Cell Stem Cell
Review

Capturing Totipotent Stem Cells

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Minority subpopulations within embryonic stem cell cultures display an expanded developmental potential similar to that of early embryo blastomeres or the early inner cell mass. The ability to isolate and culture totipotent cells capable of giving rise to the entire conceptus would enhance our capacity to study early embryo development, and might enable more efficient generation of chimeric animals for research and organ production for transplantation. Here we review the biological and molecular characterization of cultured cells with developmental potential similar to totipotent blastomeres, and assess recent progress toward the capture and stabilization of the totipotent state *in vitro*.

Introduction

The revolutionary advances in our ability to propagate human pluripotent stem cells (hPSCs) *in vitro* and differentiate them into any type of body tissue have been built on a conceptual framework established by decades of experimental embryology in model systems. Techniques for the directed differentiation of PSCs rely heavily on embryological roadmaps that help us to identify cellular intermediates along developmental pathways and to define the right cues to channel cells toward desired endpoints. Moreover, the rapid growth in the use of hPSCs as a model system for the study of human embryogenesis and its disorders is predicated on a firm understanding of the developmental status of the cells themselves. In many respects, the embryological paradigm holds the key to the successful manipulation and application of hPSCs in research and medicine.

In recent years, there has been great progress in establishing the relationship between PSCs in culture and their counterparts in the embryo (Figure 1). The first normal PSCs to be cultured *in vitro* were mouse embryonic stem cells (mESCs), which, though they are most commonly derived from the inner cell mass, are now known to resemble most nearly the pre-implantation epiblast and are referred to as naive or ground state cells (Nichols and Smith, 2012). Later, using different culture methodology, several groups managed to establish epiblast stem cell (EpiSC) lines from post-implantation mouse embryos (Brons et al., 2007; Tesar et al., 2007). These EpiSC lines resemble cells of the anterior primitive streak, at the last stage at which PSCs persist in the mouse embryo (Kojima et al., 2014). Similar to their embryonic counterparts, EpiSCs are poised for lineage specification and co-express pluripotency- and lineage-specific genes (this latter feature is called lineage priming, and EpiSCs are also referred to as a primed state). A third, transient state of pluripotency, corresponding to the early post-implantation epiblast, has been termed formative pluripotency, and it represents a state between naive mESCs and EpiSCs that has become fully competent to respond to signals that will specify formation of the three embryonic germ layers (Smith, 2017). Current studies are focusing on how naive ESCs transition to formative pluripotency and thus acquire competency for lineage specification. This work, conducted primarily in the mouse, is informing efforts to understand how human cells fit into a developmental paradigm. During the past several years, advances in molecular embryology in the human and non-human primate have provided important new benchmarks for this work (Blakeley et al., 2015; Nakamura et al., 2016; Petropoulos et al., 2016; Yan et al., 2013).

Beginning around 2010, several laboratories first reported the characterization of subpopulations of cells in mESC cultures that had some properties of pre-epiblast embryo cells, including some that resembled blastomeres of the two-cell (2C) stage of development, the stage at which the zygotic genome first becomes active in the mouse. The potential to capture cells with the capacity to form all the tissues of the conceptus would open up the possibility to study very early developmental events critical to mammalian embryogenesis, such as zygotic genome activation, rewiring of the epigenome, and the establishment of the trophoblast and primitive endoderm (PrE) lineages, under controlled conditions in cell culture systems. Moreover, it is possible that stem cells with this very early developmental potential would be more effective in forming chimeras. Human-animal chimeras formed from hPSCs could have potential important medical applications because animals whose organs have substantial human cellular content could be used for disease modeling, drug development, and transplantation (review, Wu et al., 2016a). Thus far, hPSCs have shown only minor contributions to interspecies chimeras, a limitation often attributed to their resemblance to post-implantation embryonic cells. Therefore, there has been considerable recent excitement about the possibility of capturing stem cells with expanded developmental potential.

Here we survey studies characterizing stem cells *in vitro* that represent stages of mammalian embryogenesis with greater developmental potential than naive mESCs or EpiSCs (Table S1). We consider the embryological background to this work, including molecular features of the totipotent state *in vivo*, the progress to date in isolating and characterizing these cells, and the criteria that can be applied to assess their developmental status.

Back to the Beginning: The Totipotent State *In Vivo*

Biological Basis of Totipotency

In organisms that deposit free-standing eggs into the environment, development of the embryo proper generally begins with the first cleavage divisions. Spatial patterning and lineage specification start early after fertilization and rely substantially

