS cells and EpiS cells were plated in 10-20 ng of activin a and 3 μ M Chiron. Mesoderm genes such as *T/Bra*, *Flk1* and *Mesp1* are more highly upregulated in FS than EpiS cells. Cells were also analysed by FACS at day 1 and 2. Flk1 positive, Ecadherin negative mesoderm
- 45 population were induced more efficiently from FS cells than EpiS cells.

(n) qRT-PCR and FACS analysis during mesendoderm differentiation. FS cells and EpiS cells are plated in 10-20 ng of activin a and 3 uM chiron. Endoderm genes such as *Foxa2*, *Sox17* and *Mixl1* were upregulated more in FS than EpiS cells. Endoderm cells were quantified at day 3 by FACS. Cxcr4 and Ecadherin double positive endoderm cells are efficiently induced in FS cells.

(o) Endoderm differentiation potency was examined using a directed endoderm differentiation protocol. Cells are treated with 20 ng of activin a and 3 uM chiron for 24 hours, then changed to activin only for 48 hours. Cells were analysed at 72 hours by FACS. Endoderm population was quantified by Cxcr4 and Ecadherin. FS cells differentiate into endoderm very efficiently but EpiS cells very poorly.

Figure 2. Human formative pluripotent stem cells

(a) Bright field images of human cells obtained from reset naïve Shef6 hESC after culture in A₆X (top) and A₆X+RARi (bottom).

(b, c, d) RT-qPCR analysis of naïve markers (b, in blue), primed lineage markers (c, in red) and candidate formative marker, OCT6 and general pluripotency associated marker OCT4 (d, in green) in human FS cells derived from naïve H9 and Shef6 hESC generated by transgene-free resetting.

(e) Immunofluorescence staining for endoderm specification factor SOX17 in undifferentiated FS cells or after 3 days of endoderm induction.

(f) Flow cytometry analysis for co-expression of endodermal markers cKIT and CXCR4 after endoderm induction for 3 days.

(g) Immunofluorescence staining after differentiation towards the neural lineage for 4 days.

(h) Flow cytometry analysis on day 5 of PGCLC induction.

(i) Immunostaining for BLIMP1, SOX17 and OCT4 on day 7 of PGCLC induction.

Arrowheads highlight cells co-expressing the three markers, diagnostic for PGCLC.

(j) A. Images showing a human formative cell colony formed after 26 days of reprogramming with live cell staining for Tra1-60 pluripotency marker. B. Phase contrast image of fibroblast-derived reprogrammed FS cells after 5 passages in FS cell culture medium.

Figure 3 Porcine formative pluripotent stem cells

(a) Outgrowth of pig ICM after 9 days culture in formative stem cell culture medium containing Activin A (5 ng/ml), XAV939 (2 µM) and RAR inhibitor BMS 493 (1 µM).

(b) Pig ICM-derived stem cells propagated in formative cell culture medium on laminin or on mouse MEF feeder for 5 passages.

(c) qRT-PCR showing expression of pluripotency markers in pig embryo derived stem cells (pFLSC), but not in pig embryonic fibroblasts (pEF).

Examples

Example 1 – establishing FS cells from epiblasts

5 We hypothesised that autocrine signals may be important for establishing and maintaining formative pluripotency. In the early post-implantation embryo, Nodal and the fibroblast growth factors FGF4 and FGF5 are uniformly expressed in the epiblast (Mesnard et al., 2006). These factors are candidates for supporting formative pluripotency but may subsequently contribute to lineage specification. We therefore attempted to
10 establish cell lines from E5.5 epiblasts with *low* activation of FGF and Nodal pathways that might simulate levels in the formative epiblast. Nodal is known to be down-regulated in vitro so we added activin as a nodal substitute while relying on endogenous FGF production. We included the tankyrase inhibitor XAV939 (at 2 μ M) to suppress endogenous Wnt signalling which plays a major role in primitive streak formation in the
15 embryo. We avoided use of undefined components such as feeders, serum, KSR or Matrigel commonly used for EpiS cell culture.

We microdissected E5.5 epiblasts and plated individually on fibronectin-coated 4-well plates in N2B27 medium. Cultures were propagated in 5% O₂. We tested concentrations
20 of activin from 1 to 20ng/ml. After several days, explants were treated with Accutase for 5-10 seconds at room temp and wash buffer then gently added to detach and fragment the outgrowths into small clumps. Cells were collected by centrifugation and seeded into fresh 4-well plates. Subsequently, expanding cultures were treated with Accutase briefly (30-60 seconds, at room temp) for dissociation into cell clusters by gentle trituration.
25 Established cultures were routinely passaged every 2-3 days and plated onto new fibronectin coated dishes at a split ratio of 1/10-1/15. Medium was changed every other day.

30 In passaged cultures at activin concentrations above 3ng/ml we observed appreciable expression of primitive streak markers such as Tbra, FoxA2 and Mixl1.

In activin concentrations of 1-3ng/ml, however, expression of these genes was barely detectable. We therefore focussed on conditions of low activin plus XAV (A_{lo}X). Resulting
35 cell lines displayed consistent appearance with little or no morphological differentiation (Fig. 1a). They maintained expression of Oct4 but in contrast to EpiS cells cultured in A_{hi}F, they displayed near-absence of lineage-affiliated gene expression (Fig. 1b). This observation suggests that in the absence of Wnt signal, low levels of activin/nodal and endogenous FGFs are not sufficient to induce primitive streak fates. Importantly, unlike ES cells these cultures do not survive if FGF/Erk signalling is inhibited with either the
40 MEK inhibitor PD0325901 (1 μ M) or the FGF receptor inhibitor PD173074 (0.1 μ M), confirming reliance on autocrine FGF. Furthermore, cultures can expand and maintain undifferentiated morphology in the presence of exogenous FGF (0.1, 0.5, 1.0, 2.5, 5.0 and 12.5 ng/ml) although higher concentrations may induce some specification markers. These observations indicate that developmentally appropriate minimal activation of the
45 nodal/activin pathway combined with autocrine or exogenous stimulation of the FGF/Erk

pathway may be sufficient to suspend cells in the formative phase of pluripotency when the Wnt pathway is inhibited. We term these cells formative pluripotent stem (FS) cells.

5 We tested dependency of FS cells on other pathways using small molecules antagonists. We found that FS cell cultures can withstand chemical inhibitors of Braf (SB-590885, 0.5 μ M), p38 (SB-202190, 5-30 μ M), Cdc42 (ML141, 1.0-5.0 μ M), and PTEN (bpV(HOpic), 1.0 μ M).

10 Further inhibitors which were tested were as follows: Histone deacetylase (HDAC) (Valproic Acid, 0.6 mM or Sodium Butyrate, 0.2mM), and Lysine specific demethylase 1 (Lsd1) inhibitor (Tranylcypromine hydrochloride, 1-10 μ M) and atypical protein kinase C (aPKC) (Go6983, 2uM).

15 In contrast cultures differentiated or died upon inhibition of PI3 kinase (Ly294002, 25 μ M), Src (WH-4-023, 1 μ M) or JNK (SP-600125, 10 μ M). We also observed that FS cells can withstand chemical activation of PKA pathway by 10 μ M Forskolin, Akt by 4 μ g/ml SC79 and mTor pathway by 100-400 mM L-Proline. Inhibition of GSK3 with CHIR99021 at 1 μ M induced expression of Brachyury, but this effect was not evident at 0.5 μ M.

20 In A_{lo}X we observed sporadic expression of lineage markers and intermittent differentiation. We also noted that FS cells differentiated if exposed to retinoic acid (10⁻⁷M). We therefore refined the culture system by inclusion of a pan-retinoic acid receptor inverse agonist, (RARi; BMS 493 0.1-1.0 μ M, preferably 0.5 μ M) to block retinoid signalling and reinforce suppression of neural lineage induction (Fig. 1c). RARi improves
25 FS cell derivation efficiency (Table 1) consistent with a reduction in differentiation and selective pressure. We also added the γ -secretase inhibitor, CAS 209984-56-5 at 0.1 μ M (γ Si) and observed that this further increased the homogeneity and stability of FS cell cultures by eliminating residual neural specification. Thus a preferred culture condition for FS cells comprises low activin, Wnt inhibition, retinoic acid receptor inhibition and
30 gamma-secretase inhibition (AloX+RARi+ γ Si). Optionally inclusion of a low to moderate concentration (0.1-4ng/ml) of FGF may improve consistency of expansion.

Unexpectedly we found that FS cells can tolerate activin/Tgf β receptor inhibition in the presence of RARi plus γ Si. Cultures could be passaged multiple times in AloX+RARi+ γ Si
35 in the presence of A83-01 inhibitor at 0.5-1.0 μ M. We confirmed diminished expression of Activin/Nodal target genes such as Nodal, Cripto and Lefty2 in these conditions by qRT-PCR. (Fig. 1d). This suggests that established FS cells may not be absolutely dependent on continuous nodal/activin stimulation of the Smad2/3 pathway if lineage induction signaling is fully suppressed.

40 We established multiple mouse FS cell lines from embryos of two different genetic backgrounds. Several lines have been passaged more than 20 times (60 generations) with no indication of crisis or senescence. Chromosome counts reveal predominantly
45 diploid cells even at later passages (Table 2).

Example 2 – establishing FS cells from mouse ES cells

We applied the above culture conditions to naïve mouse ES cells withdrawn from 2iLIF and could consistently establish FS cell lines. This conversion process was most efficient with the addition of RARi which enabled establishment of homogenous-looking FS cell cultures within 3 days (Fig. 1e), although some heterogeneity may transiently appear after initial passaging, ES cells in 2i or 2iLIF may be transferred directly to FS cell culture conditions or cultured in N2B27 without 2iLIF for an initial 24-48 hours then transferred. Cells are passaged in A₁₀X + RARi conditions every 2-3 days at a ratio of 1/5-1/10 for the first few passages.

Example 3 – FS cells are distinct from mouse ESs and EpiSCs cells

Whether derived from embryos or from ES cells, FS cells do not survive if cultured in 2i or 2iLIF, demonstrating that they are distinct from ES cells and cannot spontaneously revert to ES cell status.

FS cells proliferate as flat monolayer colonies of epithelial-like cells (Fig. 1a). They have a doubling time of 10-15 hours. FS differ from ES cells in absence of naïve transcription factors such as Esrrb, Tfcp2l1, Klf4 and Klf2. They differ from EpiSC in absence of early lineage markers such as Tbra, FoxA2 (Fig. 1b) and Sox1 (Fig. 1c). EpiS cells cultured in the presence of XAV (A_{hi}FX) and feeder cells have been reported to show reduced expression of lineage markers (Sumi et al., 2013). However, we observed expression of lineage markers in EpiSC cultured A_{hi}FX without feeders or undefined factors, (Fig. 1f). We conducted whole transcriptome analysis by RNA-seq to compare FS cells with EpiS cells. Principal component (PC) analysis separated FS cells from both naïve ES cells and EpiS cells. EpiS cells cultured in the presence of XAV939 locate closer to FS cells than EpiS cells maintained without XAV but do not overlap with the FS cell cluster (Fig. 1g). The transcriptome analysis also highlighted potential markers enriched in the FS cell state such as Dppa2, Dppa4 and Wdr86.

Transcriptome analyses of early post-implantation embryos (e.g. Nakamura et al, 2016; Mohammed et al, 2017) suggests the following additional markers which may distinguish formative cells from either naïve or primed pluripotency.

Example 4 – FS cells are inefficiently derived directly from EpiS cells

We investigated whether EpiS cultures may be dedifferentiated to FS cells or may contain a sub-population of FS cells. EpiS cells cultured in the presence of XAV (A_{hi}FX) transferred directly into FS cell culture conditions (A₁₀X) died or differentiated within several passages. If FGF was first withdrawn then activin reduced gradually at each passage, we observed extensive differentiation and cell death but a fraction of cells survived that could eventually be propagated similarly to FS cells.

EpiS cells (A_{hi}F) transferred to FS cell conditions showed widespread differentiation and death with only rare cells surviving. In some cases, however, the surviving cells could be

expanded. This suggests that EpiS cells may be converted at very low efficiency to FS cells by either adaptation or selection.

5 EpiS cells cultured in the presence of XAV (A_{hi} FX) then transferred directly into $A_{i0}X$ +Rari condition survived with some initial differentiation. After several passages, they could be propagated similarly to FS cells.

Example 5 – FS cells from later stage mouse epiblasts

10 We also found that FS cells could be established from later stage epiblasts, E6.5 and E7.5 (up to LS-EB stage) using $A_{i0}X$ +RARi, although cell line derivation is at lower efficiency than from E5.5 epiblast (Table 3), this may indicate that the transition between formative and primed pluripotency is gradual in vivo and may not be complete or irreversible for all epiblast cells even at relatively late stages, consistent with a recent description of the cynomolgus macaque post-implantation embryo (Nakamura et al.,
15 2016).

Example 6 – germline induction and somatic differentiation of mouse FS cells

20 In mouse, the formative phase of pluripotency is distinguished from naïve and primed phases by the competence for germline induction (Ohinata, 2009) (Hayashi et al., 2011).

Consistent with this, FS cells can be functionally distinguished from both ES cells and EpiS cells in their competence to respond directly to germ cell inductive cytokines and form primordial germ cell-like cells (PGCLC) (Fig. 1f).

25

For PGCLC differentiation, FS cells are collected by TrypLE Express and counted.

3,000 FS cells were cultured in non-adhesive U-bottom plate with hBMP2 (500 ng/ml), mSCF (100 ng/ml), hLIF (1 μ g/ml) and Egf (50 ng/ml) for 4 days in the presence of Rock inhibitor (10 μ M Y-27632) and 15% KSR in GMEM (modified from (Hayashi et al., 2011)). PGCLC were detected by expression of the surface marker genes (CD61 and SSEA1) by FACS, or by fixation and triple immuno-staining for Oct3/4, Stella and Blimp1. PGCLC induction is a consistent property of FS cells cultured in $A_{i0}X$ +RARi+ γ Si. We also tested whether EpiS cells cultured in the presence of XAV could form PGCLC but found no
35 induction of these surface markers consistent with previous failures to generate PGCLC from EpiSC (Hayashi et al, 2011).

We tested somatic lineage differentiation and found that FS cells differentiated rapidly and efficiently into the three germ layers in vitro (Fig. 1h). For the mesendoderm
40 differentiation, FS cells are directly plated in serum free medium (SF-03) or N2B27 in the presence of 10 – 20 ng/ml activin a and 3 μ M Chiron. We confirmed up-regulation of mesendoderm marker such as T/Bra, Foxa2 and Sox17 by qRT-PCR. For neural differentiation, FS cells were plated in N2B27 with Tgfb receptor inhibitor A83-01 (at 1 μ M) on laminin coated plates. We confirmed up-regulation of early neural marker genes such
45 as Sox1 and Pax6 and down-regulation of pluripotent marker Oct3/4 by qRT-PCR.

We quantified the efficiency of mesoderm and endoderm differentiation during a mesendoderm differentiation protocol applied to FS and EpiS cells. For mesoderm, T/Bra, Flk1 and Mesp1 were examined by qRT-PCR and a combination of cell surface antigen such as Flk1 and Ecadherin were used for flow cytometry analyses (Fig. 1m). For
 5 endoderm, Foxa2, Sox17 and Mixl1 transcripts were examined and Ecadherin and Cxcr4 were quantified by flow analysis (Fig. 1n). We observed that FS cells show a higher response than AFX EpiS cells to these directed differentiation signals.

We also tested an endoderm specific differentiation protocol and confirmed that FS cells
 10 make Ecadherin and Cxcr4 double positive endoderm cells more efficiently than EpiS cells measured by flow cytometry at day 3 (Fig. 1o).

Example 7 – FS cells can contribute to all lineages in chimaeras

We reasoned that formative pluripotent cells should have the potency to contribute to all
 15 lineages in chimaeras if they survive from the time of injection to early post-implantation. We therefore injected embryo-derived FS cells into blastocysts and indeed found that they consistently contribute to mid-gestation chimaeras (Fig. 1j). Significantly,
 20 contributions can extend across all germ layers and include migratory primordial germ cells. In contrast EpiSCs in general do not make substantial contributions to chimaeras unless they have been genetically modified (Masaki et al., 2016; Ohtsuka et al., 2012).

Example 8 – derivation of FS cells from human naïve or primed pluripotent stem cells

Finally we investigated the potential for deriving FS cells from human naïve pluripotent
 25 stem cells. We applied the culture conditions for mouse FS cells to naïve human stem cells propagated in t2iLGo (Takashima et al, 2014; Guo et al, 2016) and established continuous cultures. The cells grow robustly with homogeneous morphology (Fig. 2a) and
 30 a doubling time of 24-30 hours on plates coated with laminin and fibronectin. They exhibit down-regulation of naïve markers (KLF4, KLF17, TFCEP2L1) and retention of core pluripotency factor OCT4, but with little or no expression of lineage priming markers (TBRA, FOXA2) typically detected in conventional human pluripotent stem cells (Fig 2b-d).

Significantly, they show gain of early post-implantation epiblast markers OCT6 (*POU3F1*)
 35 and OTX2. OCT6 is of particular interest because this transcription factor is not normally expressed in conventional human pluripotent stem cells and thus provides a potential discriminating marker of the formative state.

