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Review

Pluripotency of embryo-derived stem cells from rodents, lagomorphs, and primates: Slippery slope, terrace and cliff

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ABSTRACT

The diverse cell states and *in vitro* conditions for the derivation and maintenance of the mammalian embryo-derived pluripotent stem cells raise the questions of whether there are multiple states of pluripotency of the stem cells of each species, and if there are innate species-specific variations in the pluripotency state. We will address these questions by taking a snapshot of our knowledge of the properties of the pluripotent stem cells, focusing on the maintenance of pluripotency and inter-conversion of the different types of pluripotent stem cells from rodents, lagomorphs and primates. We conceptualize pluripotent stem cells acquiring a series of cellular states represented as terraces on a slope of descending gradient of pluripotency. We propose that reprogramming pluripotent stem cells from a primed to a naive state is akin to moving upstream over a steep cliff to a higher terrace.

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1. Introduction

Preimplantation development of mammalian embryo proceeds with the cleavage division of the zygote into blastomeres and the allocation of these blastomeres and their descendants to the extraembryonic and embryonic cell lineages of the blastocyst (Rossant and Tam, 2009). In the mouse, the extraembryonic lineages comprise the trophectoderm that contributes to the placental trophoblasts and the primitive endoderm derived from the inner cell mass (ICM). The embryonic lineage is derived from the epiblast of the blastocyst, which forms the primary

germ layers during gastrulation. The germ layers are the progenitor of all types of cells and tissues in the body, as well as the hematopoietic tissues in the yolk sac and the vascular tissues of the foeto-maternal interface (Stephenson et al., 2012). During embryogenesis, cells in the mouse embryos transit through different states of developmental potency (Boroviak et al., 2015). In an experimental setting, single blastomeres at the early cleavage stage are able to re-constitute the whole conceptus or contribute unrestrictedly to the full suite of extraembryonic and embryonic tissues in a chimeric conceptus. Cells displaying such attributes are reputed to be totipotent. Following the segregation of the extraembryonic lineages, cells in the embryo become restricted to the epiblast fate. Lineage analysis of individual epiblast cells in chimeras revealed that they can participate in germ layer differentiation and contribute extensively to all the specialized somatic cell types and the germline of the

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embryo. The multi-lineage potential and the chimera forming capacity of the epiblast cells are hallmarks of pluripotency (Mascetti and Pedersen, 2016).

Both totipotency and pluripotency are transitory in the mouse embryo during development, with the dismantling of pluripotency by late gastrulation (Osorno et al., 2012). However, the pluripotent property can be captured in the embryonic stem cells (ESCs) that are isolated from the peri-implantation blastocyst under appropriate *in vitro* culture conditions (Evans and Kaufman, 1981; Martin, 1981). The ESCs do not only remain pluripotent in lineage differentiation and chimera development but, unlike the parental cells in the embryo, also display limitless self-renewal activity. Self-renewing stem cells that are derived from the rodent embryos under different *in vitro* conditions display discernibly different molecular properties while remaining pluripotent. Embryos of certain murine strains and rodent species are less amenable for the derivation of ESCs, and non-rodent mammalian species (such as the lagomorphs and primates) require conditions that may be substantially different from those for the mouse for isolating and maintaining the pluripotent stem cells. The diverse cell states and *in vitro* conditions for the derivation and maintenance of the mammalian embryo-derived stem cells raise the questions of whether there are multiple states of pluripotency of the stem cells of each species, and if there are innate species-specific variations in the pluripotency state that underpin the ability to procure stem cells of comparable state of cell potency *in vitro*. In this review, we will address these questions by taking a snapshot of our knowledge of the properties of the pluripotent stem cells, focusing on the maintenance of pluripotency and inter-conversion of the different types of pluripotent stem cells from rodents, lagomorphs and primates.

2. Embryonic stem cell lines from the permissive mouse strain epitomize the naïve state of pluripotency

Mouse embryonic stem cell (ESC) lines were first derived from the ICM of 129 inbred mouse (Evans and Kaufman, 1981), a permissive strain which has a genetic background known for its propensity to develop testicular teratomas (tumors with cell types of all three germ layers). Two key cell culture supplements for the derivation are leukemia inhibitory factor (LIF) and foetal calf serum (FCS) (Smith et al., 1988) (Fig. 1A); the latter can be replaced by bone morphogenetic protein 4 (BMP4) (Ying et al., 2003). LIF, via gp130, Janus kinase (JAK) 2, and signal transducer and activator of transcription 3 (STAT3) (Niwa et al., 1998), fuels the activity of a complex network of epiblast transcription factors known as the extended pluripotency network. It consists of a core pluripotency factors: *Oct4*, *Sox2*, *Nanog* and other allied factors: *Klf2*, *Klf4*, *Tcfcp2l1*, *Esrrb*, *Gbx2*, and *Sall4*, that enable the cells to acquire robust pluripotency (Aksoy et al., 2014; Bourillot et al., 2009; Dunn et al., 2014; Hall et al., 2009; Martello et al., 2012; Martello et al., 2013; Martello and Smith, 2014; Niwa et al., 2009; Qiu et al., 2015; Tai and Ying, 2013; Yang et al., 2010a; Ye et al., 2013; Yeo et al., 2014; Yuri et al., 2009). We shall call this culture condition “Serum/LIF”. The pluripotency network can be further stabilized and the self-renewal activity reinforced by blocking the differentiation-inducing signaling activity mediated by the extracellular regulated kinase (ERK) (Burdon et al., 1999) and by enhancing the metabolic activity and WNT signaling activity by inhibiting glycogen synthase kinase 3 (GSK3) (Martello et al., 2012). For this purpose, two inhibitors are conventionally used, PD0325901 and CHIR99021, which define a new culture condition called “2i/LIF” (Ying et al., 2008). These 2i/LIF ESCs can thrive in the absence of ERK activity, in contrast to those maintained in LIF and serum conditions, and are reputed to have acquired an alternative state of naïve pluripotency.

