Chapter 2
Pluripotent Cells

2.1 Different Pluripotent Cells

The defining properties of ESCs are the ability to proliferate indefinitely without commitment to any cell lineages (self-renewal) and the capacity to differentiate into cell lineages from three germ layers (pluripotency) (Evans and Kaufman 1981; Thomson et al. 1998). ESCs were the first pluripotent cells isolated from normal embryos derived from the inner cell mass (ICM) of preimplantation embryos (Evans and Kaufman 1981). Mouse ESCs (mESCs) contribute cells to the three germ layers and to the germline of chimeric animals when injected into mouse blastocysts. However, there are distinguishing molecular and biological characteristics between ESCs and their in vivo counterparts of the ICM. Cells of the ICM do not self-renew, and they have globally hypomethylated genome (Santos et al. 2002), whereas, ESCs have unlimited proliferation potential and they have characteristically highly methylated genome (Meissner et al. 2008).

Although the first mESC lines were derived 25 years ago using feeder-layer-based blastocyst cultures, subsequent efforts to extend the approach to other mammals have been relatively unsuccessful. Human ESCs (hESCs) could only be isolated in 1998 (Thomson et al. 1998). Mouse and human embryonic stem cells share similar features such as their ICM origin and pluripotency. On the other hand, they do have differences. The differences are related to their morphology, marker expression, transcription factor binding activities and growth factor requirements in culture conditions. mESCs depend on leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP), whereas hESCs rely on activin and fibroblast growth factor (FGF) (Thomson et al. 1998).

In 2007, two independent groups showed that pluripotent cells could also be derived from the epiblast of the implanted embryo (Brons et al. 2007; Tesar et al. 2007). Mouse epiblast stem cells (EpiSCs) are derived from the post-implantation epiblast of day 5.5 embryos in the presence of bFGF and activin (Tesar et al. 2007). Although EpiSCs are able to differentiate in vitro into the teratomas including tissues belonging to the three embryonic germ layers, they do not contribute to chimeras.

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Table 1 Different types of pluripotent cells and antagonistic actions of same signaling pathways on different states of pluripotency

	hESC	EpiSC	mESC
Pluripotency state	ICM-like	Post-implantation epiblast-like	ICM-like
Maturity state	Primed	Primed	Naïve
Morphology	Flattened monolayer	Flattened monolayer	Dome-shaped
Culture conditions	Activin A/bFGF	Activin A/bFGF	LIF/BMP4 2i
Pluripotency confirmation status	Teratoma formation	Teratoma formation	Tetraploid complementation Germline contribution
X chromosome inactivation	XaXi	XaXi	XaXa
BMP signaling	Induces differentiation	Induces differentiation	(+LIF) Stabilizes
TGF-β & FGF2	Support renewal	Support renewal	Induces differentiation
ERK1/2 pathway signaling	Requires	Requires	Self-renewal is enhanced by inhibition

Interestingly, hESCs share defining features with EpiSCs, yet are derived from preimplantation human embryos (Nichols and Smith 2009) (Table 1).

Pluripotent cell lines have also been derived from other embryonic and adult tissues. Embryonic germ cells (EGCs) have been derived from the primordial germ cells (PGCs) of the midgestation embryo (Matsui et al. 1992) and adult germline stem cells (male germ cells and spermatogonial stem cells) (Surani 1999) have been generated from explanted neonatal (Kanatsu-Shinohara et al. 2004) and adult (Guan et al. 2006) mouse testicular cells.

Moreover, in 2008 Chou et al. reported distinct pluripotent cells derived from blastocyst, which were defined by FGF2, activin and BIO. The authors called those cells FAB-SCs and showed that they share EpiSCs markers. However, FAB-SCs were unable to differentiate unless exposed to LIF/BMP4 (Chou et al. 2008).

With the discovery of EpiSCs, there is an emerging concept that different pluripotent states could exist, and knowledge of both transcriptional networks and signaling pathways has been vital for the precise description and dissection of the pluripotent state.