We assayed early differentiation into somatic lineages using protocols adapted from
 40 those described for human primed PSC. Human FS cells were differentiated into definitive endoderm lineage according to (Loh et al., 2014). Cells were cultured in CDM2 medium supplemented with 100 ng/ml Activin A, 100 nM PI-103 (Bio-technie, 2930), 3 µM CHIR99021, 10 ng/ml FGF2, 3 ng/ml BMP4 (Peprotech) for one day. For the next 2 days
 45 the following supplements were applied: 100ng/ml Activin A, 100nM PI-103, 20ng/ml FGF2, 250nM LDN193189. After 3 days of differentiation, cells were fixed and stained for

SOX17 or cells were analysed by either surface marker gene expression such as CXCR4 and c-KIT. We tested neural differentiation by dual SMAD inhibition (Chambers et al., 2009). Cells were fixed and stained for SOX1 at differentiation day 4. For lateral plate mesoderm differentiation, cells are differentiated by the protocol modified from (Loh et al., 2016). Cells were treated with medium supplemented with Chiron (6uM), BMP-4 (40ng/ml), activin A (30ng/ml) for 1 day, and then with BMP-4 (30ng/ml), XAV939 (10uM) and A8301 (1uM) for 2 days. The efficiency of differentiation was assessed using staining for KDR and PDGFRa quantified by FACS and qRT-PCR against MESP1, HAND2, FOXF1, IRX3, ISL1.

We also tested whether human FS cells can respond to germ cell induction signal. Human FS cells were harvested using TrypLE Express and counted. 3,000 FS cells were cultured in non-adhesive U-bottom plate with hBMP2 (500 ng/ml), mSCF (100 ng/ml), hLIF (1 µg/ml) and Egf (50 ng/ml) in the presence of Rock inhibitor (10 µM Y-27632) and 15% KSR in GMEM (modified from (Hayashi et al., 2011)). By direct treatment with the inductive cytokine cocktail, FS cells showed expression of surface markers such as TNAP and CD38 between day 4 and 7. Immuno-staining for SOX17, BLIMP1 and OCT4 confirms PGCLC formation.

Human primed pluripotent stem cells can be transiently reset to naïve status by HDAC inhibition, mRNA reprogramming, or other protocols, and short-term culture in t2iLtGö or other naïve culture condition, then converted directly to FS cells. Naïve cells are directly transferred to A₁₀X with/without RARi, or left initially for 6-10 days in basal medium (N2B27) to exit from the naïve state, then transferred to A₁₀X with/without RARi.

Conversion to FS cells proceeds without substantially increased cell death or differentiation. Human FS cells are passaged for every 4-6 days at 1/10-20. For routine passaging, human FS cells are treated with either Accutase or TrypLE for 1-2 min at room temperature. Rock inhibitor (Y-27632, 10 µM) is added after passaging and medium changed on the following day.

Human primed cells cultured on matrigel in E8 medium may also be converted to FS cells by transfer into A₁₀X with/without RARi. This may indicate that these primed cultures contain a proportion of formative-like cells, consistent with reports of hierarchical organisation (Hough et al 2014).

Example 9 – derivation of FS cells from human pre-implantation embryos

Finally, FS cells may be derived from human pre-implantation embryos either by culture of ICM explants directly in FS cell conditions or by initial expansion of naïve epiblast cells in t2iLGö with or without XAV followed by transfer to FS cell conditions either at 1st or 2nd passage, or after establishment of stable naïve cell lines.

Overall, the attributes of mouse and human FS cells are consistent with a distinct pluripotent stem cell type, transitional between previously characterised naïve (ES) and primed (EpiSC) stem cell states and showing anticipated features of formative pluripotency.

Primed pluripotent stem cells, both mouse and human, are highly dependent on exogenous stimulation of the nodal/activin/TGF β signalling and FGF pathways (Brons et al., 2007; Tesar et al., 2007; Thomson et al., 1998; Vallier et al., 2005) through ligands provided by feeder cells (the concentration of activin A secreted by feeders is 6.2-15.0 ng/ml, (Kojima et al., 2014)), serum or KSR, or as growth factor supplements. In contrast FS cells expand in the presence of much lower concentrations of FGF and activin, and even in the presence of the A83-01 inhibitor.

FS cells depend on the tankyrase inhibitor XAV or other antagonists of Wnt signalling. Canonical Wnt pathway inhibition has previously been reported to reduce mesoendodermal gene expression and shift the regional specification of EpiS cells and human PSC, but not to alter their primed identity or confer ability to colonise blastocyst chimaeras (Tsakiridis et al., 2014; Wu et al., 2015b). EpiS cells cultured in the A_{hi}F plus XAV939 and 20% KSR on feeders are reported to colonise post-implantation embryos and contribute to alkaline phosphatase positive cells (Sumi et al, 2013). EpiS cells have also been derived in A_{hi}F supplemented with the porcupine inhibitor IWP2, which blocks endogenous production of functional Wnt ligand, in the presence of serum (Kurek et al, 2015), or of KSR and feeders (Sugimoto et al., 2015). Kurek et al reported colonisation of blastocyst chimaeras, although not germ cell contribution. In the presence of serum, however, it is reported that post-implantation epiblast cells and EpiS cells can undergo epigenetic conversion to ES cells (Bao et al., 2009). EpiS cells and human PSC have also been cultured using a combination of XAV and the GSK3 inhibitor CHIR99021 (CH) without added activin or FGF, but in the presence of serum (Kim et al, 2013). This condition is also reported to reduce heterogeneity but not to cause any fundamental change in identity. Additionally XAV has been deployed in combination with 2iLIF and KSR and reported to sustain a putative naïve phenotype of human pluripotent stem cells (Zimmerlin et al, 2016). We have observed that XAV can be added to cultures of human naïve pluripotent stem cells in t2iLGo (Takashima et al, 2014) and does not alter their phenotype or identity. Collectively these reports establish that Wnt inhibition does not specifically induce or maintain formative pluripotent stem cells, but rather that it can have a stabilising effect on different pluripotent states according to the particular cell population and microenvironmental conditions.

Finally, a putative intermediate class of pluripotent stem cell, early primitive ectoderm-like (EPL), was previously reported in mouse (Rathjen et al., 1999). However, EPL cells do not form chimaeras and neither their molecular identity nor their culture conditions are well defined. EPL cells are cultured in the presence of LIF and serum with either conditioned media from HepG2 cells or the amino acid, L-Proline (Rathjen et al., 1999; Washington et al., 2010). EPL cells readily revert to ES cells, unlike FS cells or early post-implantation epiblast cells (Boroviak et al., 2014), therefore their developmental status is unclear. More recently it has been claimed that mouse ES cell differentiation in the presence of FGF, activin and CH (FAC) generates an “intermediate” pluripotent cell population that retains the ability to colonise chimaeras, including contribution to the developing germ line (Tsukiyama and Ohinata, 2014). However, FAC cultures retain high levels of naïve markers, unlike FS cells, and also express endoderm lineage markers.

Co-expression of naïve transcription factors such as *Essrb* and definitive endoderm markers has not been observed in the embryo, suggesting that the FAC cultures are either a mixture of ES cells and lineage specified cells or an in vitro artefactual cell type. Moreover, some EpiS cell derivations in the presence of KSR and feeders contain a

5 minor fraction of cells with potency to colonise blastocyst chimaeras and revert to ES cells (Bernemann et al., 2011; Han et al., 2010). Thus a minority of EpiS cell cultures, with or without XAV, may contain a sub-population of cells that either have unusually high plasticity, or may be representative of formative or naïve phases of pluripotency (Han et

10 al., 2010).

Example 10. Derivation of human formative pluripotent stem cells by reprogramming from somatic cells

As explained above, somatic cells may be reprogrammed to induced pluripotent stem cell (iPSC) status by ectopic expression of transcription factors (Takahashi and Yamanaka, Cell, 2006; Takahashi et al, Cell 2007; Yu et al, Science, 2007) or chemical treatment (Hou et al, Science, 2013; Long et al, Cell Research, 2015). We therefore tested whether formative induced pluripotent stem cells (fiPSC) may be generated and captured during the reprogramming process. We transfected human diploid fibroblasts with episomal

15 'Yamanaka factors' and after two days transferred to AFX medium for 5 days. The cells were then transferred to A5RX medium with low FGF2 (5ng/ml) until FS colonies formed and reached suitable size for picking. After colony picking cultures were expanded in FS conditions in A3RX medium without FGF2 on laminin/fibronectin.

20

25 The results are shown in Fig. 2(j).

Reprogramming Media used: AFX medium: N2B27 basal medium supplemented with Activin (5 ng/ml), FGF2 (5 ng/ml) and XAV939 (2 µM); A5RX medium: N2B27 basal medium supplemented with Activin (5 ng/ml), BMS 493 (1µM) and XAV939 (2 µM).

30

Example 11. Derivation of porcine formative pluripotent stem cells

Formative pluripotency is anticipated to be a generic feature of early mammalian development and establishment of formative pluripotent stem cells from various mammals opens up a range of biomedical and livestock applications. We therefore investigated FS cell derivation from porcine embryos. Pig embryos were flushed from the uterus and cultured until the expanded blastocyst stage (Day 6). ICMs were dissected by immunosurgery and plated in 4-well tissue culture plates coated with laminin in FS cell culture medium. Two outgrowths were obtained from three plated ICM and expanded for

35 5 passages both on laminin and on mouse embryo fibroblast (MEF) feeders (Fig 3). These cultures displayed morphology and markers of pluripotent stem cells (Fig 3).

40

Table.1 FS cell derivation efficiency			
	Number of E5.5 embryos	Lines established	Efficiency
A _{lo} X	8	4	50%
A _{lo} X + RARi	4	4	100%

5

Table.2 Chromosome counts		
Name of Line	Percentage diploid (=40)	Passage number
5a6	75.0 % (24/32)	19
NBRA3.2	87.1 % (27/31)	22

Table.3 FS cell derivation efficiency from mouse epiblast at different stages			
	Number of embryos	Lines established	Efficiency
E5.5	3	2	67%
E6.5	8	3	38%
E7.5	14	4	29%

Table 4. Properties of mouse cells in different phases of pluripotency.

	Naïve	Formative	Primed
Expression of Oct4	Yes	Yes	Yes
Expression of naïve pluripotency TFs	Yes	No	No ^a
Expression of early post-implantation TFs	No	Yes	Partial ^b
Expression of non-ectoderm lineage TFs	No	No	Yes
X inactivation	No	Partial	Yes
Germ cell competence	No	Yes	No ^c
DNA methylation	Low	Intermediate ^d	High
Epithelialisation	No	Partial	Yes
Apicobasal polarity	No	Partial	Yes
Homogeneity	Yes	Yes	No
Mitochondrial respiration	High	Low	Low
Dependence on FGF/Erk signalling	No	Moderate	High
Dependence on Nodal/activin signalling	No	Low	High
Formation of blastocyst chimaeras	Yes	Yes	No
Formation of post-implantation chimaeras	No	Yes	Yes
In vitro stem cell derivation	Yes	Yes	Yes

^a Nanog is re-expressed in pre-gastrulation posterior epiblast in the mouse egg cylinder and in EpiSCs. In the cynomolgus epiblast Nanog appears to be expressed continuously (Nakamura et al., 2016)

^b Factors such as Oct6 and Otx2 are up-regulated throughout the early post-implantation mouse epiblast but later become restricted to the anterior presumptive neuroectoderm

^c In human, a sub-set of primed cells in vitro are able to produce primordial germ cell-like cells (Irie et al., 2015; Sasaki et al., 2015)

^d In formative stem cells in vitro, global methylation may be more extensive, as seen for ES cells in serum (Ficz et al., 2013; Habibi et al., 2013; Leitch et al., 2013)

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15

Claims

1 A process for producing a formative stem cell line, from one or more precursor pluripotent stem cells, which process comprises:

- (a) providing one or more precursor pluripotent stem cells;
- (b) culturing the precursor pluripotent stem cells in formative stem cell culture media, which formative stem cell culture media comprises:
 - (i) no or < 5ng/ml activin
 - (ii) no or < 10ng/ml exogenous fibroblast growth factor (FGF);
 - (iii) a Wnt inhibitor;
 - (iv) no serum or serum substitutes;
 - (v) a retinoic acid receptor inhibitor.

2 A process for producing a formative stem cell line, from one or more precursor pluripotent stem cells, which process comprises:

- (a) providing one or more precursor pluripotent stem cells;
- (b) culturing the precursor pluripotent stem cells in formative stem cell culture media, which formative stem cell culture media comprises:
 - (i) activin at a concentration of 1 - 4ng/ml, more preferably 1.5 - 3 ng/ml;
 - (ii) no or < 10ng/ml of exogenous FGF; more preferably less than 5 ng/ml of exogenous FGF;
 - (iii) a wnt inhibitor.

3 A process as claimed in claim 2, wherein the formative stem cell culture media comprises:

- (i) no serum or serum substitutes;
- (ii) no fibroblast growth factor or other exogenous stimulator of the FGF pathway; and optionally the culturing includes:
- (iii) no feeder layer.

4 A process as claimed in any one of claims 1 to 3 wherein the wnt inhibitor is the tankyrase inhibitor XAV939, optionally at a concentration of 0.5-10 μ M.

5 A process as claimed in any one of claims 1 to 4, wherein the formative stem cell culture media further comprises a pan-retinoic acid receptor inverse agonist (RARi).

6 A process as claimed in claim 5, wherein the RARi is BMS 493, optionally at 0.1-1.0 μ M.

7 A process as claimed in any one of claims 1 to 5, wherein the formative stem cell culture media further comprises a γ -secretase inhibitor, wherein the γ -secretase inhibitor is optionally CAS 209984-56-5 at 0.0017-1.0 μ M, preferably at 0.1 μ M

8 A process as claimed in any one of claims 1 to 7, wherein the formative stem cell culture media further comprises one or more of: insulin, selenium, transferrin, anti-oxidants, lipid supplements.

9 A process as claimed in any one of claims 1 to 8, wherein the formative stem cell culture media comprises N2B27 medium

10 A process as claimed in any one of claims 1 to 9, wherein the formative stem cell culture media does not comprise any one or more of: serum; KSR; BMP; MEF feeders ; matrigel; a PI3 kinase inhibitor; an Src inhibitor; a JNK inhibitor; > 0.5 μ M of a GSK3 inhibitor, a MEK inhibitor or an FGF receptor inhibitor.

11 A process as claimed in any one of claims 1 to 10, wherein the culturing conditions in step (b) comprise one or more of: culturing on fibronectin coated plates; propagation in 7% CO₂ and 5% O₂; passaging every 2 to 3 days with a split ratio of 1/10-1/15; change of medium every other day.

12 A process as claimed in any one of claims 1 to 11 wherein the one or more precursor pluripotent stem cells are native formative stem cells obtained from pre-implantation tissue, or early post-implantation embryonic epiblast tissue.

13 A process as claimed in any one of claims 1 to 11 wherein the one or more precursor pluripotent stem cells are naïve cells.

14 A process as claimed in claim 13 wherein the naïve stem cells are from pre-implantation embryonic epiblast tissue.

15 A process as claimed in claim 14 wherein the naïve stem cells are obtained from the pre-implantation embryonic epiblast tissue by explant or dissociation.

16 A process as claimed in claim 13 wherein the naïve stem cells are from a naïve stem cell line.

17 A process as claimed in claim 16 wherein the naïve stem cell line is provided at (a) by culturing in naïve pluripotency maintenance conditions but is withdrawn from said naïve pluripotency maintenance conditions and transferred directly or indirectly to formative stem cell culture media at (b).

18 A process as claimed in claim 17 wherein the naïve pluripotency maintenance conditions comprise 2iLIF.

19 A process as claimed in claim 17 wherein the naïve stem cell line is transferred indirectly to formative stem cell culture media at step (b) via a culture medium without 2iLIF.

20 A process as claimed in any one of claims 1 to 11 wherein the one or more precursor pluripotent stem cells are primed pluripotent stem cells transferred directly or indirectly to formative stem cell culture media at (b).

21 A process as claimed in claim 20 wherein the primed pluripotent stem cells are transferred indirectly by passaging in media having reduced FGF and activin compared to the primed pluripotent stem media .

22 A process as claimed in claim 13 wherein the naïve cells are provided by resetting primed pluripotent stem cells.

23 A process as claimed in claim 22 wherein the primed pluripotent stem cells are a cultured cell line.

24 A process as claimed in any one of claims 1 to 11 wherein the one or more precursor pluripotent stem cells are provided by reprogramming somatic cells.