A naïve state of pluripotency is functionally defined by the capacity of mouse ESCs to participate in germ layer differentiation and generate germline competent chimeras following their incorporation into host blastocysts (Nichols and Smith, 2009). The naïve cells seem to mimic,

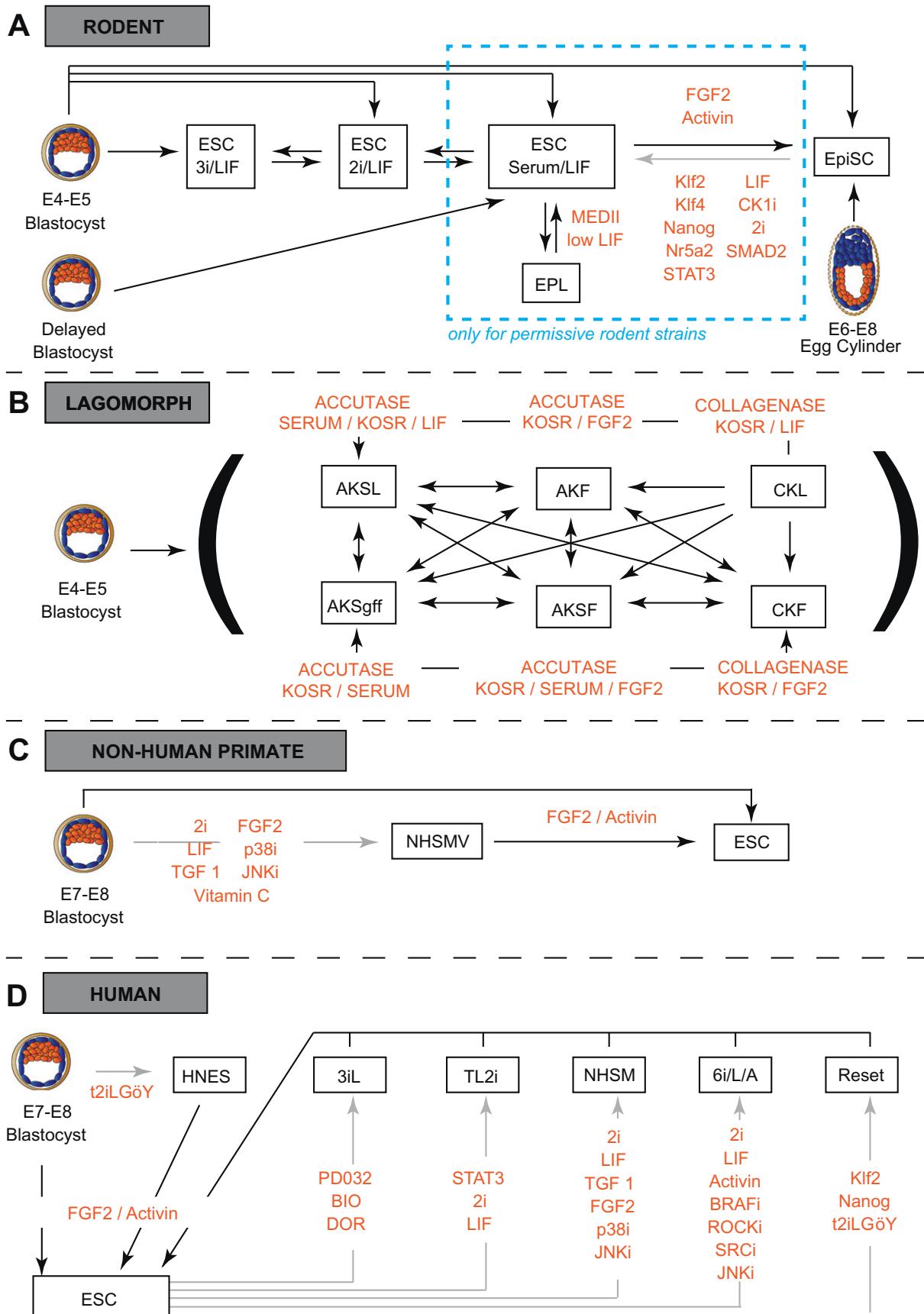
although do not completely match, the transcriptome of the pluripotent epiblast of the late blastocyst. Studies at both molecular and functional levels suggest that the epiblast of the E3.75 to E4.5 blastocyst is likely to be the founder tissue of ESCs (Boroviak et al., 2014; Brook and Gardner, 1997). The success in isolating ESCs from the epiblast of the permissive 129 mouse strain declines precipitously between E5 and E6 regardless of the culture conditions (LIF/Serum or 2i/LIF) used for derivation (Boroviak et al., 2014; Gardner and Brook, 1997). Gene knockout studies have demonstrated that none of the components of the LIF signaling pathway, *i.e.*, LIF receptor, signal transducer gp130, and STAT3, are required for pre-implantation embryo development (Li et al., 1995; Nakashima et al., 1999; Takeda et al., 1997; Ware et al., 1995; Yoshida et al., 1996). Activation of LIF signaling is, however, required for blastocyst survival during diapause (Nichols et al., 2001), a physiological process that momentarily prevents implantation to delay pregnancies in mice. LIF signaling prevents apoptosis in the epiblast until implantation takes place and the epiblast resumes cell division. In sharp contrast to the *in vivo* situation, LIF signaling stimulates the cell cycle of ESCs in LIF/serum culture (Coronado et al., 2013). Therefore, the activation of LIF signaling may be a cellular response to the drive to cell immortalization *in vitro*.