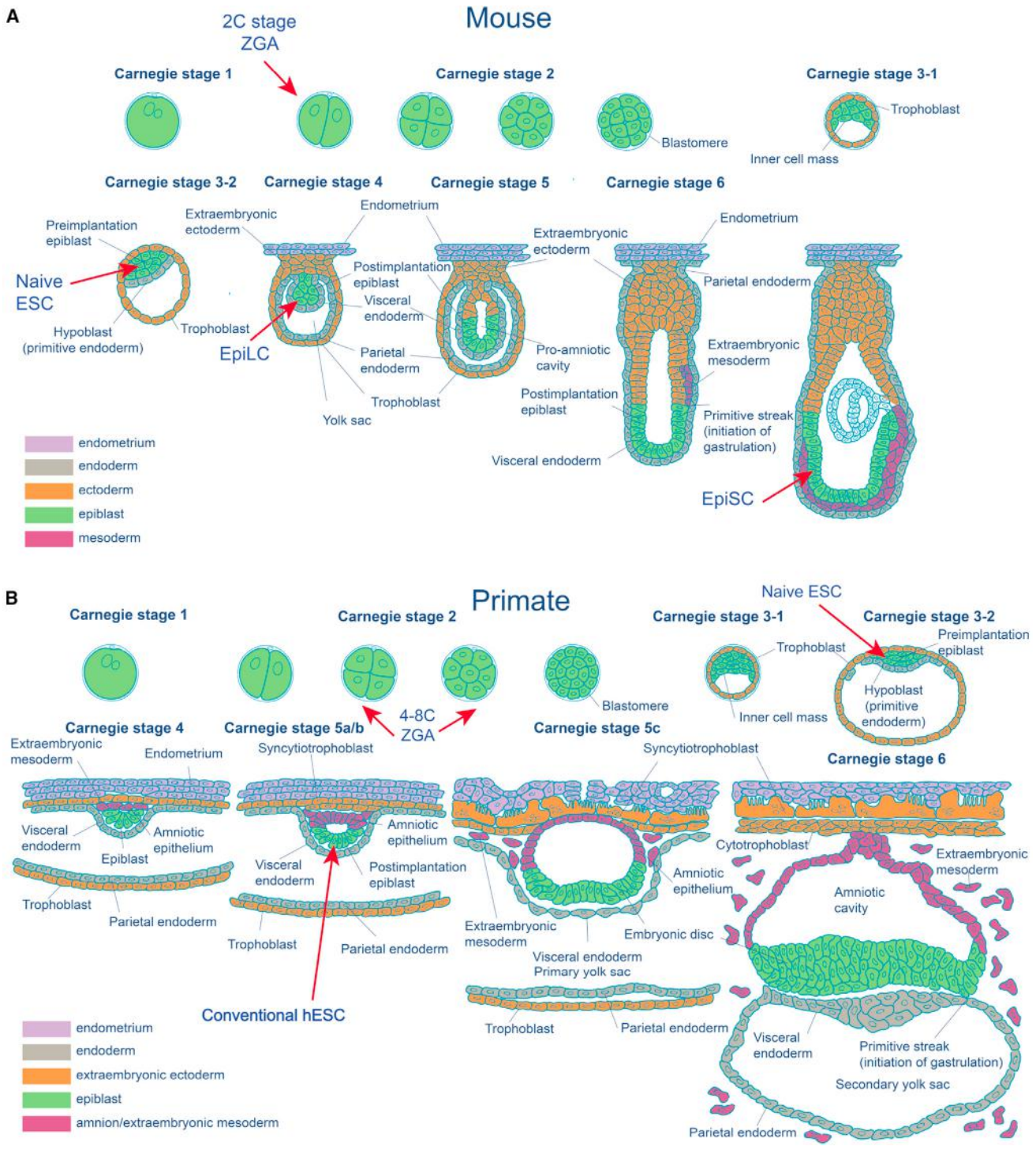

Figure 1. Schematic Illustration of Early Mouse and Primate Embryonic Development

Overview of mouse (A) and primate (B) development. The zygotic genome is activated at the 2C stage in the mouse and the four- to eight-cell stage in the human. Totipotency, measured by the ability of a single isolated cell to give rise to live born offspring, persists only to the 2C stage in the mouse, but single blastomeres from up to the 16-cell stage of development can give rise to live young in aggregation chimera assays. Naive mESCs are most similar to the pre-implantation epiblast stage, as are human naive ESCs. Mouse EpiSCs correspond to the epiblast at the late primitive streak stage of development. Conventional hESCs resemble the post-implantation epiblast, but at an earlier stage than mouse EpiSCs. A transient mouse pluripotent cell type not yet stabilized *in vitro*, the EpiLCs, represents a counterpart of the early post-implantation stage of development, referred to as the formative state of pluripotency. This figure is adapted from Boroviak and Nichols (2017).

on maternally derived factors in the egg to establish the body plan. By contrast, the development of the embryo proper in mammals does not begin until many cell divisions after fertilization. As a consequence of adaptation to development in utero, the mammalian egg contains little in the way of nutrient stores and does not communicate directly with the external environment. This means that mammals must rapidly establish the functional interface between the embryo and the mother that facilitates uptake and processing of nutrients and exchange of fluids and gases (review, Frankenberg et al., 2016). To achieve this, the early stages of mammalian development are devoted to the specification of two critical extraembryonic membranes, trophectoderm (TE) and PrE, differentiated tissues that are derived from the conceptus, play supporting roles in development, and are discarded at or before birth. Since the progenitor cells of these two extraembryonic tissues are committed to form one lineage only, there must be a mechanism to set aside a group of cells that retains the capacity to form all the tissues of the embryo proper, the pluripotent cell population. Once established, the pluripotent cell population must undergo expansion to a sufficient critical size to form the embryo proper, and it must have a capacity to respond to naturally occurring cell loss, or to physiological stimuli that modulate the developmental clock, such as diapause. The existence of a regulative state of pluripotency throughout early development can be considered an innovation of mammalian evolution (Cañon et al., 2011; O'Farrell, 2015).