25 A process as claimed in any one of claims 1 to 24 wherein the formative stem cells are mammalian or avian.

26 A process as claimed in claim 25 wherein the formative stem cells are rodent or pig cells.

27 A process as claimed in claim 25 wherein the formative stem cells are human cells.

28 A process as claimed in claim 26 wherein the formative stem cells are mouse formative stem cells are characterised by one or more of the following phenotypes:

- (i) lack expression of naïve transcription factors such as Esrrb, Tfcp2l1, Klf4 and Klf2, or early lineage markers such as Tbra, FoxA2 and Sox1;
- (ii) display robust multi-lineage differentiation in vitro;
- (iii) respond directly to germ cell induction;
- (iv) widespread contribution to all lineages in chimaeras after blastocyst injection.
- (v) can be propagated in the presence of the activin receptor (ALK5) inhibitor A-8301 in the presence of RARi and gamma secretase inhibitor;
- (vi) maintain expression of Oct4 in all cells
- (vii) display near-absence of lineage-affiliated gene expression;
- (viii) do not survive if FGF/Erk signalling is inhibited with either the MEK inhibitor PD0325901 (1µM) or the FGF receptor inhibitor PD173074 (0.1µM);
- (ix) can expand and maintain undifferentiated morphology in the presence of exogenous FGF (0.1, 0.5, 1.0, 2.5, 5.0);
- (x) proliferate as coherent monolayer colonies of epithelial-like cells;
- (xi) show enriched expression of markers: Dppa2 and Dppa4;
- (xii) differentiate if exposed to serum or KSR or BMP

29 A process as claimed in claim 27 wherein the formative stem cells are characterised by one or more of the following phenotypes:

- (i) down-regulation of naïve markers KLF4, KLF17, TFCEP2L1.
- (ii) retention of core pluripotency factor OCT4;
- (iii) little or no expression of lineage priming markers TBRA and FOXA2.
- (iv) gain of early post-implantation epiblast marker OCT6 (*POU3F1*).
- (v) capable of differentiation into neural, mesodermal and endodermal lineages;
- (vi) responsive to germ cell induction signal by direct treatment with the inductive cytokine cocktail. FS cells show expression of surface markers TNAP and CD38 between day 4 and 7 and immuno-staining for SOX17, BLIMP1 and OCT4 confirming PGCLC formation.

30 A process for propagating a formative stem cell line, which process comprises:

- (A) providing one or more formative stem cells;
- (B) culturing the formative stem cells in a formative stem cell culture media, which formative stem cell culture media as defined in any one of claims 1 to 10.

31 A process as claimed in claim 30 wherein the formative stem cells are obtained by the process of any one of claims 1 to 29.

32 A process as claimed in claim 30 or claim 31 wherein the formative stem cells are cultured in the formative stem cell culture conditions defined in claim 11.

33 A process as claimed in any one of claims 30 to 32 wherein the formative stem cells are native formative stem cells obtained from early post-implantation embryonic epiblast tissue.

34 A process as claimed in claim 33 wherein where the native formative stem cells are rodent epiblast cells between E5 and E6, preferably E5.5, or are cells from human ICM explant culture.

35 A process as claimed in any one of claims 1 to 34 which maintains a substantially homogeneous population of FS cells in continuous proliferative culture for at least 30 days, wherein substantially homogeneity comprises more than 90% of cells expressing OCT4 protein as detected by immunostaining, and less than 5% of cells immunopositive for Brachyury, SOX1, FOXA2, or Sox17.

36 A formative stem cell line obtained or obtainable by the process of any one of claims 1 to 35.

37 A substantially homogeneous population of formative stem cells having the characteristics defined in claim 28 or claim 29.

38 A process for producing a primed pluripotent stem cell from a formative stem cell or line of claim 36 or claim 37, which process comprises withdrawal from FS cell culture medium and transfer to primed pluripotent stem cell culture medium.

39 A process for producing primordial germ cell-like cells (PGCLC) from a formative stem cell or line of claim 36 or claim 37, which process comprises exposing said formative stem cell line to one or more inductive cytokines.

40 A process as claimed in claim 39 wherein said formative stem cell line is exposed to hBMP2, mSCF, hLIF, and Egf.

41 A process for producing somatic lineage differentiated cells from a formative stem cell or line of claim 36 or claim 37, which process is initiated by withdrawal from FS cell culture medium and exposure of the cells to differentiation conditions.

42 A process as claimed in claim 41 wherein the somatic lineage is mesendoderm, and the formative stem cell line is exposed to serum free medium in the presence of activin a and Wnt or a GSK3 inhibitor such as CH99021.

43 A process as claimed in claim 41 wherein the somatic lineage is neural, and the formative stem cell line is exposed to a Tgfb receptor inhibitor.

44 A process for producing a chimaeric organism, which process comprises introducing a formative stem cell or line of claim 36 or claim 37 into a host embryo at pre-implantation or early post-implantation stages.

45 A formative stem cell culture media which is as defined in any one of claims 1 to 10.

46 A formative stem cell culture media comprising:

- (i) activin at a concentrations of 1 - 4ng/ml, more preferably 1.5 - 3 ng/ml;
- (ii) less than 10ng/ml of exogenous FGF; more preferably less than 10ng/ml of exogenous FGF;
- (iii) a Wnt inhibitor.

47 Use of the formative stem cell culture medium of claim 45 or claim 46 to produce or propagate a formative stem cell line.