3. Modulation of pluripotency reveals a progressive poising for lineage differentiation

Several studies have reported the conversion of ESCs from the naïve state to other states of pluripotency (Fig. 1A). ESCs cultured in a medium conditioned by the human HepG2 hepatocarcinoma cells (known as MEDII) convert to a morphologically distinct population, the early primitive ectoderm-like (EPL) cells (Rathjen et al., 1999). EPL cells could differentiate into derivatives of the three germ layers *in vitro*, indicating that they may retain pluripotency. However, EPL cells have lost chimera-forming ability (Rathjen et al., 1999). The establishment of EPL cells is accompanied by changes in gene expression pattern such as the down-regulation of the early epiblast markers *Gbx2* and *Rex1* and the up-regulation of the late epiblast marker *Fgf5*, suggesting that the EPL cells resemble the epiblast population of the post-implantation mouse embryo (Pelton et al., 2002; Rathjen et al., 1999). We currently lack a complete transcriptome characterization to benchmark EPL cells against an epiblast from the implanting blastocyst stage to the gastrula stage. Moreover, whether EPL cells can be derived directly from the epiblast of a postimplantation embryo under the MEDII condition is presently not known.

ESCs can be converted into the cells that are similar to the epiblast stem cells (EpiSCs) that are derived directly from the epiblast of postimplantation embryo. This is accomplished by culturing ESCs as small colonies in a chemically defined culture medium supplemented with knockout serum replacement factors (KOSR), FGF2 and Activin A (Guo et al., 2009). The transition is accompanied by gain of FGF2/ERK and Activin A/Smad signaling dependency, down-regulation of early epiblast markers and up-regulation of late epiblast markers (Osteil et al., 2016a). Like the EPL cells, the converted cells retain the capacity to differentiate into derivatives of the three germ layers *in vitro* but lose chimeric competency, suggesting that ESC-derived EpiSCs may be developmentally similar to the epiblast population of the post-implantation mouse embryo.

EpiSCs can be derived directly from the epiblast of E6 to E8 post-implantation mouse embryos. These cells display a global gene expression profile similar to that of the epiblast of the post-implantation embryo, but distinct from that of the ESCs (Brons et al., 2007; Kojima et al., 2013; Tesar et al., 2007). The characteristic features of EpiSCs have led to the notion of a primed state of pluripotency, which is presumably closer to the commitment of lineage differentiation (Tesar, 2016). Atop transcriptomic reconfiguration, the shift from naïve to primed pluripotency is accompanied by genome-wide hyper-methylation, enhanced activity of DNA methylation, ATP-dependent chromatin



modification and nucleosome remodeling (Ficz et al., 2013; Habibi et al., 2013; Leitch et al., 2013), differential expression of miRNA clusters (Jouneau et al., 2012) and selective enhancer activity (Factor et al., 2012), and a transition from oxidative phosphorylation to glycolysis for energy production (Zhou et al., 2012).

2i/LIF ESCs, serum/LIF ESCs, EPL cells, and EpiSCs may represent different states of pluripotency, with 2i cells placed at the naïve end of the hierarchy and EpiSCs at the primed end. Depending on the *in vitro* conditions for derivation, epiblast cells of a late blastocyst will settle on different positions along a descending gradient of pluripotency (conceptualized as a series of terraces on the slope) (Fig. 2 – permissive rodents). 2i/LIF conditions appear to have captured the cells at the state of pluripotency of the epiblast of the E3.75 to E4.5 blastocyst, as revealed by the similarity in gene expression profiles (Kolodziejczyk et al., 2015). However, it is unclear without interrogating the molecular properties of individual cells, if there is an admixture of heterogeneous cell types in the 2i/LIF colonies. A case in point is the presence of a minor population of apparently “totipotent” stem cells with the propensity of extraembryonic cell lineages in the LIF/Serum and the 2i/LIF ESC colonies (Macfarlan et al., 2012; Morgani et al., 2013). Whether these “totipotent” cells are generated specifically in conditions that promote a naïve state of pluripotency is not known. LIF and serum might capture cells at another state of naïve pluripotency, which may correspond to the pluripotency of cells in the diapause (delayed) blastocyst (Fig. 1A). EPL cells are positioned further along the gradient (the slope) than the LIF/Serum ESCs. FGF2 and Activin arrest the cells at an even further stage of pluripotency corresponding to that of the epiblast of gastrula-stage embryos (Kojima et al., 2013). In essence, the progression from a naïve state to the primed state of pluripotency in the stem cells brings forth the commitment to, or the poising for, lineage differentiation.