2.2 Transcriptional Networks and Signaling Pathways of Pluripotency

Different pluripotent cell types can be characterized and classified by their different growth requirements, developmental properties and pluripotency states (Table 1):

1. ICM-like pluripotent state: ESCs derived from ICM, embryonic germ cells and male germ cells or spermatogonial stem cells.
2. The post-implantation epiblast-like state: EpiSCs

These two states depend on signaling pathways that often antagonize each other (Hanna et al. 2010b). On the other hand, Nichols and Smith designated pluripotent cells as na and primed according to their maturity state of pluripotency (Nichols and Smith 2009). Isolated from the ICM of preimplantation blastocyts in the presence of LIF and BMP, mESCs fulfill all criteria of pluripotency. Therefore, they have been accepted as they are in "na" pluripotent state. On the other hand, EpiSCs are referred to as "primed" pluripotent cells because they exhibit only some pluripotency criteria.

Apart from the contribution to blastocyst chimeras, there are more differences between na and primed pluripotent states (Table 1): While na pluripotent cells show low susceptibility for primordial germ specification and high single-cell clonogenicity, primed cells display the reverse. While mESC colonies display compact dome-shaped morphologies, both hESCs and EpiSCs grow as a flat monolayer and the positive regulators of both the states are WNT and IGF. Na pluripotent cells require LIF/Stat3 and BMP4 signaling, whereas primed pluripotent cells need TGF-β, activin, FGF2, ERK1/2 signals. Interestingly enough, the two signals antagonize each other: BMP4 in EpiSCs and TGF-β, activin, FGF2, ERK1/2 in na cells induce differentiation. Na pluripotent cells generally do not express lineage specification markers (FGF5, Blimp1, Cer 1). However, these markers are positive in primed state cells with heterogeneous expression pattern. Moreover, primed pluripotent cells express MHC class I antigen, while na state cells do not. Na ESCs carry two active X chromosomes (XaXa) in female cells; in contrast primed EpiSCs have already undergone X chromosome inactivation (Nagy et al. 1990; Thomson et al. 1998; Ying et al. 2003; James et al. 2005; Brons et al. 2007; Tesar et al. 2007; Ying et al. 2008).

These studies proved that extrinsic stimuli are dispensable for the derivation, propagation and pluripotency of ESCs. They also showed that the cells have an innate program for self-replication. Ying et al. demonstrated that LIF and BMP could be dispensed with inducing inhibitors of particular signaling pathways. LIF and small molecule inhibitors of two protein kinases, ERK 1/2 and GSK3β (termed "2i") can replace serum by stimulating the WNT pathway. Therefore, 2i allows the maintenance of ESCs in fully defined medium without embryonic feeder cells. The results have been interpreted as the indication that the pluripotent and self-renewing state of ESCs is a "ground state", that is, a natural default state that need not be actively maintained (Ying et al. 2008).

Taken together, hESCs, EpiSCs and mESCs manifest themselves as potentially distinct cell types and hESCs may be most closely related to the post-implantation human epiblast (Wilmut et al. 2011). The generation of na hESCs will allow creating new opportunities for patient-specific researches. On the other hand, Guo et al. (2009) examined interconversion between mESCs and EpiSCs. They showed that when mESCs exposed to bFGF they could readily become EpiSCs. However,

EpiSCs do not change into ESCs with defined culture environment. Only forced expression of Klf4 in the presence of LIF and BMP could promote the conversion of EpiSCs into ESC-like cells (Guo et al. 2009). Another group, Bao and colleagues (2009) showed the reprogramming of advanced epiblast cells from embryonic day 5.5–7.5 mouse embryos to ESC-like cells in response to LIF-STAT3 signaling. The authors also reported that those reprogrammed ESCs could contribute to somatic tissues and germ cells in chimaeras unlike EpiSCs (Bao et al. 2009). Taken together, it is obvious that modulation of signaling by environmental changes are sufficient to interconvert these closely related cell types indicating that extrinsic growth factors could be dispensable for sustaining the pluripotent state (Ying et al. 2008). Moreover, Hanna et al. (2010a) converted ESCs into a more immature state with an active X-chromosome (XaXa) by ectopic induction of Oct4, Klf4 and Klf2, combined with LIF, GSK3β inhibitor and MEK inhibitor (Hanna et al. 2010a). These converted hESCs have similar growth properties, gene expression profiles and signaling pathway dependence with mESCs. Intriguingly enough, the recent establishment of preimplantation-derived EpiSCs cultured in human ESCs culture conditions supports this idea (Najm et al. 2011). It was also shown that hESCs with two active X chromosomes (XaXa) could be generated under hypoxic (5% oxygen) conditions (Lengner et al. 2010).