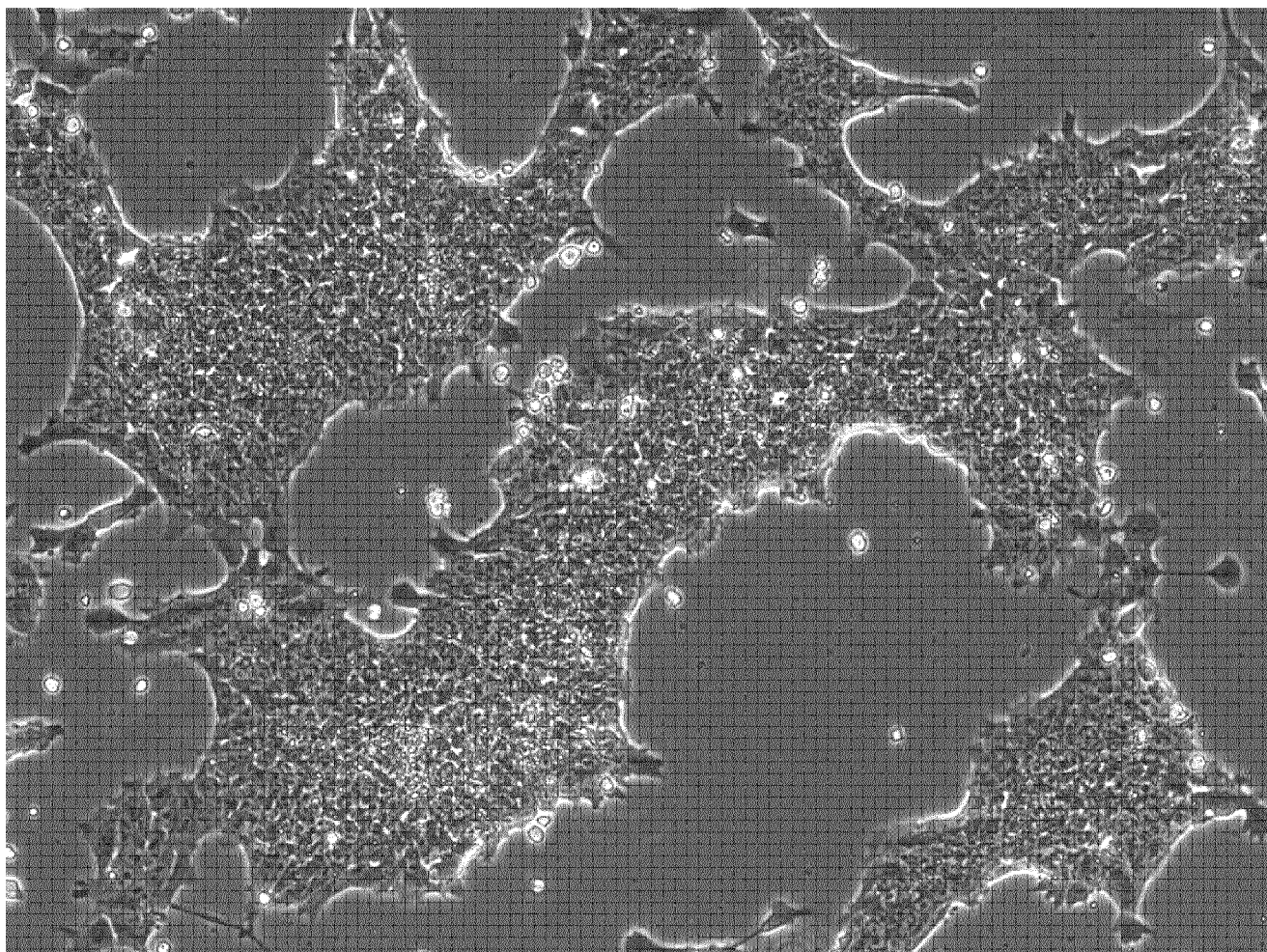


Fig. 1a

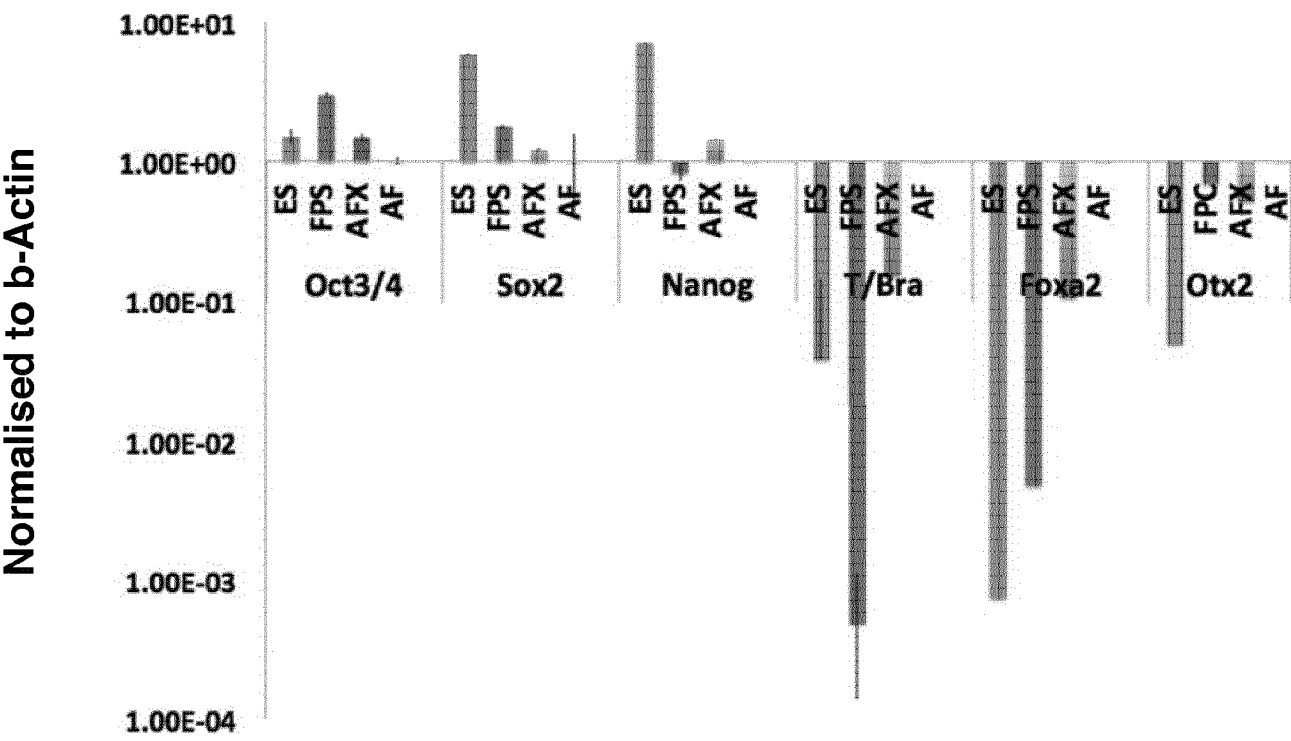


Fig. 1b

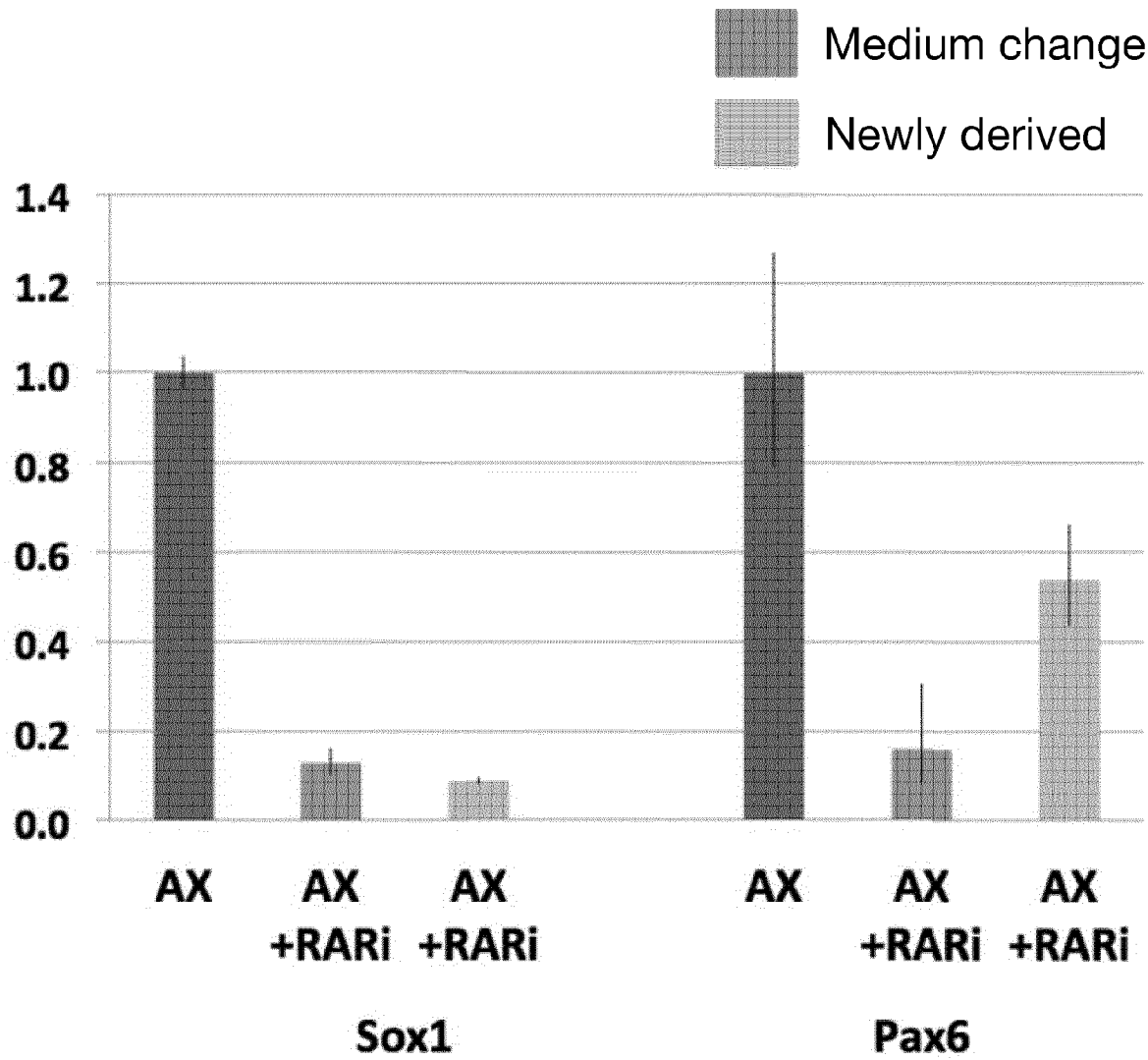


Fig. 1c

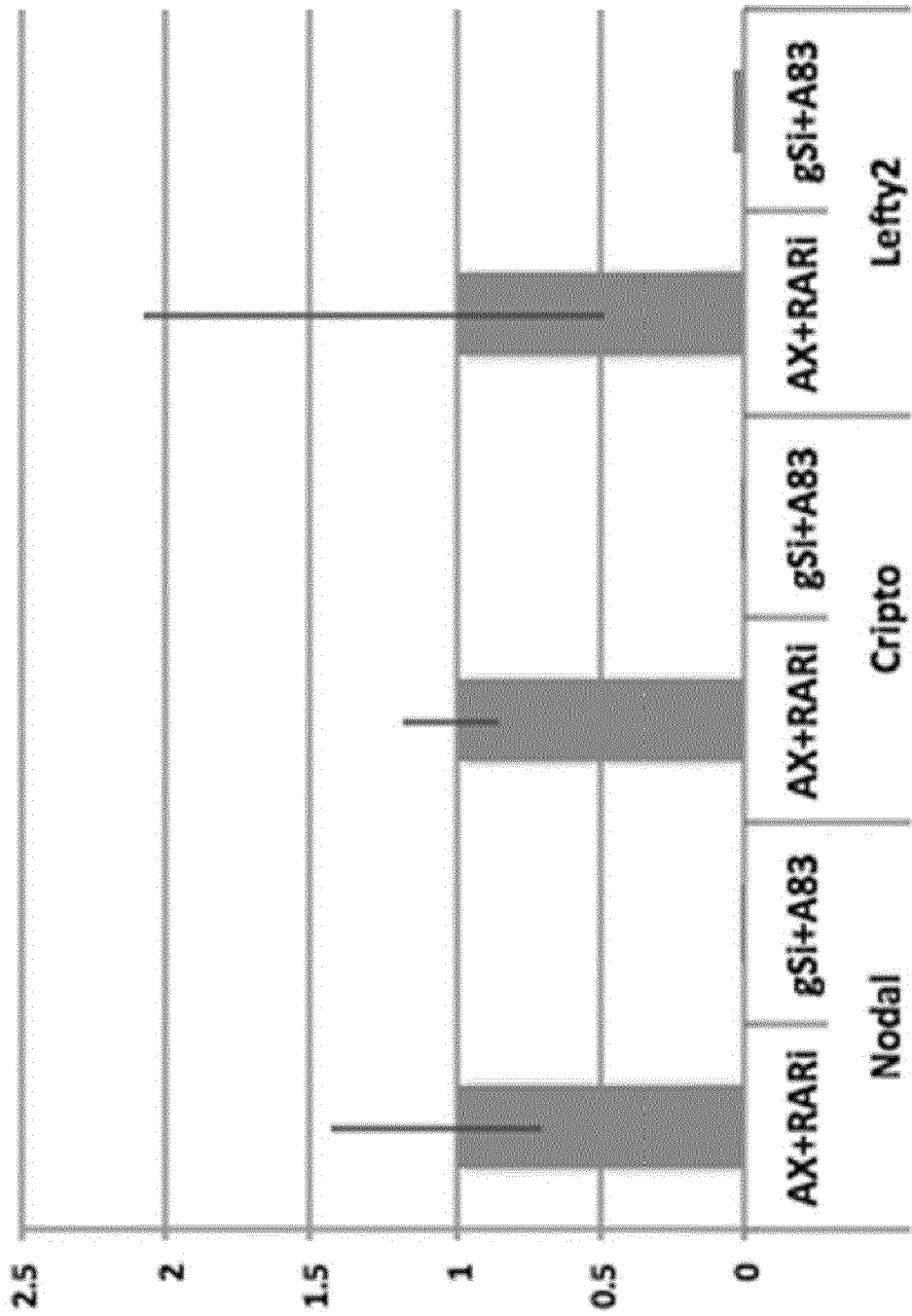


Fig. 1d

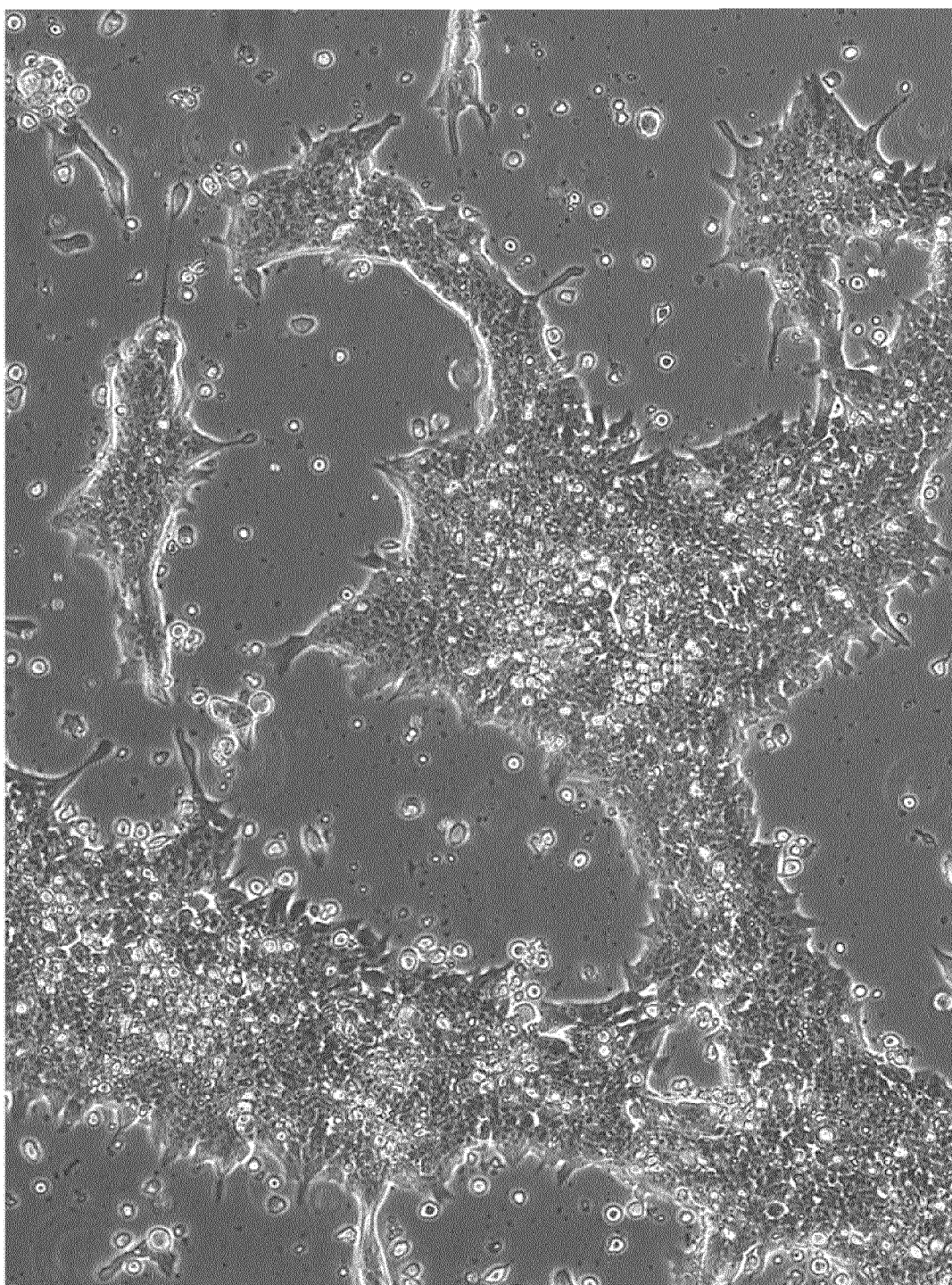


Fig. 1e

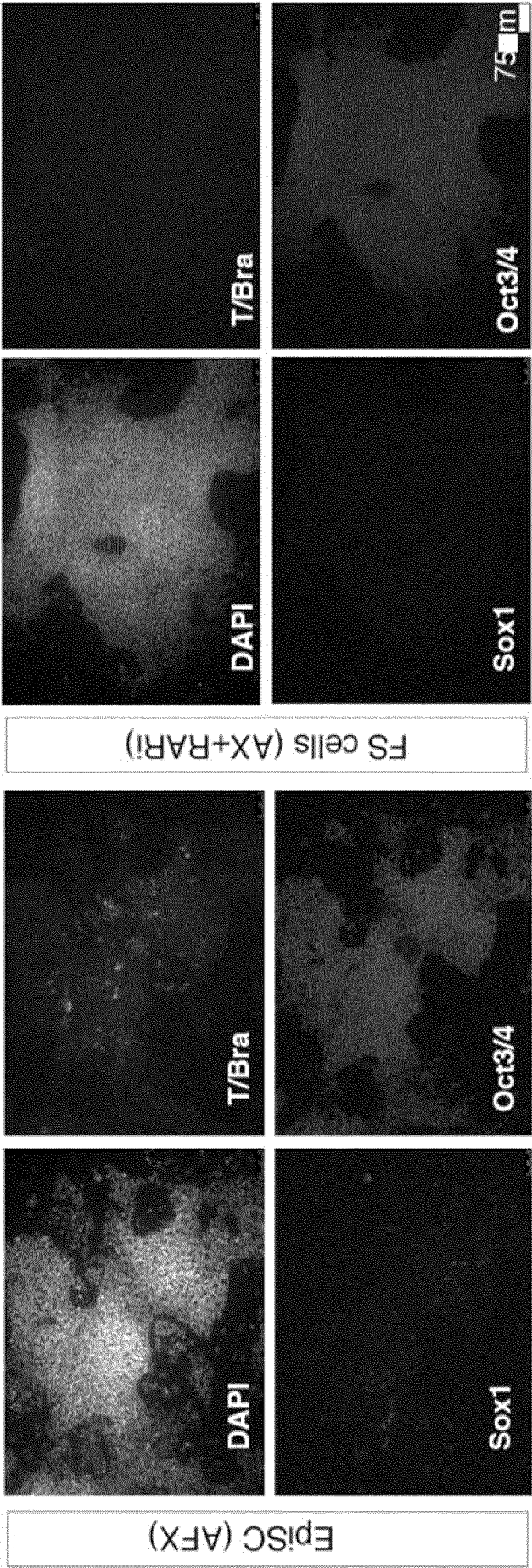


Fig. 1f

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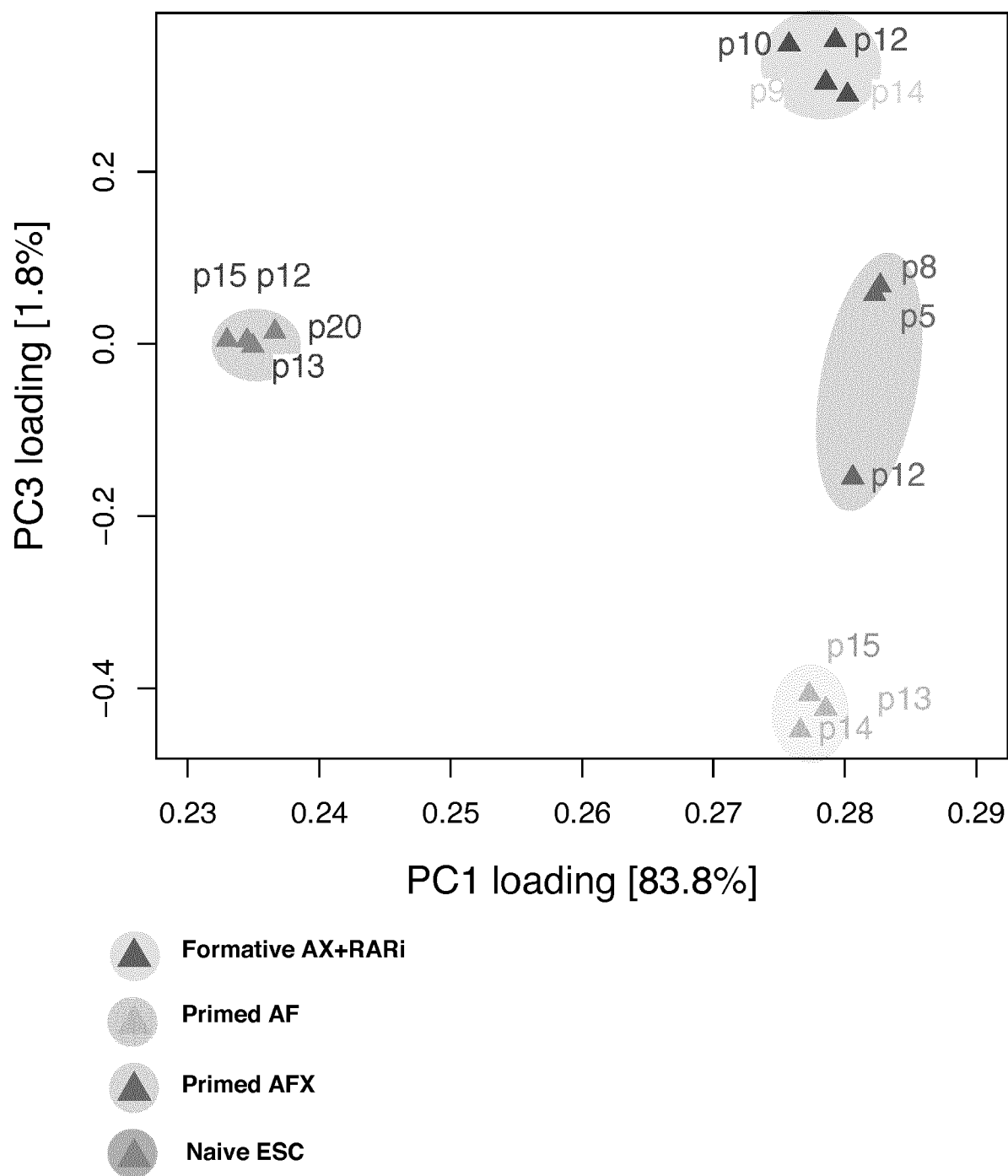
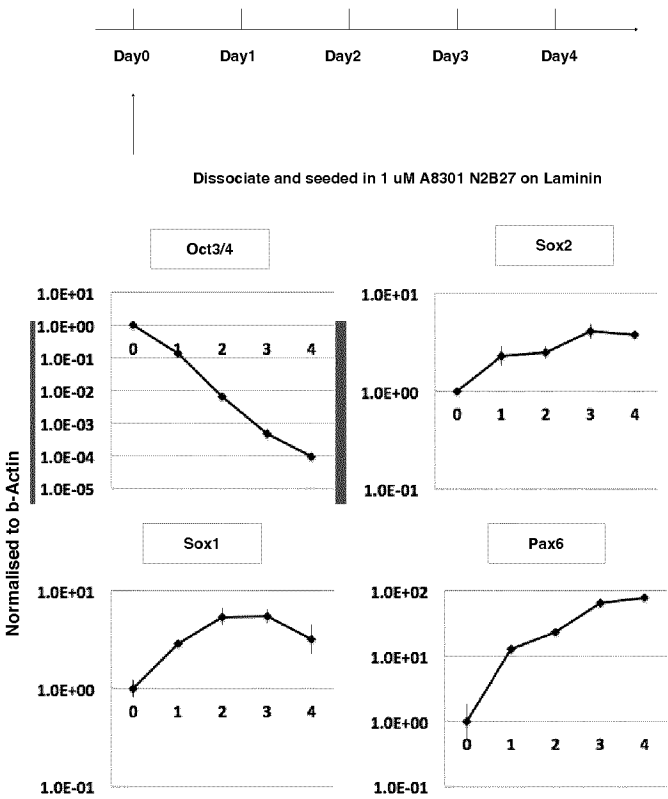