Pluripotent cells arise around the time of the formation of the inner cell mass, when the TE lineage is established. Before this point, the cells of the mammalian embryo are said to be totipotent. However, it is important to recognize that there are two widely used definitions of totipotency. The most stringent definition states that a totipotent cell is a single cell that can give rise to a new organism given appropriate maternal support. A less stringent definition is that a totipotent cell is one that can give rise to all the extraembryonic tissues plus all the tissues of the body and the germline. In assessing recent studies that describe totipotent cells, it is instructive to recall how totipotency was originally defined experimentally. The original (and most stringent) test of totipotency is performed by isolating a single blastomere, placing it into an empty zona pellucida to ensure its survival in utero, returning the construct to the oviduct, and monitoring its further development to live born young (Tarkowski, 1959). By this experimental criterion, totipotency extends only to the 2C stage in the mouse, or the four- or eight-cell stage in sheep, cattle, and monkey (review, Suwińska, 2012). However, the failure of later stage blastomeres to support chimera development in this assay can be viewed as a consequence not of restricted developmental potential, but of the limited size of the inner cell mass of the reconstructed embryo, as postulated originally by Rossant (1976). Thus, in the mouse, failure of chimeras formed from later stage blastomeres to develop can be overcome by combining marked individual cells from later developmental stages with carrier (tetraploid) blastomeres. This rescue might be attributed to the tetraploid carrier cell contribution to the trophoblast, which would enable the grafted blastomere to contribute chiefly to a larger inner cell mass. In this fashion, individual cells from as late as the 16-cell stage were shown to give rise to all tissues in chimeras, including epiblast, PrE, and TE (Tarkowski et al., 2010). Thus, in assessing developmental capacity in chimera assays, it is essential to note that factors that limit incorporation, survival, and growth of individual cells and their descendants can influence the outcome of experiments and obscure developmental potential (or cellular plasticity). Recent studies have shown that overexpression of anti-apoptotic genes in grafted EpiSCs or hESCs can overcome stage- and species-specific barriers to chimera formation (Masaki et al., 2016; Wang et al., 2017).

Molecular Features of Totipotent Cells in the Embryo

What molecular features define the totipotent state? One of the hallmarks of early preimplantation embryos, for both mouse and human, is global epigenetic reprogramming. A characteristic example of this reprograming is the transient DNA demethylation that occurs during the early cell divisions through establishment of the inner cell mass. Loss of DNA methylation is observed rapidly after fertilization, with most CpGs becoming hypomethylated by the two- to eight-cell stage in human embryos when they are still in a totipotent state (Guo et al., 2014; Smith et al., 2014). DNA methylation remains low in the inner cell mass before increasing to normal somatic levels post-implantation. Interestingly, repetitive elements show differences in level of methylation, with evolutionarily older repeats becoming more hypomethylated compared to new repetitive elements (Guo et al., 2014). This dynamic epigenetic reprogramming is accompanied by specific changes in transcriptional programs as the cells develop from the zygotic stage through morula (Xue et al., 2013), and transient expression from some families of repetitive elements, the dynamics of which are highly dependent on type of repetitive element. While preimplantation development occurred normally in mouse cells lacking DNA methyltransferases, cells with loss of DNA methylation were unable to contribute to embryonic tissue, whereas DNA methylation is dispensable for development of extraembryonic tissue (Sakaue et al., 2010).

Two key features of 2C cells described to date include the activity of components derived from transposable elements and an open state of chromatin. The mammalian genome has been shaped and populated by a myriad of self-replicating transposable elements. One specific subclass of ancient retroviral elements is that flanked by long terminal repeats (LTRs), known as endogenous retroviral elements (ERVs) (reviewed in Chuong et al., 2017; Gerdes et al., 2016; Gifford et al., 2013). ERVs are specifically upregulated during zygotic genome activation through transcription driven from functional enhancers within the LTR. ERVs are maximally activated around the 2C stage in mouse and between the four- to eight-cell stage in humans. This early developmental time period of ERV activity coincides with the stage at which cells are still totipotent and undergoing major epigenetic reprogramming. Generally, ERVs are broadly silenced subsequently in most cell lineages through epigenetic mechanisms that can include both DNA and histone methylation. It is still an open question whether expression of ERVs during zygotic genome activation is specifically upregulated, or whether it is a byproduct of the inactivity of the pathways that act to silence these elements later in development.

The most common form of ERV sequence in mammalian genomes is a solitary LTR. These *cis*-regulatory sequences are left behind when ERVs become mobilized, and retain their

biochemical enhancer activity, subsequently becoming incorporated into local gene regulatory networks. Indeed, the permissive expression of ERVs during early development may be in part tolerated as a consequence of the co-option of transcription factor binding sites found in the LTR into the regulatory elements of the pluripotent network. In fact, up to 25% of *Pou5f1* and *Nanog* target sites in mESCs and hESCs are found in transposable elements; the highest percentage is ERVs (Kunarso et al., 2010), although only about 5% of targets are orthologous between species.

Evolutionarily distinct ERVs share the similar, restricted expression pattern in early embryo development in both mouse and humans, suggesting that the chromatin environment during this developmental window is permissive for ERV activity independent of the origin of the transposable element (Chuong et al., 2017).

Chromatin mobility is higher in the 2C-stage embryo, when cells are still totipotent, versus a few divisions later in the eight-cell stage (Bošković et al., 2014). This increased histone mobility may be independent of global histone modifications, either activating or repressive marks, as there is no change in mobility with inhibition of histone acetylation or histone H3 lysine 9 methylation. While in general the overall histone mobility is decreased during development, pluripotent cells found in the inner cell mass retain higher mobility relative to differentiated TE cells.