Other pluripotent stem cells with intermediate phenotypes have been generated *in vitro*. Pluripotent stem cells with dual responsiveness to LIF/STAT3 and Activin A/Smads and a mixed gene expression pattern, known as intermediate epiblast stem cells (IESCs), are comparable to a state in between ESCs and EpiSCs (Chang and Li, 2013). Activin or FGF2 could maintain ESCs derived from the mouse inner cell mass cells (Ozawa et al., 2014). ESCs derived with FGF2 could contribute to germline chimeras, attesting their naïve-like characteristics. The ability to generate *bona fide* EpiSCs directly from the mouse blastocyst suggests that an intermediate 2i/LIF step for the transit through the naïve state may not be a prerequisite for acquiring primed pluripotency *in vitro* (Najm et al., 2011). Whether these atypical states of pluripotency reflect intermediate states that are native to the epiblast or are the results of response of the embryonic cells to the experimental conditions is not known.

4. Pluripotent stem cells from refractory/non-permissive mouse strains and rats

The 129-related mouse strains are amenable for the derivation of germline-competent ESC lines from E4 blastocysts using LIF and selected batches of serum or BMP4, while many other mouse strains are refractory to the generation of ESCs under this condition. Epiblast cells of refractory rodents differentiate rapidly and die when grown in Serum/LIF condition. ESCs can be derived, albeit at a low efficiency, for some of the refractory strains, such as C57BL/6, DBA/1lacJ, CBA/Ca, and

CD1, through the induction of implantation delay (that mimics embryonic diapause) to activate LIF signaling, and the isolation of an epiblast free of the primitive endoderm prior to *in vitro* culture in Serum/LIF on fibroblast feeders (Gardner and Brook, 1997; Brook and Gardner, 1997). Other mouse strains (C57BL/6 X CBA, CBA, MF1, and NOD) and several strains of rats are completely refractory to ESC derivation in Serum/LIF (or BMP4) conditions (Blair et al., 2011; Gardner and Brook, 1997) (Fig. 1B). The basis of the refractory nature of these mouse strains and the rats is not fully understood. However, the successful derivation of ESCs in 2i/LIF or 3i/LIF conditions (see below) suggests that autonomous FGF4-ERK signaling activity and/or strong activation of differentiation-promoting ERK signaling by LIF are probably the counterproductive factors. Using the serum-free 2i/LIF regime that eliminates most of the differentiation-induced signaling from ERK1/2, stable ESC lines can be generated with high efficiency from many strains of mouse, including the aforementioned refractory strains (Nichols et al., 2009; Ying et al., 2008). Germline-competent ESC lines have also been obtained from rat blastocysts (Buehr et al., 2008; Hirabayashi et al., 2010; Kawamata and Ochiya, 2010; Li et al., 2008). Rat ESCs have also been generated using the serum-free 3i condition (Buehr et al., 2008; Li et al., 2008), a condition based on the 2i/LIF medium supplemented additionally with a chemical inhibitor of the FGF4 receptor, SU5402. This synthetic medium was designed to block the inductive stimuli transduced by FGF/ERK signaling that drive cell differentiation. The 2i/LIF and 3i/LIF culture regime led to the notion of a ground state of pluripotency, a pristine state characterized by the self-renewing of rodent pluripotent stem cells in the absence of any known exogenous signaling activity (Ying et al., 2008) (Fig. 1B, Fig. 2 – non-permissive rodents). Provision of 2i/LIF or 3i/LIF condition, in particular the abolition of ERK activity, therefore enables capturing ESCs at the naïve pluripotency state for refractory rodent strains. However, culturing the epiblast cells from these strains in defined culture medium supplemented with KOSR, FGF2, and Activin A captures the stem cells at the primed state of pluripotency (Brons et al., 2007; Tesar et al., 2007). Cells from the refractory rodent species may be inherently permissive for the derivation of primed pluripotent stem cells.

5. Pluripotent stem cells from non-rodent mammals acquire a more downstream potency level

Successful derivation of preimplantation embryo-derived pluripotent stem cell lines was reported for rabbit [*Oryctolagus cuniculus*] (Honda et al., 2008; Osteil et al., 2013; Osteil et al., 2016b; Wang et al., 2006), dog (Hayes et al., 2008; Vaags et al., 2009), mink [*Neovison vison*] (Menzorov et al., 2015), hamster [*Mesocricetus auratus*] (Doetschman et al., 1988), marmoset [*Callithrix jacchus*] (Thomson et al., 1996), cynomolgus macaque [*Macaca fascicularis*] (Suemori et al., 2001), rhesus macaque [*Macaca mulatta*] (Mitalipov et al., 2006; Wianny et al., 2008), baboon [*Papio anubis*] (Simerly et al., 2009) and human (Thomson et al., 1998). Attempts have been made, with little success, to derive pluripotent stem cell lines from pre-implantation embryos of cow, sheep and pig (Notarianni et al., 1991; Sims and First, 1994). These cell lines exhibited poor self-renewal and/or failure to differentiate into cells characteristic of the three germ layers.