Fig. 1g

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Neural Differentiation



Mesendoderm Differentiation

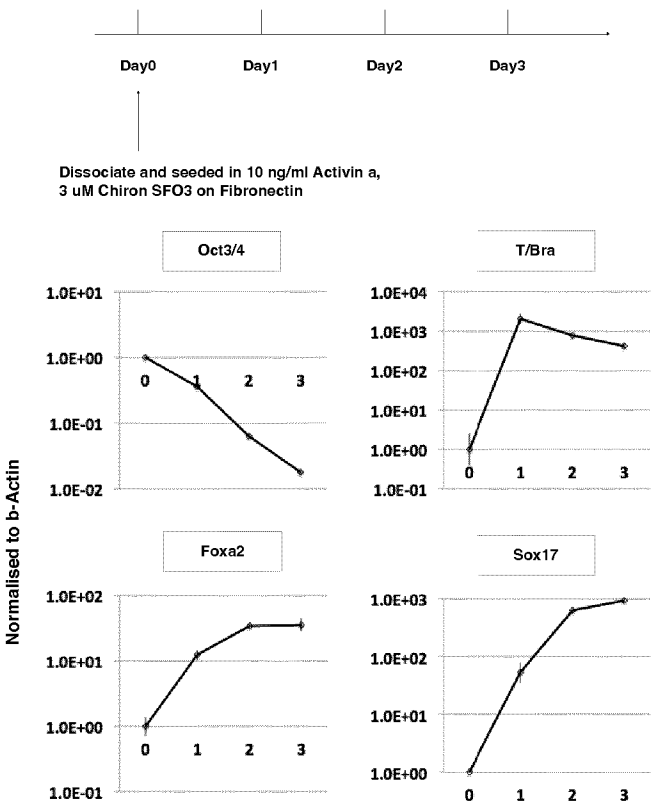


Fig. 1h

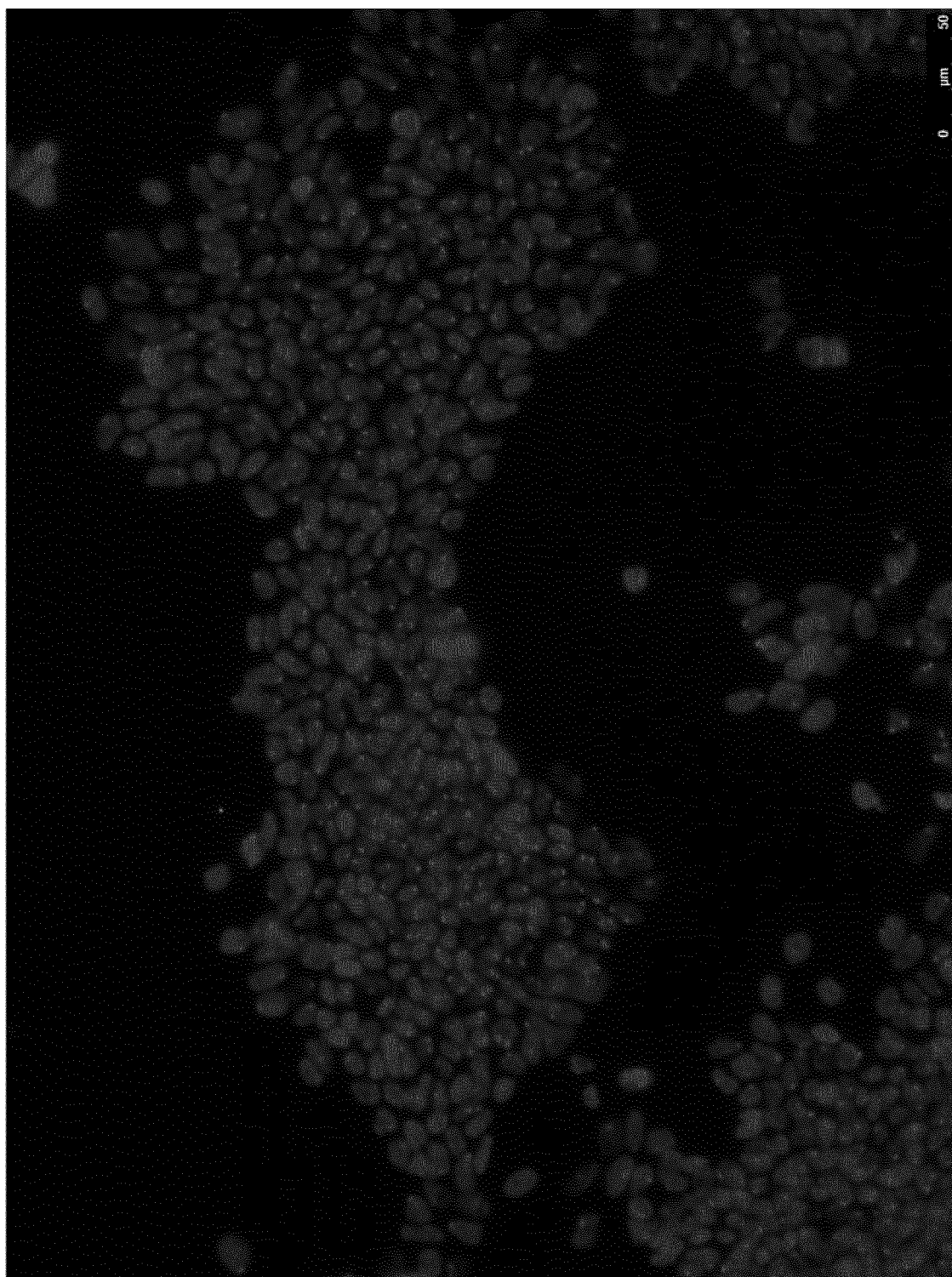


Fig. 1i

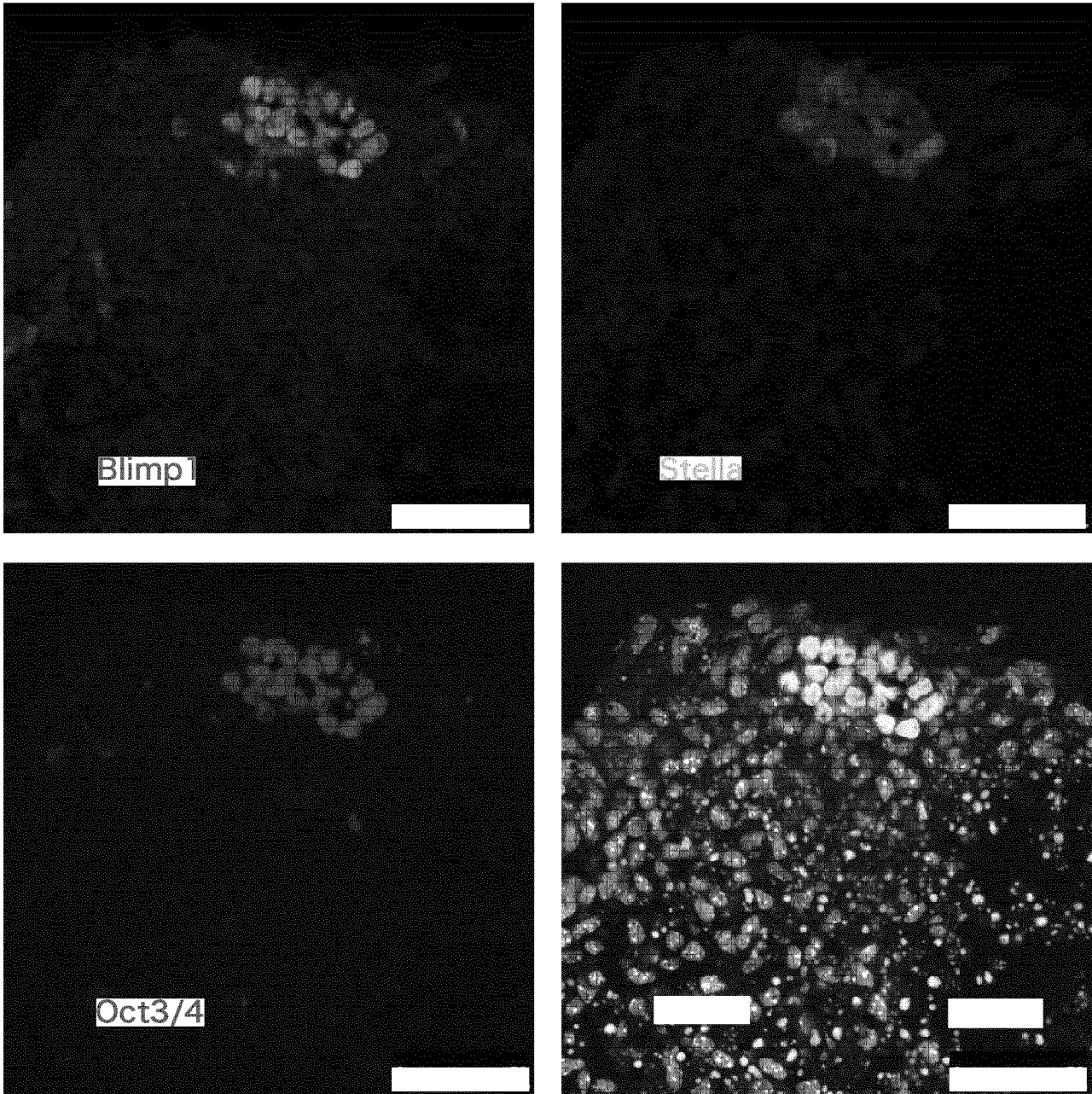


Fig. 1j

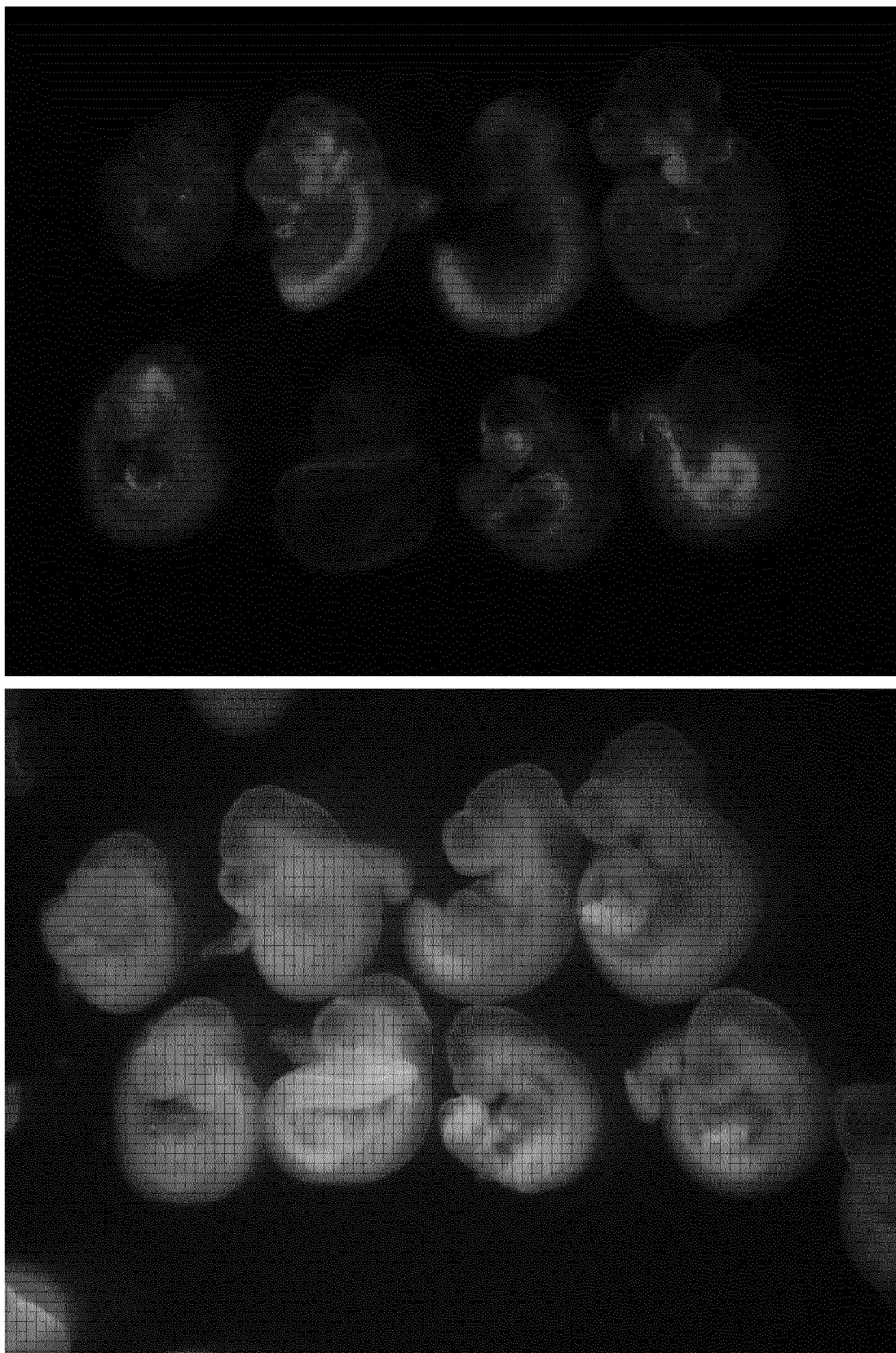


Fig. 1k

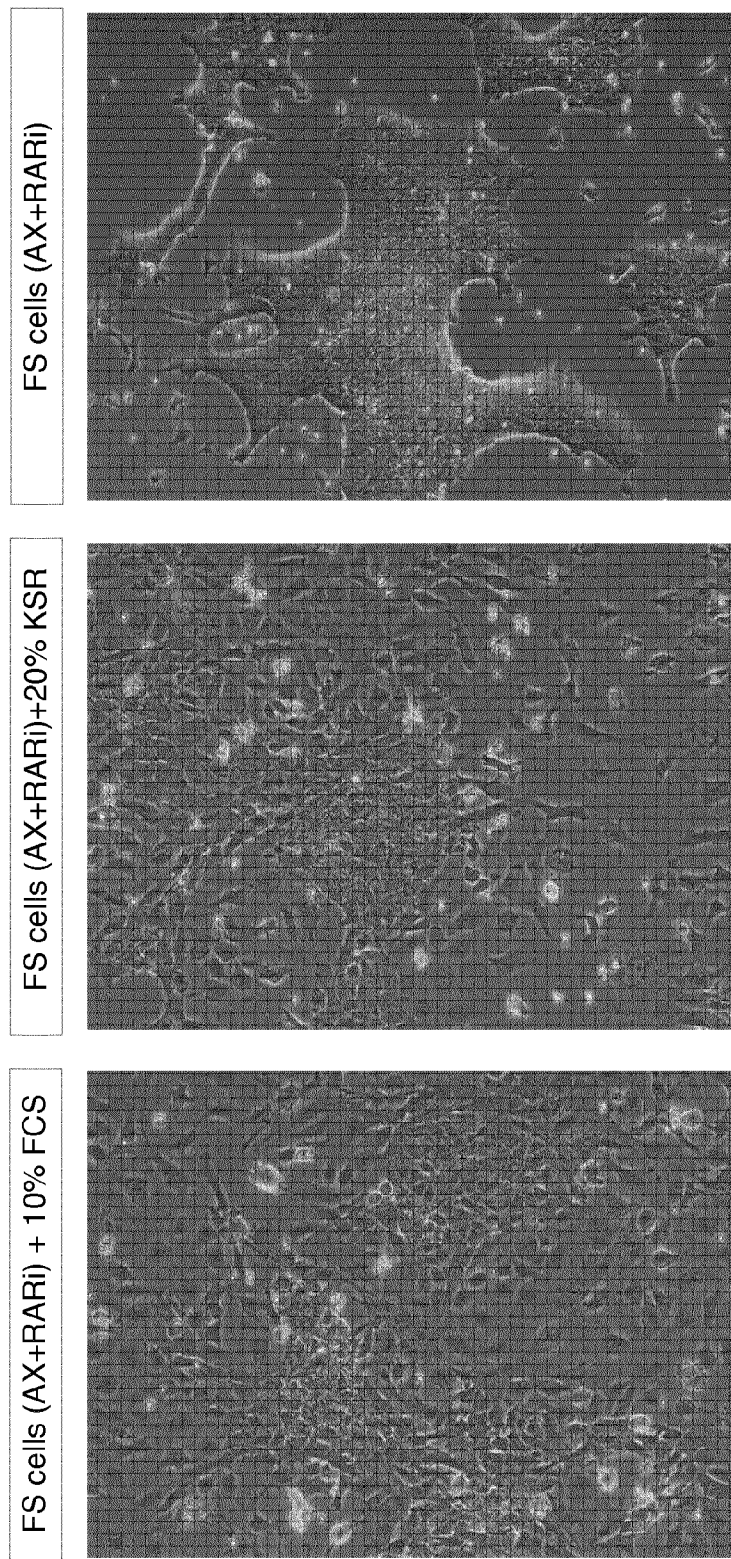
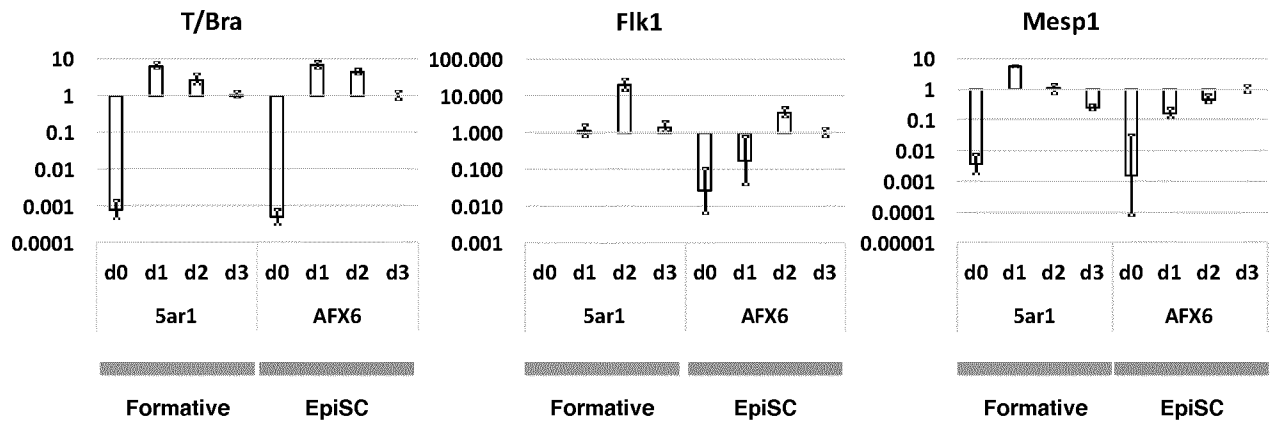
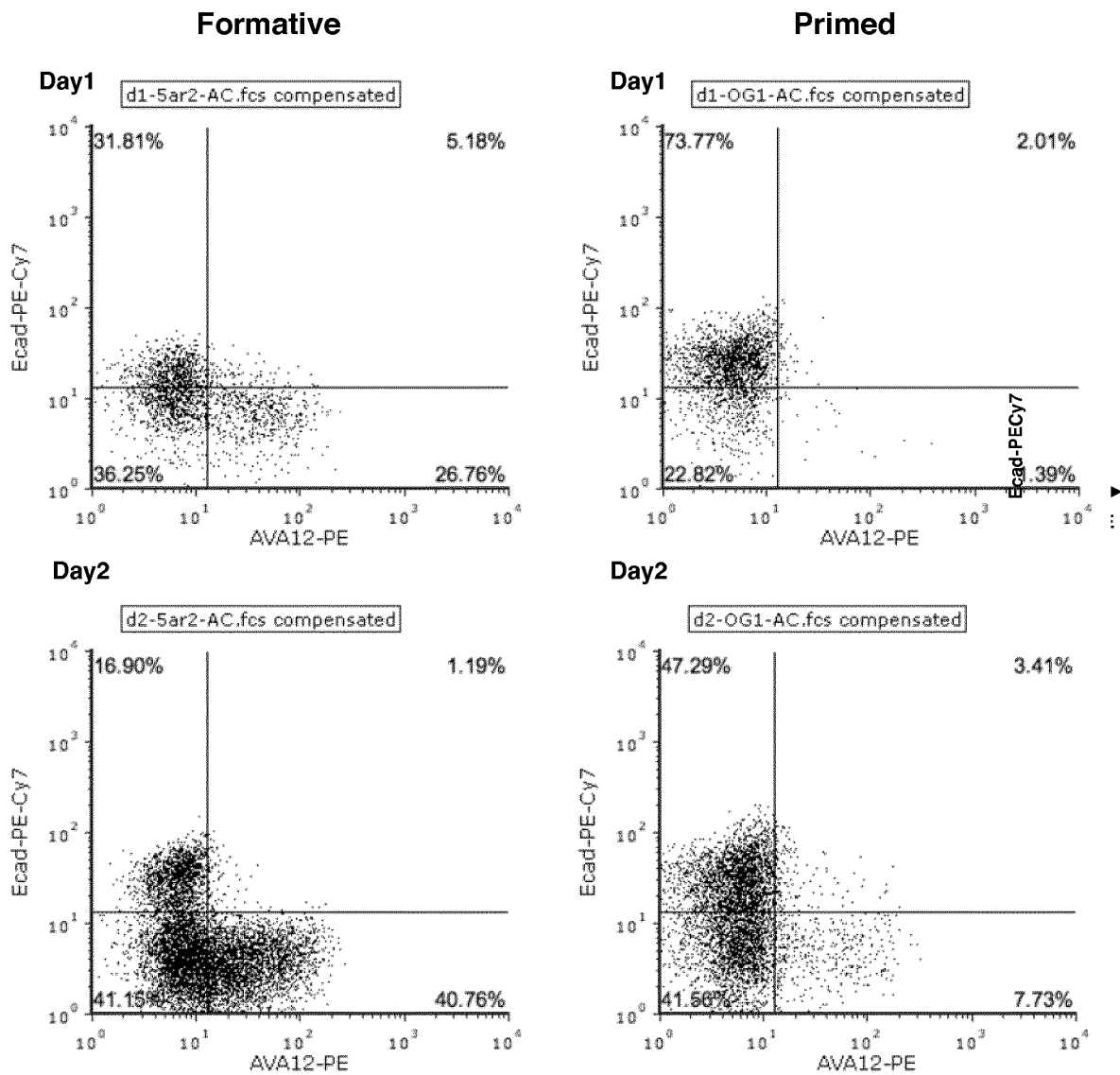