Deciphering Developmental Potential in mESC Cultures

Subsets of Cells Resembling Inner Cell Mass, Not Epiblast, in ESC Cultures

It is widely held that naive mESCs cultured under conditions that suppress differentiation (2i plus LIF [leukemia inhibitory factor]) correspond to the pre-implantation epiblast (Boroviak et al., 2014). The epiblast emerges from the inner cell mass after the TE and PrE lineages have been specified, and on this basis it is not surprising that naive ESCs do not contribute to these extraembryonic lineages in chimeras. However, it has long been known that mESCs are capable of differentiating into extraembryonic endoderm, at least *in vitro* (Bradley and Robertson, 1986; Doetschman et al., 1985). A number of reports over the past decade have highlighted the phenomenon of heterogeneity in mESC cultures (Chambers et al., 2007; Dietrich and Hiiragi, 2007; Hayashi et al., 2008; Singh et al., 2007; Toyooka et al., 2008). Further investigation led to the prospective isolation and characterization of minority populations of cells with broader developmental potential from mESC cultures, through the use of reporter cell lines that identify either cells expressing markers of extraembryonic lineages or retroviral regulatory elements that are activated in the early stages of mouse development.

An early exploration of PrE differentiation showed that extraembryonic endoderm progenitors exist in mESC cultures in equilibrium with cells that are pluripotent but lack the capacity to form yolk sac precursors. Canham et al. (2010), using a *Hex* reporter cell line, identified precursors of extraembryonic endoderm in mESC cultures that co-expressed some canonical markers of pluripotency and PrE, and interconverted with Nanog expressing, reporter-negative PSCs. The reporter-positive cells were able to contribute to both visceral and parietal endoderm in aggregation chimeras, and were able to give rise to an outer layer in embryoid bodies (EBs) expressing PrE markers. The PrE progenitors did not, however, contribute to the embryo proper, unlike the reporter-negative fraction, despite the fact that the two populations could interconvert *in vitro*, as shown by reconstitution of mixed cultures following isolation and propagation of single cells representing either subpopulation.

Morgani et al. (2013) then examined the features of *Hex* reporter-positive cells in mESC cultures propagated in 2i medium. A substantial proportion of cells grown in 2i medium expressed the *Hex* reporter and could differentiate into TE as well as PrE, in contrast to reporter-positive cells found in cultures maintained in serum plus LIF, discussed above, which formed only PrE. These *Hex*+ 2i cells contributed to all three preimplantation lineages in aggregation chimeras. Single-cell gene expression analysis revealed that the *Hex*-positive cells propagated in 2i demonstrated lineage priming, or co-expression of pluripotency- and lineage-specific markers, and confirmed their tri-lineage developmental potential in chimeras. Thereafter, Martin Gonzalez et al. (2016) further evaluated the effects of culture conditions on the makeup of mESC populations. Cells grown in 2i or KSOR (Knockout Serum Replacer, a proprietary serum substitute) resembled the cells of the embryonic day 3.5 (E3.5) inner cell mass cells most nearly in their transcriptome, though there was some overlap in gene expression with the 2C-stage embryo. Injection of single cells grown in 2i or KSOR into morula resulted in robust chimera formation (100% chimeric mice), but injection of single mESCs grown in serum, which resembled the E4.5 epiblast in gene expression, did not. Cells cultured in 2i or KSOR cells could contribute to TE in chimeras generated with 2C-stage embryos. Culture in 2i or KSOR also induced the appearance of cells expressing a *Zscan4* reporter, a marker for the 2C stage (below).

Subsequently, Lo Nigro et al. (2017) identified another marker, *Pdgfra*, that could be used to isolate PrE primed progenitors. *Pdgfra* reporter-positive cells also exist in mESC cultures maintained in the presence of LIF plus KSOR, though they were not observed in cultures grown in 2i. Using the marker Pecam1, it was possible to distinguish PrE specified cells, epiblast, and double-positive cells (Pecam-Pdgfra+, Pecam1+Pdgfra−, and Pecam+Pdgfra+, respectively). Double-positive cells were considered an intermediate between the PrE and epiblast populations, contributed to epiblast derivatives and extraembryonic endoderm in chimeras, and mapped transcriptionally to the early-mid epiblast stage of development.

Taken together, these results indicate that under appropriate conditions, mESC cultures can contain both naive state cells corresponding to pre-implantation epiblast and cells with the broader developmental potential of the early inner cell mass. Activation of LIF signaling, inhibition of MEK kinase and Gsk3b, and undefined components in KSOR and serum strongly impact the proportion of cells with expanded developmental potential within mESC cultures.

Further evidence for the existence of mouse PSCs with trophoblast differentiation capacity came from a somewhat surprising source. Abad et al. (2013) studied the outcome of reprogramming to pluripotency *in vivo*, using a mouse bearing a dox-inducible cassette containing the factors *Pou5f1*, *Sox2*, *Klf4*, and *c-Myc*. Transient induction of expression of the four factors caused the development of teratomas in multiple tissues of the mice, and induced PSCs (iPSCs) could be isolated from

the blood of animals subjected to *in vivo* reprogramming. These iPSCs gave rise to TE tissue in teratoma assays and, on intraperitoneal injection, produced *in vivo* EBs that contained TE and could give rise to trophoblast stem cells *in vitro*. The cells formed placenta in chimera assays (morula stage to blastocyst or E14.5 placenta stage) and formed embryo-like structures containing cells expressing markers of all three germ layers as well as TE and extraembryonic endoderm. However, there was no evidence that these cells expressed the 2C markers *Zscan4* or murine endogenous retrovirus with leucine tRNA primer binding site (MERVL) elements. This work is consistent with the possibility that *in vivo* reprogramming supports the emergence of cell populations with expanded developmental potential.