Pluripotent stem cell lines that are isolated from human blastocysts require only FGF2 and Activin A for maintenance of pluripotency (Levenstein et al., 2005; Ludwig et al., 2006; Vallier et al., 2005; Vallier

Fig. 1. The *in vitro* conditions for the isolation of pluripotent stem cells and the inter-conversion of cell types in (A) the rodent strains, (B) the lagomorphs (rabbit), (C) non-human primates (rhesus monkey) and (D) human species. Color of pointer lines reflects the degree of efficiency (black = more efficient) or technical complexity (gray = more complex and less efficient) in converting the cell types in the indicated direction. Abbreviations: 2i, MEK + GSK3 β inhibitors; 3i, MEK + GSK3 β + FGFR inhibitors; 6i/L/A, MEK + GSK3 β + BRAF + JNK + ROCK + SRC inhibitors + LIF + Activin; AKF, Accutase + KOSR + FGF2; AKSF, Accutase + KOSR + Serum + FGF2; AKSgf, Accutase + KOSR + Serum; AKSL, Accutase + KOSR + Serum + LIF; BIO, Bromoindirubin-3-oxime; BRAFI, B-Raf Proto-Oncogene inhibitor; DOR, Dorsomorphin; CKF, Collagenase + KOSR + FGF2; CKL, Collagenase + KOSR + LIF; EPL, Early Primitive ectoderm-like; EpiSC, Epiblast Stem Cell; ESC, Embryonic Stem Cell; FGF2, Fibroblast Growth Factor 2; GSK3 β , Glycogen Synthase Kinase 3 inhibitor; JNK1, JUN N-Terminal Kinase inhibitor; Klf2, Krüppel Like factor 2; Klf4, Krüppel Like factor 4; LIF, Leukemia Inhibitory Factor; MEDII, Medium conditioned by the human HepG2 hepatocarcinoma cells; MEKi, MAP2K1 inhibitor; NHSM, Naive Human Stem cell Medium; Nr5a2, Nuclear Receptor Subfamily 5, Group A, Member 2; p38i, p38 MAPK inhibitor; SRCi, Src family kinases inhibitor; STAT3, Signal Transducer and Activator of Transcription 3; t2iLG δ Y, 2i + G δ 6983 + Y-27632; TL2i, Tamoxifen + LIF + 2i.

