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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 naive 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*, *Tfcp2l1*, *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 naive pluripotency.

A naive 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 naive 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 naive to primed pluripotency is accompanied by genome-wide hyper-methylation, enhanced activity of DNA methylation, ATP-dependent chromatin