2C-like Cells in ESC Cultures

Another subset of cells in mESC cultures with a distinct developmental profile was discovered independently by several groups studying the control of expression of ERVs in the early embryo. Cellular flux between the pluripotent and totipotent states gives rise to the appearance of 2C-like cells in mESC cultures. These cells characteristically show high expression of ERV, in a pattern reminiscent of the 2C stage.

Genetic manipulation of chromatin-modifying enzymes in mESCs to either increase active chromatin marks, such as H3K4me1/2, or reduce repressive chromatin marks, such as H3K9me2/3, results in expanded fate potential. Macfarlan et al. (2011) showed that deletion of lysine demethylase 1 Lsd/Kdm1a in the mouse resulted in the activation of endogenous MERVL elements, along with a number of very early embryonic genes (normally activated at the zygotic genome activation stage) that featured MERVL sequences or cryptic retroviral LTRs in their regulatory regions. mESCs derived from the *Lsd1* knockouts formed EBs and teratomas with a much higher expression of transcripts for extraembryonic endoderm and trophoblast stem cell markers compared to wild-type cells. Later, using a reporter construct containing the MERVL LTR, Macfarlan et al. (2012) found that a minority of cells in mESC or iPSC cultures were labeled (the finding that positive cells were found in iPSC cultures ruled out contamination from extraembryonic tissues as a source for the TE and extraembryonic endoderm cells). These cells expressed genes characteristic of the 2C stage of development. Though transcripts for *Pou5f1*, *Nanog*, and *Sox2* were found in the MERVL reporter-positive cells, the corresponding proteins were absent. These 2C-like cells existed in flux with the reporter-negative cells, demonstrated by Cre/LoxP lineage tracing of cells that expressed MERVL genes. Reporter-positive cells contributed to somatic, germline, and extraembryonic lineages when injected into blastocysts, while negative cells contributed only to embryonic tissues.

A separate line of investigation also led to identification of a minority subpopulation of 2C-like cells in mESC cultures and provided hints at a functional role for the cycling of mESCs in and out of the 2C state. A search for genes specifically expressed at the 2C stage identified the transcription factor *Zscan4* as a highly specific marker of totipotent cells, but also found that it was expressed in a small fraction of mESCs (Falco et al., 2007). Zalzman et al. (2010) showed that although only 5% of cells in mESC cultures activate a *Zscan4* reporter at any given time, when this minority population was labeled continuously with *Zscan4*-induced Cre/LacZ, the entire culture was eventually labeled, showing that all ESCs passed through the Zscan4-positive state. *Zscan4* knockdown led to apoptosis, chromosome instability, and telomere shortening in mESC cultures. Subsequently it was shown that *Zscan4* induces global demethylation through degradation of two key components of the maintenance methylation pathway, *Uhrf1* and *Dnmt1*, and that DNA demethylation was required for telomere extension (Dan et al., 2017). Amano et al. (2013) used an expression construct to increase the frequency of Zscan4-positive cells in mESC cultures and showed that these cells have greater chimera potential in blastocyst or tetraploid aggregation chimeras compared to naive mESCs, as measured by rates of chimerism. Contribution to extraembryonic lineages was not reported. Unexpectedly, *Zscan4* reporter-positive cells from control mESC cultures showed much less ability to contribute to chimeras than reporter-negative cells. This suggests that Zscan4-positive cells may not be equivalent to the MERVL positive cells discussed above.

It is curious that ESCs cycle in and out of a 2C state, and notable that they do not do so through the intervening stages of embryonic development, but instead appear to undergo direct transition from the naive epiblast to 2C stages and back. If indeed occasional entry into a Zscan4-positive 2C state is necessary for telomere stability, as suggested above, it is possible that this transition is an essential adaptation to PSC maintenance *in vitro*.

Stabilizing and Creating Cell States with Expanded Developmental Potential

Genetic Manipulation of Early Developmental Regulators

These studies above analyzed transient, preexisting subpopulations within mESC cultures that showed expanded developmental potential relative to naive mESCs. More recent work has focused on methods to stabilize or create cells with expanded potential, through analyzing the gene regulatory networks active at the earliest stages of mammalian embryogenesis, or by a nonbiased screening approach.

Ishiuchi et al. (2015) investigated the role of chromatin structure in conversion in and out of the 2C state. These investigators depleted either the p60 or p150 subunits of CAF-1, an enzyme complex responsible for deposition of histone H3 and H4 during DNA synthesis, in ESCs containing an MERVL reporter. The knockdown cultures had a greater proportion of reporter-positive cells (up to 10% relative to 1.0% in controls) and showed activation of 2C-stage transcripts, including MERVL transcripts and *Zscan4*. Chromatin structure in knockdown cells resembled the 2C state. Loss of CAF-1 in ESCs results in chromatin decondensation, increased histone mobility, activation of MERVL repeat elements, and loss of chromocenters. These results suggest that chromatin compaction or assembly might be an essential step in the transition from totipotency to pluripotency. To assess developmental potency, the authors compared how well the knockdown cells compared to control cells as nuclear donors during somatic cell nuclear transfer. Somatic cell nuclear transfer embryos derived from the knockdown cells or 2C-like cells expressing the MERVL reporter spontaneously showed higher frequency of development to the 2C, morula, or blastocyst stage, compared to those formed with mESC donors.