While the na and primed pluripotent states are inconvertible into each other and can be stabilized by appropriate culture conditions, these states have not been observed to coexist stably in the same culture conditions in both mouse and humans. However, it has been proposed that specific extrinsic and intrinsic factors can induce transitions between the states. Although the na state captured by Hanna et al. could be maintained only for limited passages, this study giving clear ideas about growth conditions should be improved in order to resume na ground state in genetically unmodified human cells (Hanna et al. 2010b).

Considerable research effort has been exerted to dissect the molecular functions of core pluripotency factors in the maintenance of pluripotency and establishment of a molecular association among pluripotent cell types. As already mentioned, since there has been important progress in dissecting culture-mediated signaling pathways; now the time is ripe to understand how signaling can be integrated to the transcriptional network.

2.2.1 Transcriptional Network of Pluripotency

ESCs have been investigated by very large-scale genomics and protein-DNA interaction studies to mechanistic studies of individual transcription factors. In addition to signaling requirements, particular transcription factors play major roles in establishing and maintaining pluripotency.

The most critical transcription factors of pluripotent state in ESCs are Oct4, Sox2 and Nanog and they are called the core transcription factors (Silva and Smith 2008). Transcription factors recognize specific DNA sequences and either activate

or prevent transcription. They bind both to promoter-proximal DNA elements and to more distal regions (Young 2011).

Initial specification of pluripotent cells in vivo requires Oct4 expression (Nichols et al. 1998). While losing Oct4 expression leads to trophoectoderm differentiation, higher levels induce differentiation to mesoderm and endoderm (Niwa et al. 2000). Oct4 functions by forming heterodimer with Sox2 in ESCs. Sox2 binds to DNA sequences adjacent to the Oct4 binding sites (Avilion et al. 2003; Chambers and Tomlinson 2009). Sox2 is required for epiblast maintenance. Nanog, on the other hand, promotes a stable undifferentiated ESC state and it is needed for pluripotency to develop in ICM cells (Silva and Smith 2008). ESCs deficient in Nanog genes are more prone to differentiate but do not lose pluripotency per se. Nanog is essential for pluripotent cell specification during normal development and induction of pluripotency to finalize somatic cell reprogramming during induction of pluripotency (Theunissen and Silva 2011). Theunissen and Silva proposed that Nanog acts as a molecular switch to turn on the na pluripotent program in mammalian cells (Theunissen and Silva 2011).

Young has also suggested recently that there are two dominated concepts for our understanding of the core transcription factors in control of ESC state (Young 2011):

(1) The core transcription factors function together to positively regulate their own promoters, forming an interconnected autoregulatory loop.
(2) The core factors co-occupy and activate expression of genes necessary to maintain ESC state, while contributing to repression of genes encoding lineage-specific transcription factors whose absence helps prevent exit from the pluripotent state.

This interconnected autoregulatory loop could generate a bistable state for ESC: when the factors are expressed at appropriate levels positive-feedback-controlled gene expression program takes action. Alternatively, when any one of the core transcription factors is unavailable, functionally differentiation program is activated (Young 2011).

In recent years, there have been considerable efforts to understand pluripotency in a genome-wide manner as well as on a systems level to provide a global understanding of the ground state of ESC state and differentiation. Thus, the discovery of reprogramming of somatic mammalian cells into pluripotent state by overexpression of only four transcription factors had a tremendous effect in understanding the basic cell biology.

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