Fig. 11

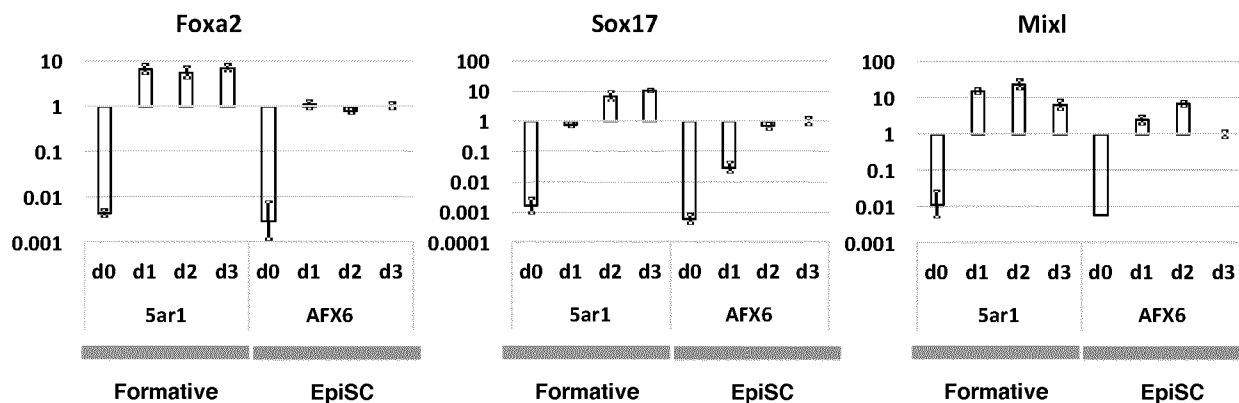
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Time course qPCR analysis of mesoderm genes**Time course FACS analysis of mesoderm population****Fig. 1m**

Ecad-647
↑
Flk1-PE
→

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Time course qPCR analysis of endoderm genes



FACS plot of Endoderm at day 3

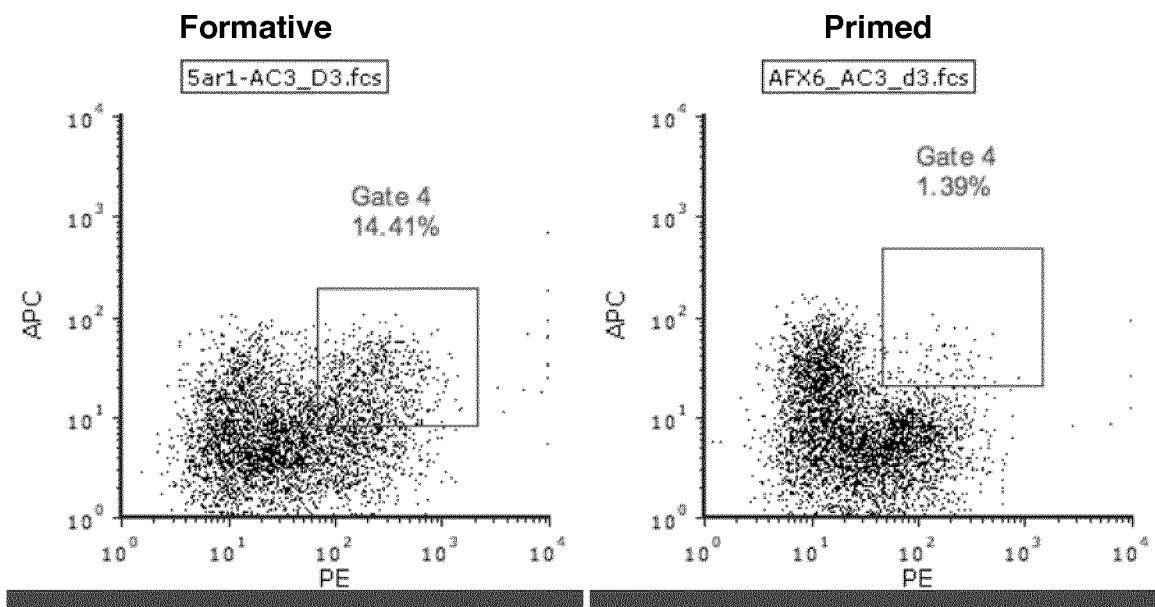


Fig. 1n

Ecad-647
Cxcr4-PE

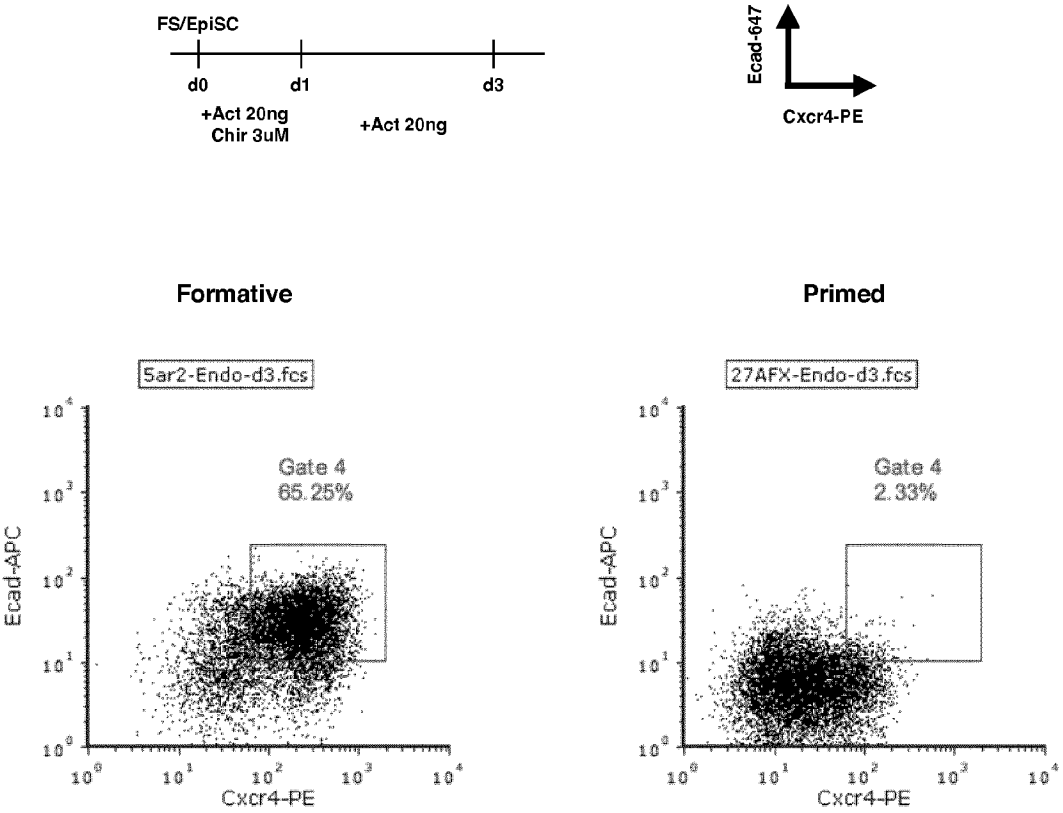
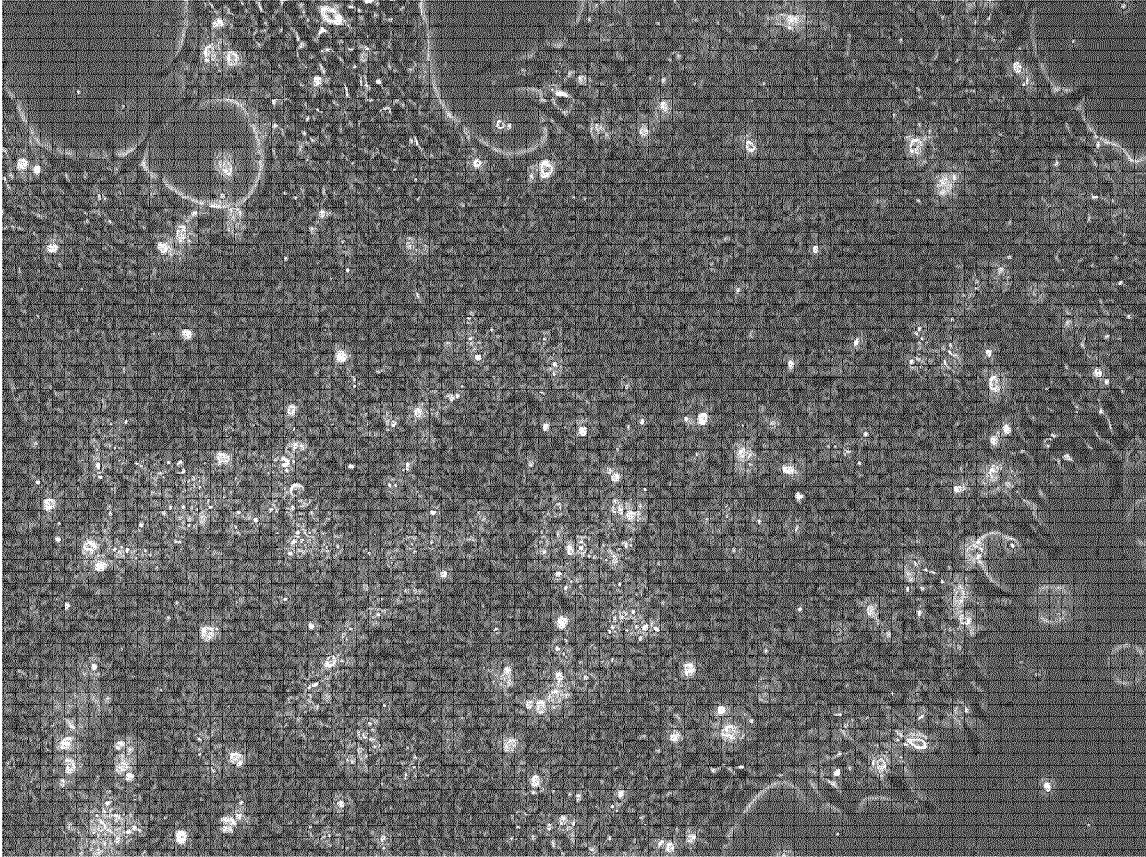


Fig. 1o

FS cells (AX)



FS cells (AX+RARi)