miR-34a was originally identified as a target of p53 and was subsequently shown to inhibit cellular reprogramming to pluripotency. Teratomas from *miR-34a*$^{-/-}$ mESCs and iPSCs expressed markers of TE and extraembryonic endoderm, as did EBs derived from these cells (Choi et al., 2017). In morula aggregation chimeras grown to the blastocyst stage, *miR-34a* knockout cells contributed to both yolk sac and placenta. Single-cell chimera assays confirmed that at least a significant proportion of cells in these cultures had such expanded developmental potential. Blastocyst stage injection chimeras allowed to develop to mid-gestation also showed contribution of homozygous *miR-34a* null cells to placenta and yolk sac. Among genes differentially expressed in the knockout cells were the MERVL family (10% of cells in *miR-34a*$^{-/-}$ ESC cultures expressed an MERVL reporter compared to less than 1% in wild-type cultures); overall, their gene expression profile mapped closer to 2C-stage cells than to canonical ESCs, though the degree of heterogeneity of the cultures was not directly assessed. Despite the phenotypic alterations observed in *miR-34a*$^{-/-}$ mESCs, *miR34a* knockout embryos develop normally.

Loss of *mir-34a* (Choi et al., 2017) did not result in large changes in the histone modification profile at MERVL loci. Expression from MERVL elements is silenced by overexpression of *mir-34a*, even in cells lacking *Kdm1a*. This finding suggests that an increase in epigenetic marks associated with open chromatin alone is not sufficient to induce 2C-like genes and MERVL expression, and indicates that the activity of *mir-34a* is likely mediated through post-translational inhibition of a *trans*-acting factor that directly regulates 2C-stage genes. This study identified *Gata2* as a target for *mir-34a*-mediated repression. Members of the Gata family of transcription factors have pioneer factor function (Iwafuchi-Doi and Zaret, 2014), a property associated with the ability to overcome chromatin barriers and directly create open chromatin at their target loci. Nonetheless, *Gata2* overexpression was not sufficient to drive expression of MERVL loci and other 2C genes, suggesting that other factors are necessary for the regulation of the totipotency network.

Several recent studies have assessed the action of *Dux*, an activator of early zygotic gene expression specifically expressed at the 2C stage, in the regulation of PSCs (De Iaco et al., 2017; Hendrickson et al., 2017). *Dux* (DUX4 in human) activates a wide family of cleavage-stage-specific transcripts in mouse and human, including *Zscan4*/ZSCAN4, and many endogenous retroviral MERVL/HERVL elements. Dux was highly expressed in the MERVL reporter-positive subpopulation of mESC cultures (De Iaco et al., 2017; Hendrickson et al., 2017). Using a dox-inducible *Dux* expression construct, these authors were able to convert up to 70% of cells in ESC cultures to an MERVL-positive state. The Dux-positive cells lacked Pou5f1 protein and had lost chromocenters. Overexpression of *Dux* also resulted in conversion of mESC chromatin to a more open state resembling that of the 2C stage (below). Chromatin accessibility is a surrogate marker for regulatory elements that drive cell-type specificity, and similar chromatin accessibility profiles can indicate similar regulatory architecture between cell types being compared. Overexpressing *Dux* is sufficient to create 2C-like cells with similar chromatin accessibility landscape to early 2C embryo (Hendrickson et al., 2017; Wu et al., 2016b), suggesting these two cell types have similar regulatory architecture. The majority of the newly acquired open chromatin regions after *Dux* expression are in MERVL elements and correlate with increased expression of genes regulated by these repeat elements. Conversely, *Dux* knockdown inhibited spontaneous conversion of mESCs to the 2C state and blocked the previously observed conversion of mESCs to 2C cells induced by CAF1 knockdown. A second study confirmed that *Dux* deletion using CRISPR/Cas9 prevented spontaneous conversion of mESCs to a 2C-like state (De Iaco et al., 2017). The heterochromatin inducing factor *Trim28* directly binds to the Dux locus (De Iaco et al., 2017). Loss of *Trim28* leads to a local decrease of H3K9me3 and subsequent increase in *Dux* expression, as well as other 2C genes. It is possible that the high histone mobility found in 2C embryos allows expression of *Dux* and subsequent activation of repeat elements. Later, as heterochromatin begins to form, *Dux* expression is reduced and MERVL regulatory elements are silenced. This interpretation is supported by the observation that the 2C-specific gene expression seen after *Trim28* knockdown is attenuated in *Dux*-depleted ESCs (De Iaco et al., 2017). The developmental potential of *Dux* knockdown cells was not assessed in either of these studies.

Taken together, these studies show that changes in chromatin status are critical, but not sufficient, for transition in and out of the 2C state.

Manipulation of Intercellular Signaling Pathways

The above studies all relied on manipulation of gene expression to achieve higher proportions of 2C cells in equilibrium with mESCs. Another approach that has been widely used in attempts to stabilize the naive state is the screening of small molecules for the ability to modulate stem cell gene expression. Yang et al. (2017b) carried out a chemical screen to identify culture conditions that would stabilize the naive state, and they arrived at a specific combination of pathway inhibitors that appeared to achieve this for mouse or human cells. However, in chimera assays, the authors noted the incorporation of the mouse cells cultured in the new media into extraembryonic tissues. Therefore, they called the cells expanded potential stem cells, or EPSCs. The authors went on to perform further chimera analyses, assessing the ability of single cells to contribute at the blastocyst and post-implantation stages as well as to support development to term. In all chimeras, descendants of the EPSC were found in the embryo and placenta and yolk sac. The EPSCs passed the rigorous test of the tetraploid complementation assay, giving rise to germline competent chimeras. The human EPSCs also showed significant contribution to both embryonic and extraembryonic lineages in chimeric mouse embryos, though the extent of contribution was much less than that seen with the mouse cells. When the transcriptome of the cells was analyzed and compared to naive ESCs and EpiSCs, 2C cells, and later stages of preimplantation development, it became apparent that the EPSCs were unique. EPSCs shared some patterns of gene expression with blastomeres at the 2C stage of development but otherwise were quite distinct from any of the developmental stages to which they were compared. A similar set of observations pertained to the human cells, which showed gene expression patterns that were distinct from naive or primed cells but had some overlap with those of the zygote to morula stages. Notably, this study showed that the culture

medium containing the chemical cocktail could be used to derive the EPSCs directly from mouse embryos.