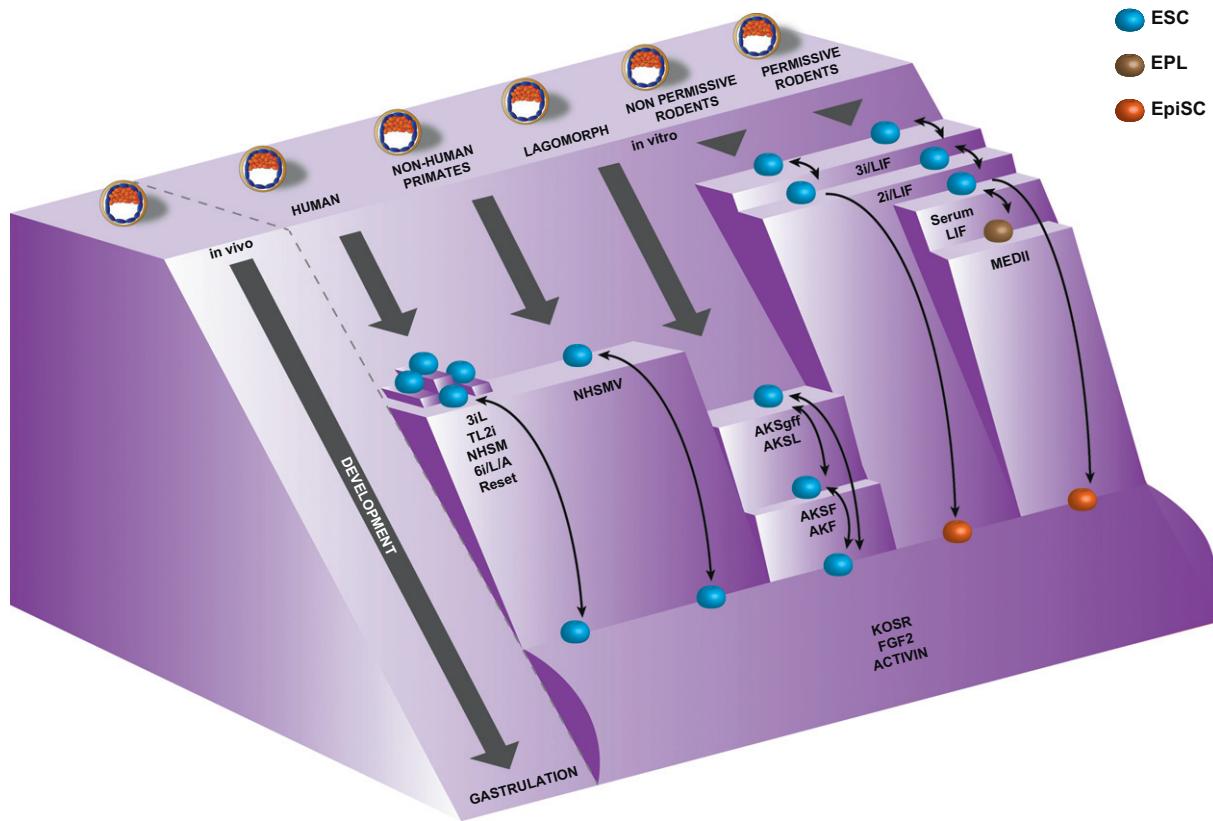


Fig. 2. The conceptual framework of changes in lineage potency during mouse embryogenesis (the slope with naïve pluripotency upstream to primed pluripotency downstream) and the attainment of pluripotency state (the terrace) in embryo-derived stem cells of humans, lagomorphs (rabbit), refractory/non-permissive rodents (rat and non-129 mouse strains) and permissive murine strains (129 strain). The slippery slope with no terrace is not conducive for the stem cells to acquire a stable pluripotency state. The gradient and height of the cliff reflect the stringent requisite for reprogramming the stem cells towards naïve pluripotency. Abbreviations: 2i, MEK + GSK3 β inhibitors; 3i, MEK + GSK3 β + FGFR inhibitors; 3i, 3i and LIF; 6i/L/A, MEK + GSK3 β + BRAF + JNK + ROCK + SRC inhibitors + LIF + Activin A; AKF, Accutase + KOSR + FGF2; AKSF, Accutase + KOSR + serum + FGF2; AKSgff, Accutase + KOSR + Serum, growth factor free; AKSL, Accutase + KOSR + Serum + LIF; CKF, Collagenase + KOSR + FGF2; CKL, Collagenase + KOSR + LIF; EPL, Early Primitive Ectoderm-Like; EpiSC, Epiblast Stem Cell; ESC, Embryonic Stem Cell; HNES, human Naïve Stem Cell; LIF, Leukemia Inhibitory Factor; MEDII, medium conditioned by human HepG2 hepatocarcinoma cells; t2iGöY, 2i + Gö6983 + Y-27632; TL2i, Tamoxifen + LIF + 2i.

et al., 2009; Xu et al., 2005) (Fig. 1C). They display molecular and functional features of rodent EpiSCs. In particular, they do not express many of the transcription factors in the naïve pluripotency network of mice, but express some early lineage markers instead. Moreover, in a recent study describing the transcriptome of pluripotent cells in the cynomolgus embryo from early blastocyst to gastrula, conventional human pluripotent stem cell lines exhibited a transcription profile highly similar to that of the late post-implantation epiblast (Nakamura et al., 2016). Similar to mouse EpiSCs (Huang et al., 2012), human pluripotent stem cells fail to colonize the mouse blastocyst (Masaki et al., 2015), whereas they can colonize the late epiblast and participate in germ layer differentiation in postimplantation chimeras (Mascetti and Pedersen, 2016). Although, pluripotent stem cell lines obtained in non-human primates (NHPs; i.e., marmoset, cynomolgus and rhesus macaque) are less well characterized, they also seem to display characteristics of mouse EpiSCs. In particular, when the transcriptome is benchmarked against an epiblast from the early blastocyst stage to the gastrula stage, cynomolgus pluripotent stem cells display strong similarity to the late post-implantation epiblast (Nakamura et al., 2016). However, they remain distinct from gastrulating cells whereas mouse EpiSCs express early differentiation markers of the anterior primitive streak indicating that these stem cells are primed for lineage differentiation (Kojima et al., 2013). All these features indicate that the primate pluripotent stem cells are self-renewing cells at a state of primed pluripotency.

The early embryos of lagomorphs share similar developmental properties with primate embryos (Fischer et al., 2012), including the timing of embryonic genome activation (Christians et al., 1994), DNA methylation (Reis Silva et al., 2011), and X chromosome inactivation (Okamoto

et al., 2011). In contrast to the egg-cylinder configuration of rodent pre-gastrula embryos, rabbit embryos like their human counterparts are organized as a flattened disk at the surface of the conceptus (Idkowiak et al., 2004). Similar to primate pluripotent stem cells, preimplantation embryo-derived rabbit pluripotent stem cells rely on FGF2 and Activin A/Nodal/TGF β but not on LIF signaling for the maintenance of pluripotency (Honda et al., 2009; Intawicha et al., 2009; Osteil et al., 2013; Osteil et al., 2016b; Wang et al., 2006; Wang et al., 2008), and they express transcription factors associated with primed pluripotency in rodents (Osteil et al., 2013; Schmaltz-Panneau et al., 2014). In addition, they do not colonize the epiblast after injection into rabbit pre-implantation embryos (Osteil et al., 2013). Thus, similar to primate pluripotent stem cells, rabbit pluripotent stem cells appear to be inherently primed. Remarkably, depending on the *in vitro* conditions for derivation, rabbit pluripotent stem cell lines exhibit variable transcriptomes. In particular, when cultured as single cells in FGF2-free medium, rabbit ICM cells give rise to stem cell lines with pluripotency gene expression profiles more closely aligned with those of the rabbit ICM/epiblast (Osteil et al., 2016b). Whereas conventional FGF2-dependent rabbit pluripotent stem cells exhibit DNA damage checkpoints in both the G1 and G2 phases of the cell cycle like differentiated cells, rabbit stem cells derived from single ICM cells lack the G1 checkpoint when derived in the absence of FGF2 (Osteil et al., 2016b), like the primed and naïve pluripotent stem cells of rodents and primates (Aladjem et al., 1998; Filipczyk et al., 2007; Fluckiger et al., 2006; Momcillovic et al., 2009). This latter finding indicates that the lack of DNA damage checkpoint in the G1 phase is not an absolute mark of pluripotency in cell culture systems. Moreover, this suggests that the