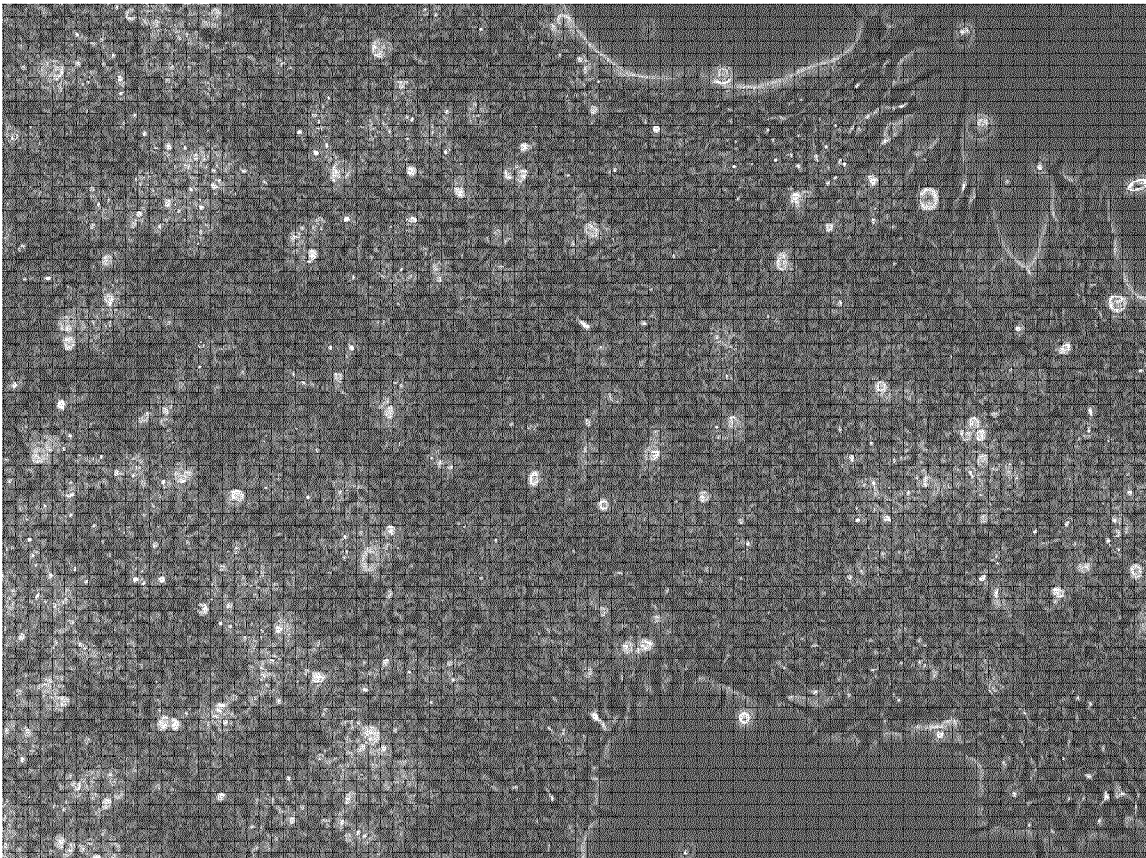


Fig. 2a

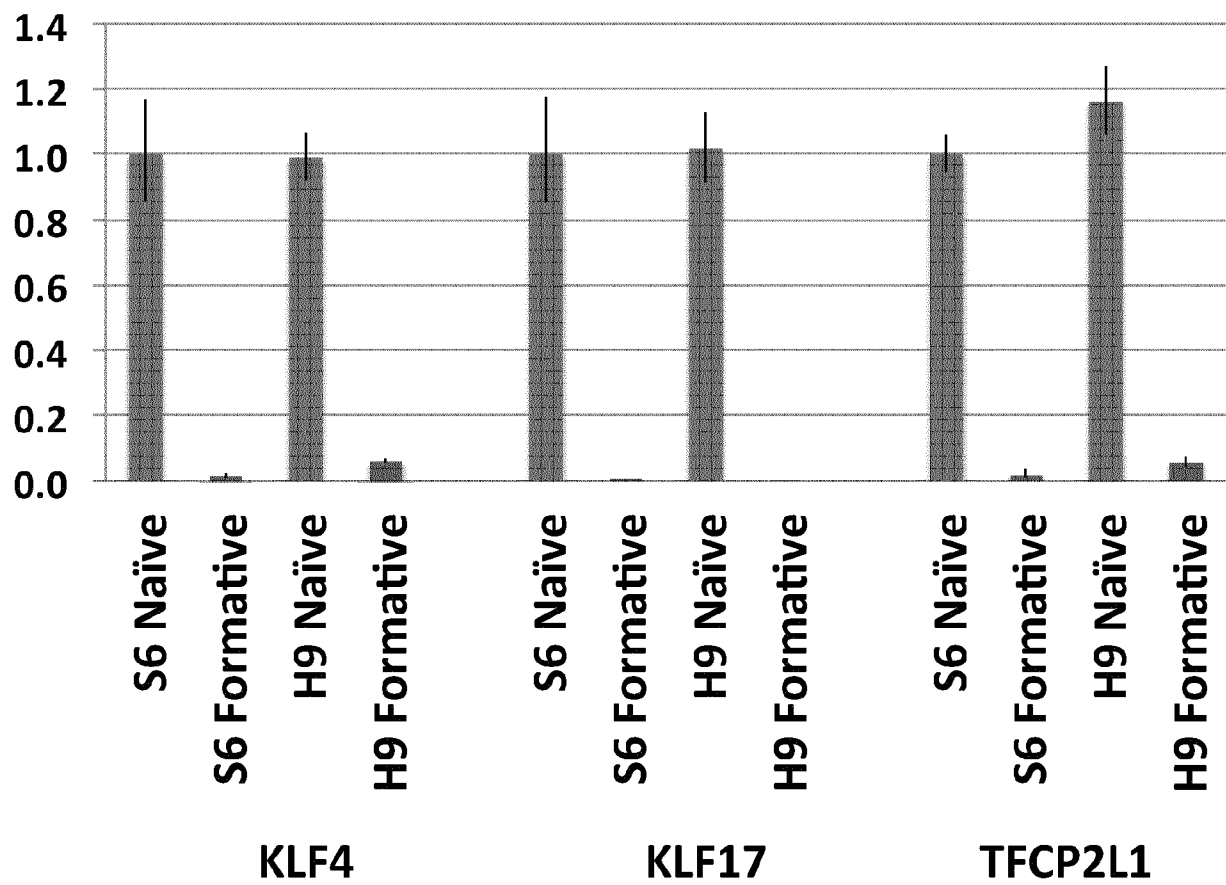


Fig. 2b

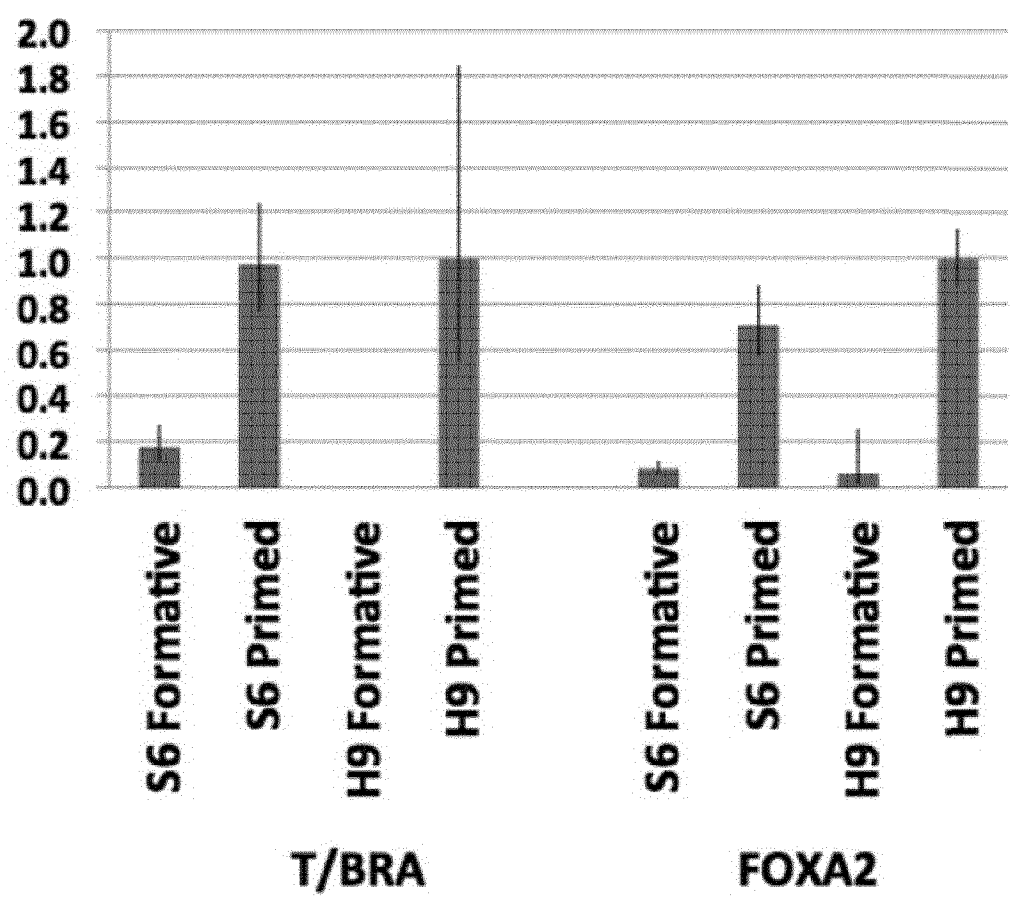


Fig. 2c

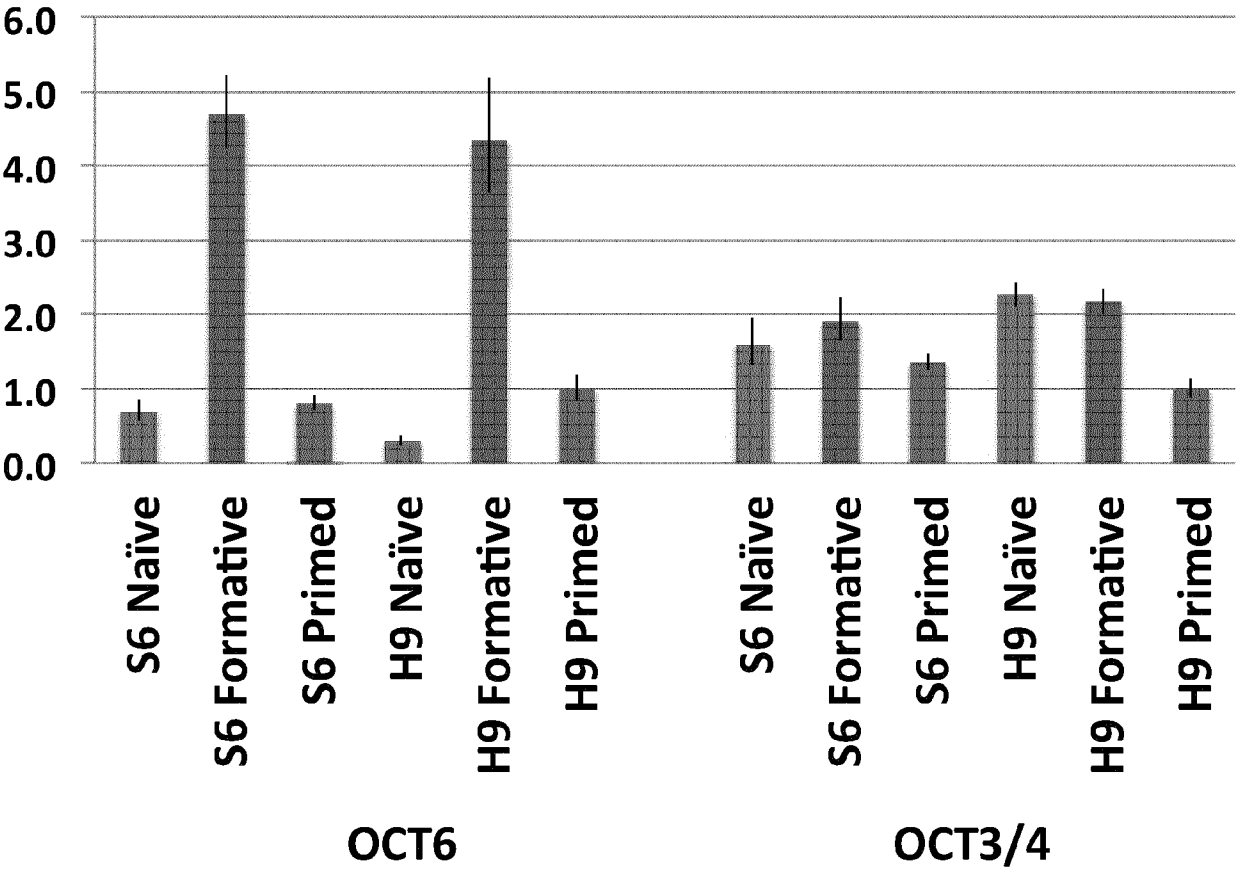


Fig. 2d

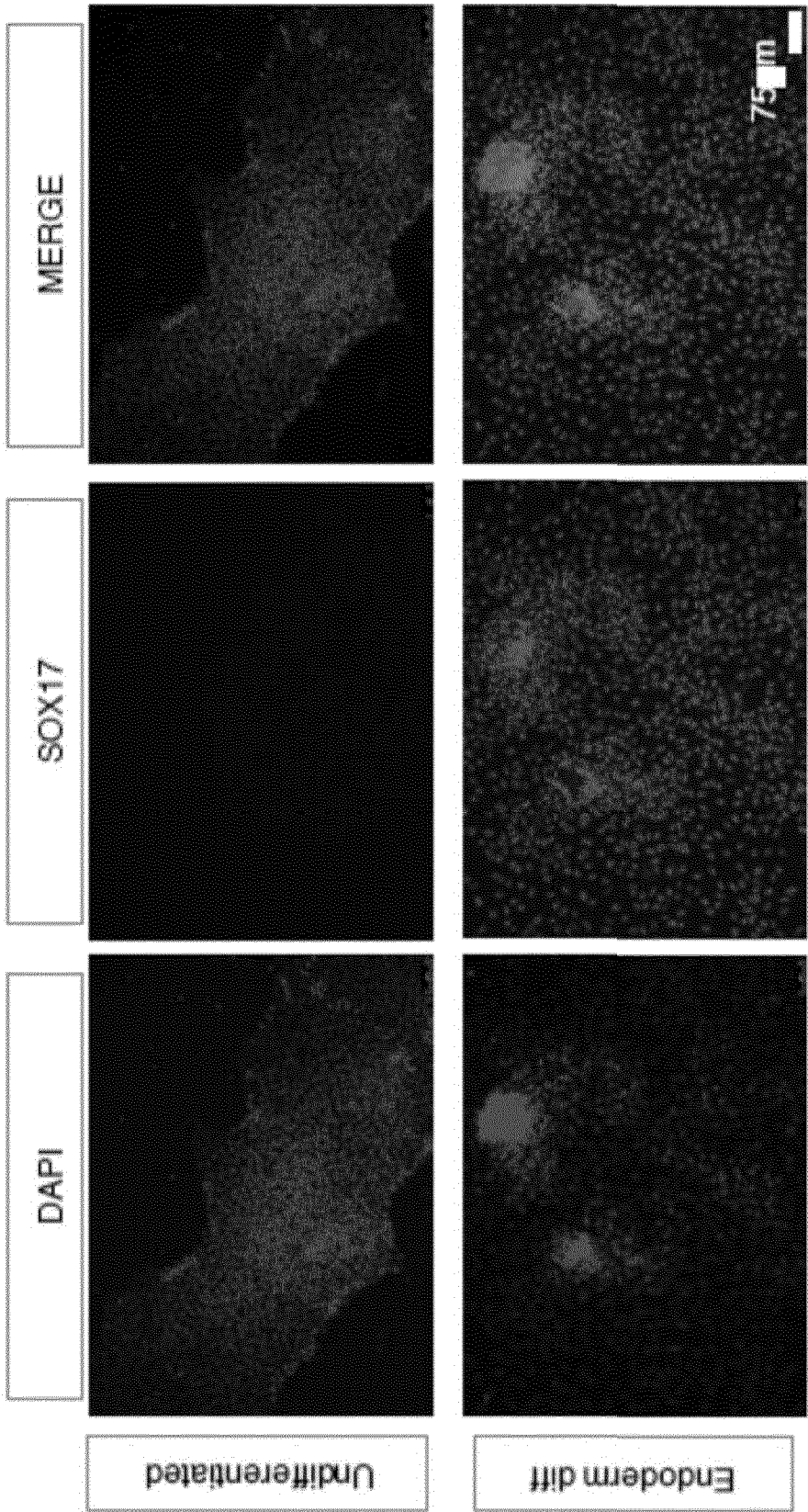


Fig. 2e

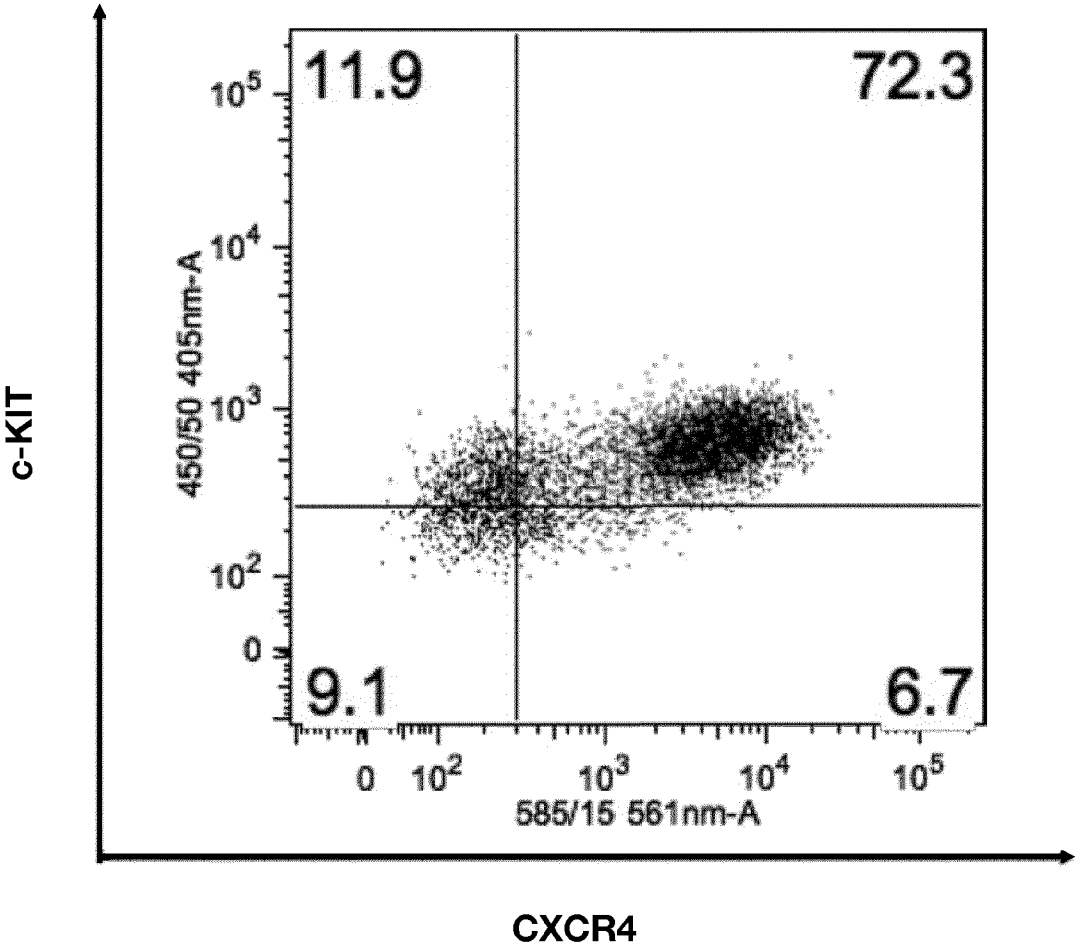


Fig. 2f

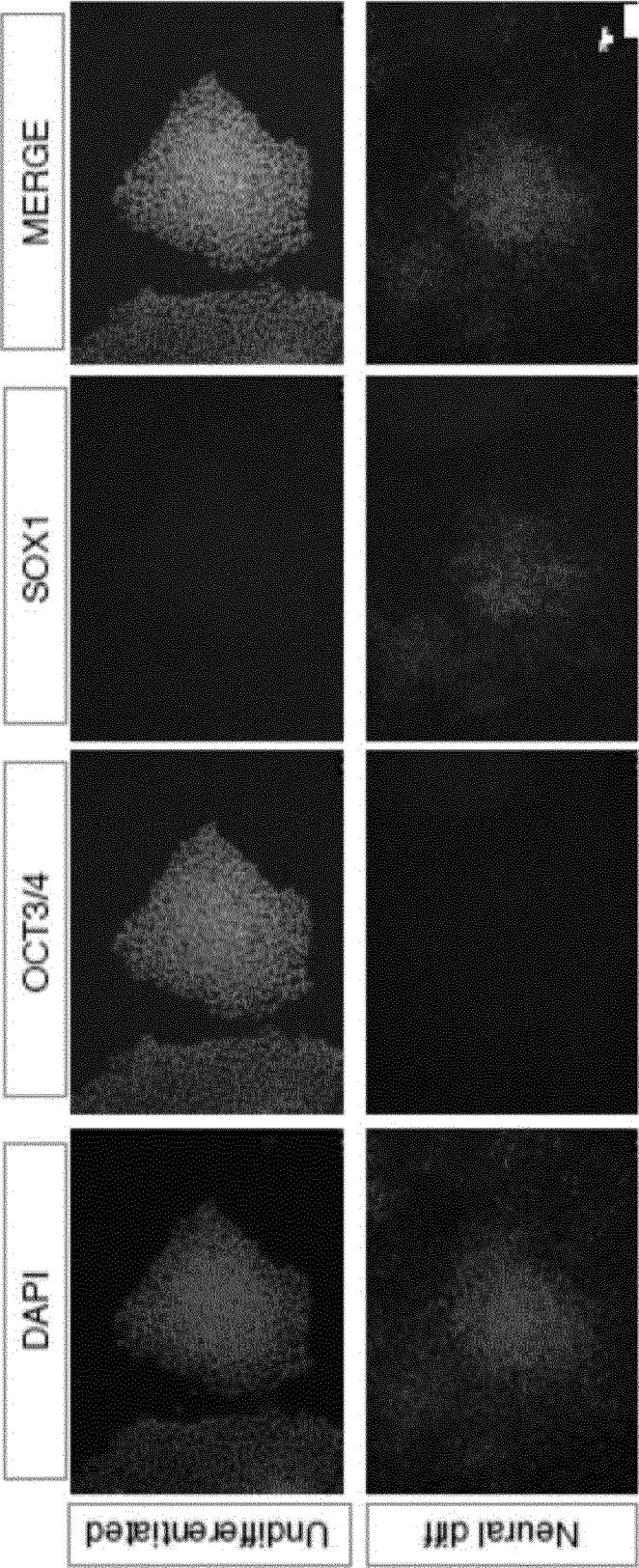


Fig. 2g

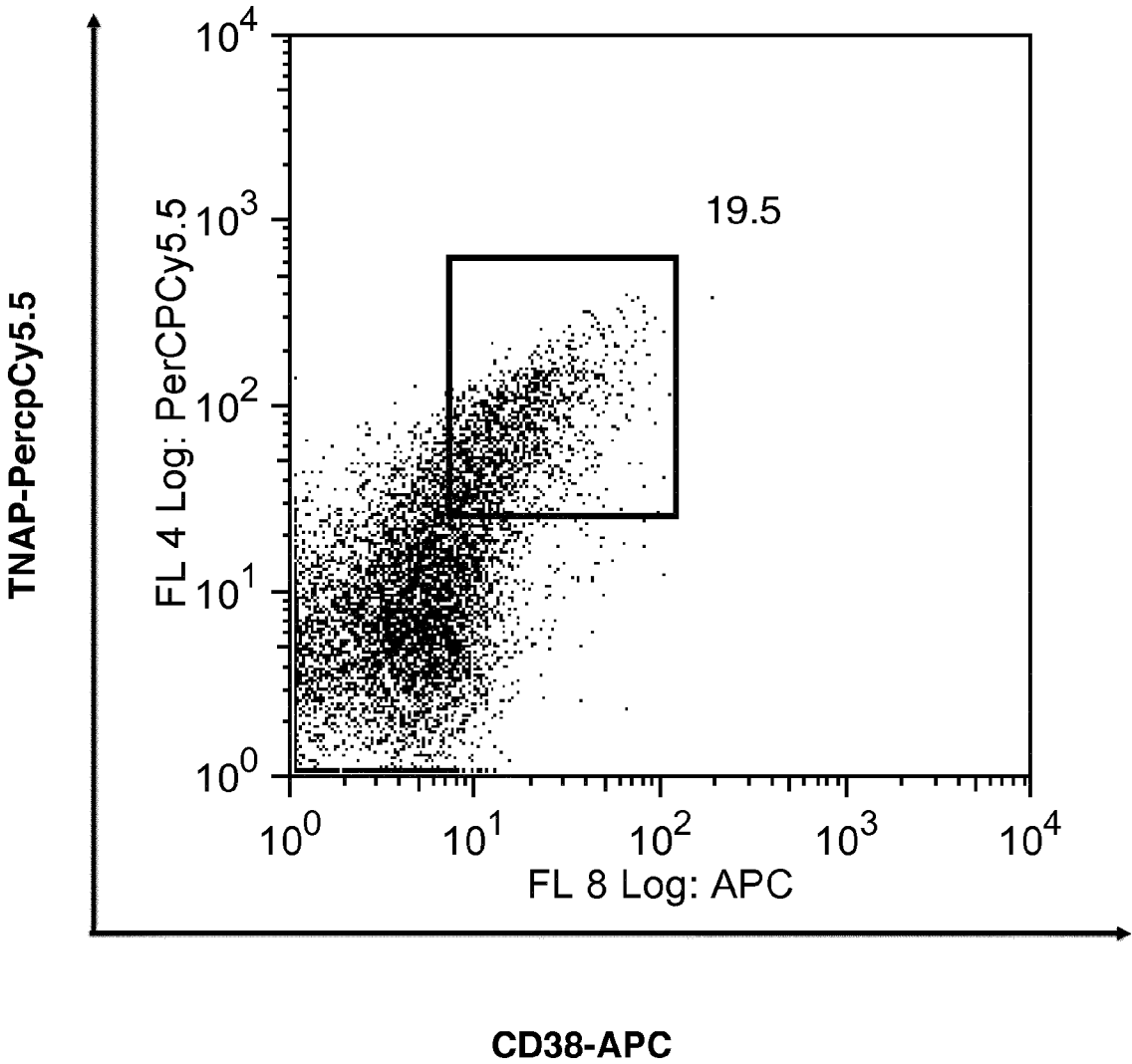


Fig. 2h

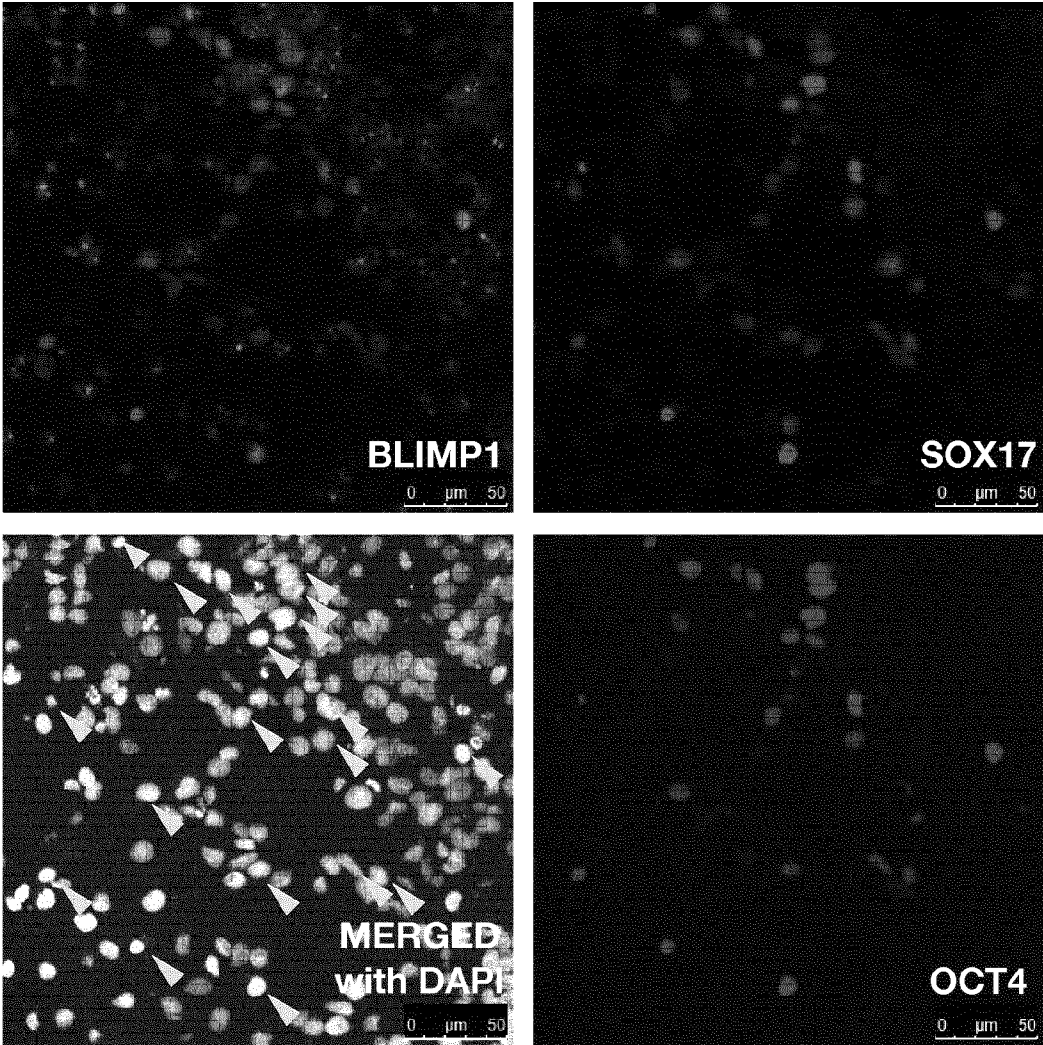


Fig. 2i

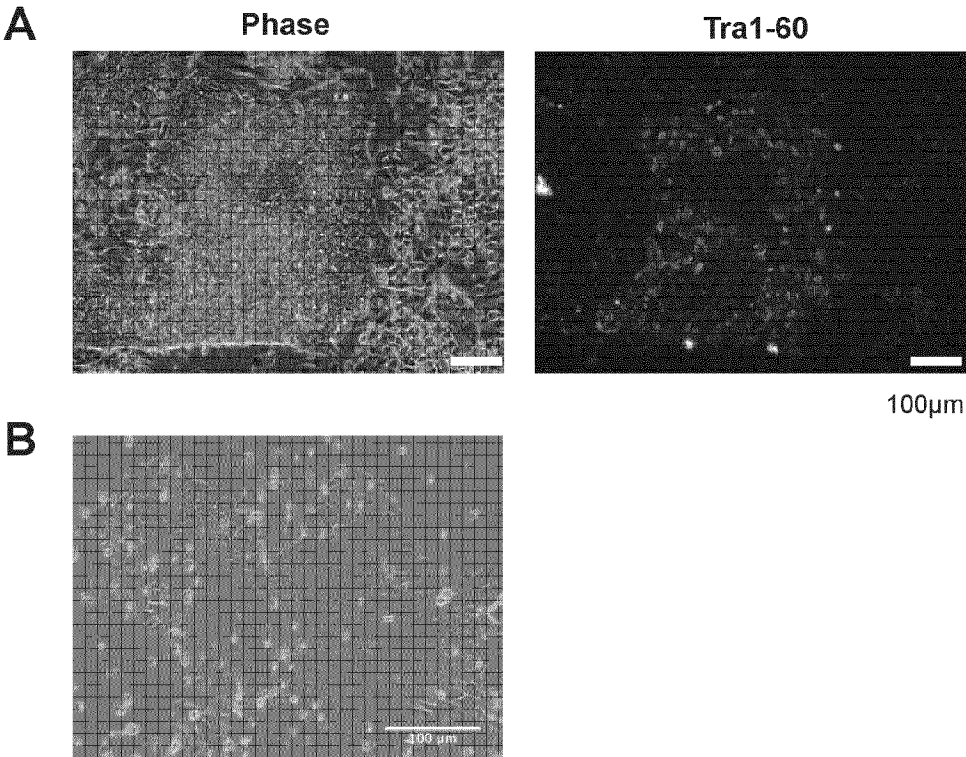


Fig. 2j

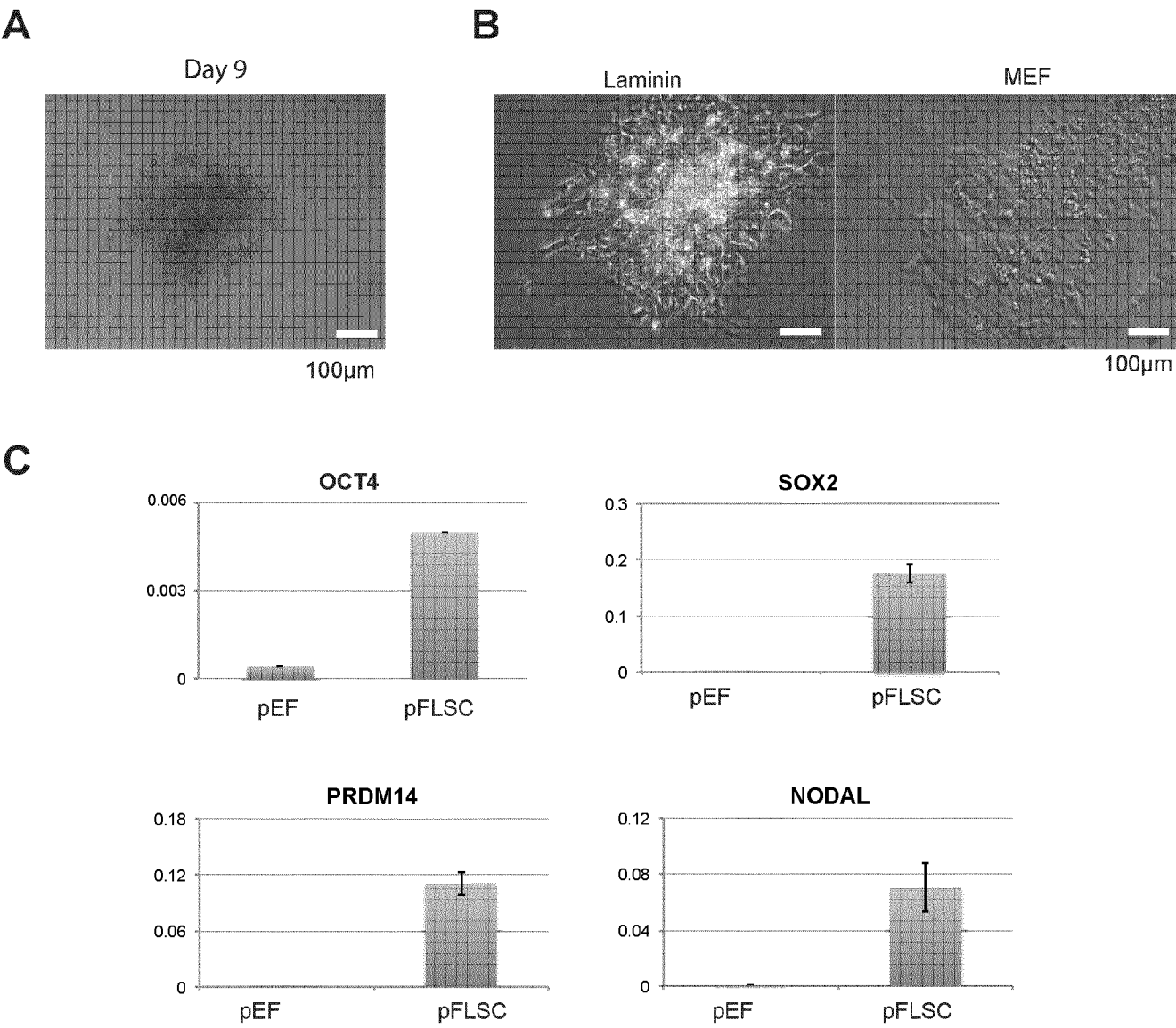


Fig. 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/051990

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/0735
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, EMBASE, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TOMOYUKI SUMI ET AL: "Epiblast Ground State Is Controlled by Canonical Wnt-&bgr;-Catenin Signaling in the Postimplantation Mouse Embryo and Epiblast Stem Cells", PLOS ONE, vol. 8, no. 5, 14 May 2013 (2013-05-14), page e63378, XP055224866, DOI: 10.1371/journal.pone.0063378 abstract EpiSC culture; page e63378 ----- -/--	1-46



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"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

1 March 2018

Date of mailing of the international search report

19/03/2018

Name and mailing address of the ISA/

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Fax: (+31-70) 340-3016

Authorized officer

Loubradou-Bourges, N

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/051990