A second study employed a similar approach to obtain cultures of EPSCs via conversion of mESCs or iPSCs, or through derivation directly from single eight-cell stage mouse blastomeres. Yang et al. (2017a) developed a culture medium containing six small-molecule pathway inhibitors (several with multiple effects on different protein kinases) designed to block trophoblast and PrE differentiation, along with LIF, and used it to derive EPSC lines at high efficiency from the four- or eight-cell stage blastomeres, or from conventional ESCs or iPSCs via extended passage in the new medium. The cells contributed to embryonic and extraembryonic lineages (both TE and PrE) in chimeras. Transcriptionally, the cells mapped close to conventional naive mESCs in a principle component analysis; they were distinct from four- or eight-cell blastomeres, and while they expressed some genes characteristic of early preimplantation development, the EPSCs could further be distinguished from the 2C-like cells, early inner cell mass-like cells, or *in vivo* reprogrammed iPSCs described above. These EPSC cultures were able to give rise to trophoblast stem cell or extraembryonic endoderm stem cell lines *in vitro*.

Assessing Totipotency

The goal of isolating and propagating EPSCs *in vitro* has been achieved by two groups using small-molecule inhibitors of signaling pathways. It remains to be seen whether cultured cells equivalent to normal embryonic totipotent cells can be maintained in isolation. Studies to date have established some criteria that might be considered in assessing future attempts to capture totipotent cells. The cells should have gene expression characteristic of the appropriate stage of development. 2C-stage cells in the mouse lack the pluripotency proteins Pou5f1, Sox2, and Nanog; have active MERVL elements; and express *Zscan4*, *Dux*, *Eifa*, *Zfp352*, *Tcstv1/3*, and *Tpodz1-5*. There are a number of other marker genes that are relatively specific for this stage of embryonic development, and studies of the human embryo provide similar stage-specific markers. The degree of heterogeneity within the cultures is important and should ideally be assessed by single-cell transcript analysis because while it is possible through multiple means to enhance the content of 2C-like cells in mESC cultures, the 2C state is metastable under these conditions. As with other PSC culture methodologies, it is important to ensure that a system for totipotent cell propagation enables maintenance of a normal genetic makeup over long-term culture, and that the cellular epigenome does not undergo radical changes, since chromatin structure is key to the definition of this developmental state.

EPSCs should be able to differentiate *in vitro* into cells representative of the three embryonic germ layers, as well as TE and PrE and their derivatives. Although the endpoint of such analysis is often gene expression, a mouse totipotent cell might also be expected to give rise to mESCs as well as trophoblast and extraembryonic endoderm stem cells. Criteria for the assessment of mESCs and extraembryonic lineage stem cells are well established. In the human, a recent study laid out criteria for assessing TE differentiation (Lee et al., 2016), and a system for amnion formation *in vitro* was recently published (Shao et al., 2017); the extraembryonic endoderm, amnion, and mesoderm lineages are not well described in molecular terms in human, but further studies of the post-implantation non-human primate embryo should provide more guidance here. The *in vivo* test of chimera formation through mid-gestation or preferably to term should reveal EPSC contribution to all somatic tissues as well as the germline and extraembryonic derivatives. Several groups have performed rigorous single-cell chimera assays to achieve this, though it is yet to be demonstrated that a single putative totipotent cell can give rise to a newborn animal when transferred in isolation to a foster mother. There has been good progress in assessing developmental contribution in interspecies chimeras, but the extent of incorporation of human cells into mouse chimeras remains modest. It is possible that the differences in development between mouse and human present a barrier that is difficult to cross, and in any case, there are ethical challenges to a rigorous demonstration of totipotent developmental potential in animal human chimeras. Perhaps a less controversial test would be to assess the contribution of human cells to particular tissues in animal hosts with genetic deficiencies in organ formation.

Overview and Conclusions

It is important to distinguish the various cell types that display expanded developmental potential (Figure 2). The permanent cultures of EPSCs reported by Yang et al. (2015) do not appear to have a normal developmental counterpart and are dependent for their maintenance upon inhibitors of muscarinic and histaminergic signaling, two pathways with unknown roles in early development. Likewise, the established EPSC cultures recently described by Yang et al. (2017a) appear to be distinct from any other cell type yet described. The *Hex*-positive cells described by Morgani et al. (2013), and those studied by Martin Gonzalez et al. (2016), co-express epiblast and extraembryonic markers, and by gene expression correlate to an early inner cell mass stage (E3.25). These cells express the conventional pluripotency transcription factors. MEK inhibition and LIF, or 2i and KSOR, help to maintain a population with extraembryonic potency in a flux with cells with conventional properties of the naive state. Thus, these cells likely depend on some modification of the gene regulatory networks that maintain naive cells. Their stabilization *in vitro* depends upon modulation of the extrinsic signaling networks that support naive pluripotency, and they are distinct from cells in the 2C state described by other authors, which lack the proteins encoded by the pluripotency genes *Pou5F1*, *Sox2*, and *Nanog*.