gain of the G1 checkpoint might take place in the post-implantation epiblast, *i.e.* before lineage commitment. Together, these findings indicate that stem cells derived from ICM/epiblast cells of the rabbit blastocyst can settle at different positions (terraces) along the gradient (slope) of pluripotency state depending on the *in vitro* conditions for derivation and culture (Fig 2). However, it is not clear where each of these rabbit pluripotent stem cells positions against an epiblast of the early blastocyst stage to gastrula stage. Current knowledge of the transcriptome of the rabbit epiblast is limited (Schmaltz-Panneau et al., 2014). A detailed characterization from morula stage to gastrula stage at the single-cell level is required for benchmarking against pluripotent stem cells.

All attempts to generate ESC lines in primates and rabbits using the Serum/LIF, 2i/LIF, or 3i/LIF culture regimes that work for the derivation of ESCs from permissive and refractory rodent strains have been unsuccessful. This finding suggests that, in primates and rabbits, the inhibition of MAPK activities is not sufficient to capture the stem cells at naïve pluripotency and block cell differentiation. Single-cell RNA-seq analysis has revealed that the pluripotency network in the human epiblast collapses shortly after the cells are placed in culture (Yan et al., 2013). It is likely that these cells transit rapidly to a state of FGF2-dependent inhibition of differentiation before being stabilized as self-renewing pluripotent stem cells. Capturing human embryo-derived stem cells at naïve pluripotency is a challenge. One first step to achieve this objective is the use of a t2iLGöY culture regime: serum-free 2i/LIF medium supplemented with the PKC inhibitor Gö6983, ascorbic acid and ROCK inhibitor, Y-27632 (Fig. 1D). The human naïve ES (HNES) cells so generated expressed naïve state-specific transcription factors and displayed features of mitochondrial respiration, global gene expression, and genome hypo-methylation that are distinct from those of conventional pluripotent stem cells. However, upon transferring the HNES cells to culture medium supplemented with FGF2 and KOSR, they readily acquire primed pluripotency (Guo et al., 2016). It may be noted that the single-cell transcriptome of HNES cells does not match that of human epiblast cells, suggesting that while the t2iLGöY culture regime blocks pluripotent stem cells from spontaneous transition from naïve to primed pluripotency, it has yet to replicate the pluripotency state of the human epiblast or the naïve mouse ESCs.

6. Conversion from primed to naïve pluripotency: moving upstream

The stability of the pluripotency state acquired by the embryo-derived stem cells of the rodents and primates has been tested by manipulating the transition between cell states. In the mouse, ESCs can be converted to EPL by culturing in MEDII medium, and EPL cells can be reverted efficiently to ESCs by withdrawal of MEDII (Rathjen et al., 1999). Such facile inter-conversion highlights a relatively close proximity of the two cell types. Other transitions turned out to be much less efficient. Unlike EPL cells, reversion of primed EpiSCs to naïve ESCs cannot be achieved by switching culture conditions between FGF2/Activin A to LIF/serum or 2i/LIF, as can be achieved in the opposite direction. Reversion of EpiSC to ESC occurs in <1 in 1×10^{-6} cells (Guo and Smith, 2010). The reversion can be enhanced by enforced expression of genes such as *Klf2*, *Klf4*, *Nanog*, *STAT3* or *Nr5a2* (Guo and Smith, 2010; Guo et al., 2009; Hall et al., 2009; Hanna et al., 2009; Silva et al., 2009; Yang et al., 2010b), or by inhibiting CK1, MEK and SMAD2 activity and enhancing WNT activity (Illich et al., 2016). The reverted ESCs acquired the characteristics of naïve pluripotency, including transcriptome and epigenome reconfiguration and chimeric competency. However, the efficiency of “enhanced” conversion still remains low at <0.1%–1% of the EpiSCs being re-programmed. Reversion can be achieved in approximately 10% of EpiSCs by the synergistic action of *Klf4* and *Nr5a2* that re-activates the naïve pluripotency network (Guo and Smith, 2010). Therefore, naïve ESCs can easily be driven downstream to EpiSCs (down a slippery slope), whereas the reversion (moving upstream) requires reprogramming of the extended pluripotency network and extensive reset of the epigenome. This raises the possibility that maintenance of naïve pluripotency demands more of the functional output from the

gene regulatory network (GRN) than that of the primed state. In a similar context, the extent of requirement of GRN function may underpin the highly strain/species dependent efficiency of derivation of naïve stem cells but generally not that of primed stem cells.