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KATSUHIKO HAYASHI ET AL: "Reconstitution of the Mouse Germ Cell Specification Pathway in Culture by Pluripotent Stem Cells", CELL, CELL PRESS, AMSTERDAM, NL, vol. 146, no. 4, 28 June 2011 (2011-06-28) , pages 519-532, XP028383021, ISSN: 0092-8674, DOI: 10.1016/J.CELL.2011.06.052 [retrieved on 2011-07-16] abstract	1-43,46
X	----- OHAD GAFNI ET AL: "Derivation of novel human ground state naive pluripotent stem cells", NATURE, vol. 504, no. 7479, 30 October 2013 (2013-10-30), pages 282-286, XP055128176, ISSN: 0028-0836, DOI: 10.1038/nature12745 abstract	1-43,46
X,P	----- TÜZER KALKAN ET AL: "Tracking the embryonic stem cell transition from ground state pluripotency", DEVELOPMENT, vol. 144, no. 7, 7 February 2017 (2017-02-07), pages 1221-1234, XP055455247, GB ISSN: 0950-1991, DOI: 10.1242/dev.142711 abstract	36-43
A,P	----- SOPHIE MORGANI ET AL: "The many faces of Pluripotency: in vitro adaptations of a continuum of in vivo states", BMC DEVELOPMENTAL BIOLOGY, vol. 17, no. 1, 13 June 2017 (2017-06-13), XP055455295, DOI: 10.1186/s12861-017-0150-4 abstract	1-35
X,P	----- AUSTIN SMITH: "Formative pluripotency: the executive phase in a developmental continuum", DEVELOPMENT, vol. 144, no. 3, 31 January 2017 (2017-01-31), pages 365-373, XP055455303, GB ISSN: 0950-1991, DOI: 10.1242/dev.142679 abstract	36-43
A,P	----- ----- -/--	1-35

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/051990

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	MASAKI KINOSHITA ET AL: "Pluripotency Deconstructed", DEVELOPMENT GROWTH AND DIFFERENTIATION., vol. 60, no. 1, 1 January 2018 (2018-01-01), pages 44-52, XP055455321, US	36-43
A,P	ISSN: 0012-1592, DOI: 10.1111/dgd.12419 abstract -----	1-15