Thus, it is now apparent that within mESC cultures, there are subpopulations of cells that display molecular features and developmental potential of 2C-stage blastomeres, or early inner cell mass cells, and two new EPSC types with expanded developmental potential have been generated using novel culture methodologies. Although it is certainly reasonable to describe cells with the ability to form extraembryonic and somatic tissues in chimeras as totipotent, no one has yet demonstrated that they can pass the most stringent test of totipotency, the ability to give rise to a newborn mouse when placed in a zona pellucida and returned to a foster mother. Indeed, it has not yet been established whether EPSCs that are equivalent to an early totipotent embryonic stage can be maintained in isolation, or that such cells

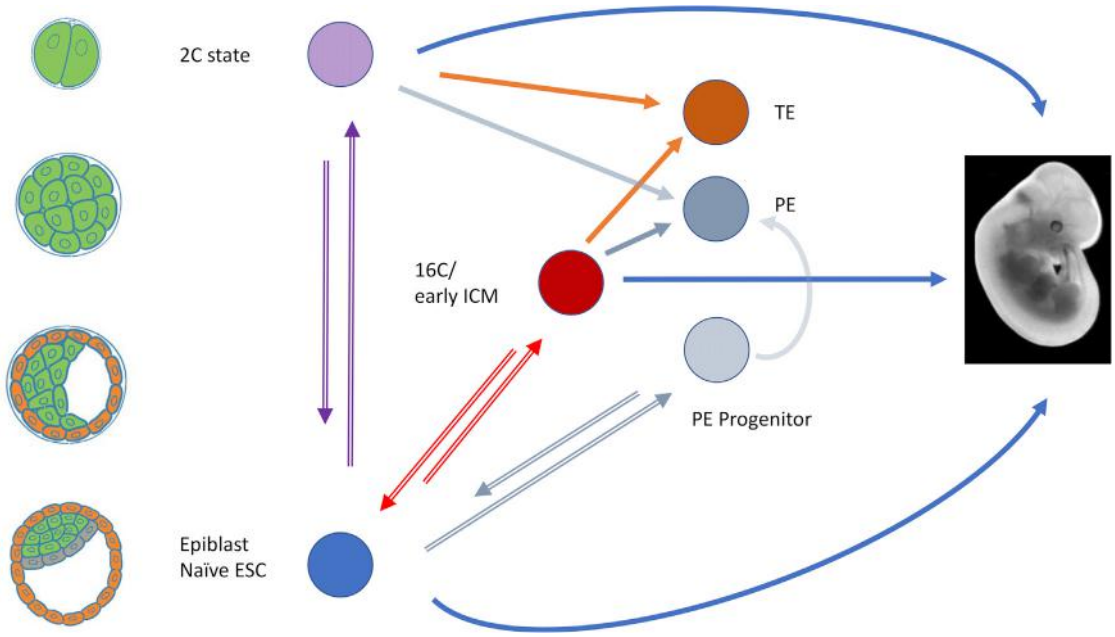

Figure 2. Relationships between Pluripotent Naive mESCs *In Vitro* and Cells with Expanded Developmental Potential
Depending on the conditions under which they are maintained, mESC cultures may contain minority subpopulations of cells with the properties of PrE progenitors, or cells with the molecular features and developmental capacity of 16-cell/early inner cell mass or 2C-stage blastomeres (which can form PrE and TE *in vitro* and *in vivo* along with all the tissues of the embryo proper). There is reversible interconversion between these cell states and mESCs.

can be derived directly from an embryo. The cells described by Yang et al. (2017b) and Yang et al. (2017a) can be maintained in apparent isolation (though these studies did not directly assess the degree of heterogeneity in the cultures) and can both be derived directly from the embryo, but it is not clear that either EPSC equates to any normal embryological counterpart. In considering the use of small-molecule inhibitors in stem cell culture systems, it is apparent that it is not possible to saturate chemical space, and one could envision that high-throughput screens might identify a range of combinatorial culture additives that support an array of artificial cell states *in vitro* with unusual developmental potential. Such artificial cells might have very important practical applications, but their potential role as models for embryo development will need to be carefully scrutinized.

The relationship between the 2C-like cells and the early inner cell mass-like cells remains to be defined, though both exist in mESC cultures. Although some molecular features of totipotent cells have been identified, the gene network that would be required to maintain and expand such a state remains unknown. Likewise, the requirement for passage through a 2C-like state for stability and long-term maintenance of ESCs (Zalzman et al., 2010) merits further study.

Stable cultures of EPSCs could provide access to the study of embryological stages that have not been previously captured *in vivo*, and they might yield more robust chimera formation relative to ESCs. While expanded potential cells have clearly been found to contribute more widely to extraembryonic tissues in intraspecies chimeras compared to mESCs, the evidence that they will provide a definitive working advantage in interspecies chimeras is still lacking. Whether human cells corresponding to four-cell stage totipotent cells or the early inner cell mass will be discovered also remains to be seen. Single-cell gene expression analysis has identified cells co-expressing pluripotency markers alongside extraembryonic endoderm markers in hESC cultures (Hough et al., 2014), but their developmental potential has not been explored. Though many issues remain unresolved, the emerging field of totipotent stem cell culture is already helping to illuminate the molecular regulatory networks that govern cell fate decisions in early mammalian development, and is liable to empower our efforts to exploit stem cells in animal biotechnology and medicine in the future.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table and can be found with this article online at https://doi.org/10.1016/j.stem.2017.12.011.

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