The situation in primates is complex. Several studies have reported the reversion of conventional human ESCs to naïve-like pluripotency (Fig. 1D), using different combinations of transcription factors (KLF2, NANOG, and STAT3), growth factors (LIF, Activin, and FGF2), and chemical inhibitors of various kinases (ROCK, BRAF, p38^{MAPK}, GSK3, MEK1/2, SRC, and JNK) (Fig. 1C). The reverted cells, known variously as NHSM (Gafni et al., 2013), 3iL (Chan et al., 2013), Reset (Takashima et al., 2014), 6i/L/A (Theunissen et al., 2014) and TL2i (Chen et al., 2015a), display some characteristic features of naïve state pluripotency of rodent stem cells, displaying reconfigured transcriptome and epigenome, alterations in mitochondrial respiration (Takashima et al., 2014), loss of FGF2 and ERK dependency (Chen et al., 2015a; Takashima et al., 2014), and gain of LIF/STAT3 dependency (Chan et al., 2013; Chen et al., 2015a; Gafni et al., 2013; Takashima et al., 2014). It is worthy of note that none of these studies explored the cell cycle of the reprogrammed cells to determine whether they have acquired all the characteristic features of the mouse ES cell cycle including the loss of retinoblastoma protein-dependent control of the G1/S transition (Savatier et al., 1996; Savatier and Malashicheva, 2004; Conklin et al., 2012). A comparison of the different protocols used to reprogram human ESCs and iPSCs to naïve-like pluripotency and the characteristics of the resulting cells have been discussed in several recent reviews (Huang et al., 2014; Manor et al., 2015; Nakamura et al., 2016; Theunissen et al., 2016; Weinberger et al., 2016). Since the reverted cell lines do not display uniform features, they appear to have settled at different positions along the pluripotency gradient. However, the variability may also be related to the genetic heterogeneity of the cell lines. The efficiency of reversion from primed to naïve-like pluripotency was not reported, but it seemed relatively low as the reverting cells were often described as isolated primary colonies. Moreover, reversion induced by cocktails of growth factors and small molecules (without eliciting any transgene activity), is accompanied by extensive cell death and the reverted cells have to be identified by selective marker expression (Theunissen et al., 2014). This corroborates that the occurrence of transgene-free conversion is extremely rare in rodent stem cells. Reversion of cynomolgus macaque ESCs to naïve-like pluripotency using the NHSM reprogramming protocol has been reported (Chen et al., 2015b). When introduced into host embryos, these reverted cells seemed capable of colonizing the epiblast and differentiate into cells characteristic of the three germ layers in the fetus. However, the frequency of chimerism was low, possibly reflecting that very few cells have truly acquired a naïve state (despite the expression of the appropriate core pluripotency factors) and their weak competitive edge against the host cells in contributing to tissue differentiation.

The barrier for primed-to-naïve state reprogramming may be conceptualized as that reversion is akin to moving upstream on a steep cliff to a higher terrace (Fig. 2). Those that made it appear to have reconfigured transcriptome and epigenome, reinstalled oxidative phosphorylation for energy production, and reset a rapid cell cycle. Whether they represent a rare cell type naturally existing in an otherwise primed cell population or emerging stochastically is not known. In primates and non-rodent mammals, the situation is more complex as reverted cells seem to exist in various naïve-like states (*i.e.*, at different positions on the slope) when they are benchmarked against the mESCs and EpiSCs. The natural tendency to acquire a primed pluripotency state by the non-rodent stem cells would reflect the inability to attain a naïve state by gaining a foothold upstream on a slippery slope (Fig. 2).

7. Conclusions

The rodent pluripotent stem cells can be broadly attributed to the naïve and the primed state of pluripotency, while there may be other intermediate states in between. The transition in either direction between

the naïve and primed states suggests that the demand on the activity of the gene regulatory network maybe more stringent for maintaining the naïve state than the primed state, which may be reflected by the lower efficiency of the “upstream” conversion of the pluripotency state. Embryos from permissive and refractory mouse strains differ radically in their propensity to attain the upstream naïve pluripotency. In the permissive mouse strain, LIF activity is sufficient to prevent the exit of pluripotency. In the refractory mouse strains and rats, MAPK activity appears to drive the stem cells downstream to exit pluripotency, which could be impeded by inhibiting MAPK activity. In primates, inhibition of both MAPK and PKC activities are required to block the stem cells from exiting pluripotency, but is not sufficient to capture a naïve-like state of pluripotency.

Capturing the *bona fide* naïve state of pluripotency of primate embryonic cells in the context of ESCs is a major challenge. One reason for the difficulty to achieve this goal is the scarcity of human and nonhuman primate embryos for benchmarking the states of pluripotency *in vivo*. As a result, little is known of the innate pluripotency state of the primate epiblast cells, be it inherently naïve or primed. Besides that, rabbit embryos are more readily available for experimentation, and the early embryos of lagomorphs share similarities with primate embryos in their developmental properties (Fischer et al., 2012). Moreover, the pluripotent stem cells isolated from rabbit preimplantation embryos display the characteristics of primed pluripotency (Osteil et al., 2013). As a surrogate model, the rabbit may be useful for exploring the cellular functions and mechanisms of acquisition and maintenance of pluripotency in the embryonic cells and the embryo-derived stem cells for other non-rodent mammals including the primates.

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