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Capturing and Interconverting Embryonic Cell Fates in a Dish

Jennifer Watts^{*,†,‡,2}, Alyson Lokken^{*,2}, Alexandra Moauro^{*,‡}, Amy Ralston^{*,†,1}

^{*}Michigan State University, East Lansing, MI, United States

[†]Program in Reproductive and Developmental Sciences, Michigan State University, East Lansing, MI, United States

[‡]Graduate Program in Physiology, Michigan State University, East Lansing, MI, United States

Abstract

Cells of the early embryo are totipotent because they will differentiate to produce the fetus and its surrounding extraembryonic tissues. By contrast, embryonic stem (ES) cells are considered to be merely pluripotent because they lack the ability to efficiently produce extraembryonic cell types. The relatively limited developmental potential of ES cells can be explained by the observation that ES cells are derived from the embryo after its cells have already begun to specialize and lose totipotency. Meanwhile, at the time that pluripotent ES cell progenitors are specified, so are the multipotent progenitors of two extraembryonic stem cell types: trophoblast stem (TS) cells and extraembryonic endoderm stem (XEN) cells. Notably, all three embryo-derived stem cell types are capable of either self-renewing or differentiating in a lineage-appropriate manner. These three types of embryo-derived stem cell serve as paradigms for defining the genes and pathways that define and maintain unique stem cell identities. Remarkably, some of the mechanisms that maintain the specific developmental potential of each stem cell line do so by preventing conversion to another stem cell fate. This chapter highlights noteworthy studies that have identified the genes and pathways that normally limit the interconversion of stem cell identities.

1. INTRODUCTION

Very early in mammalian embryogenesis, cells make decisions to produce either the fetus or the extraembryonic tissues of the placenta and yolk sac. Failure to properly execute cell fate decisions can result in miscarriage, birth defects, and can even lead to long-term health issues in the adult. It is now widely appreciated that cell fates must be actively maintained, and that a failure to maintain cell fate can lead cells to adopt aberrant phenotypes. Progress toward elucidating genes important for directing cell fate decisions and maintaining cellular phenotypes has been provided by analyses in embryo models. Additionally, our understanding of the molecular underpinnings of cell fate has been substantially advanced by the study of stem cell lines that represent the fetal and extraembryonic lineages in vitro. As an experimental system, stem cell lines provide many of the benefits of studying embryos because they can be differentiated to a variety of mature endpoints. Yet, stem cells provide

¹Corresponding author: aralston@msu.edu.

²Equal contribution.

an advantage over embryo models because they can be expanded to provide massive cellular quantities, which are more limited in embryos. Accordingly, stem cell lines have been used to identify factors that normally initiate and maintain cell identities during embryogenesis.

Some of the first paradigms for capturing and preserving specific developmental cell fates in vitro included pluripotent stem cell lines, such as embryonal carcinoma (EC) (Kelly & Gatie, 2017) and embryonic stem (ES) cell lines (Evans & Kaufman, 1981; Martin, 1981). These pluripotent cell lines made possible the expansion of largely pure populations with which to perform controlled studies of differentiation. Additionally, pluripotent cell lines provided precedent that specific embryonic cell states could indeed be captured and preserved in vitro. The subsequent derivation of epiblast stem cells (EpiSCs) from later-stage embryos (Brons et al., 2007; Tesar et al., 2007) demonstrated that pluripotent stem cell progenitors could be propagated from multiple developmental stages.

While pluripotent stem cells can differentiate into any mature cell type of the body, they are incapable of efficiently producing extraembryonic cell types of the trophoblast and extraembryonic endoderm lineages (Beddington & Robertson, 1989). Nevertheless, this limitation is mitigated by the existence of extraembryonic stem cell lines, including trophoblast stem (TS) and extraembryonic endoderm stem (XEN) cells, which have been derived from pre- and postimplantation stage embryos (Kunath et al., 2005; Lin, Khan, Zapieć, & Mombaerts, 2016; Tanaka, Kunath, Hadjantonakis, Nagy, & Rossant, 1998). Like ES cells, TS and XEN stem cells are capable of either self-renewing or differentiating to more mature, lineage-appropriate endpoints in response to extrinsic cues. Extraembryonic stem cell lines have enabled researchers to learn critical lessons regarding how extraembryonic cell fates are specified and maintained during development, and provide key insight into the mechanisms that enable stem cells to maintain their lineage-specific developmental potential.

2. MECHANISMS REPRESSING TS CELL FATE IN ES CELLS

During embryonic development, the trophoblast lineage is the first lineage to be specified, beginning as the trophoderm of the blastocyst, and then gradually differentiating to produce multiple types of differentiated cell. The ultimate goal of the trophoblast lineage is to connect with extraembryonic mesoderm-derived umbilical cord and produce a functioning placenta (Fig. 1). Given the fundamental importance of the placenta in fetal health, understanding the origins of the trophoblast lineage has been a central goal in reproductive and developmental biology. In this regard, the mouse has an advantage over many mammalian models because self-renewing, multipotent TS cells can be derived from mouse embryos (Tanaka et al., 1998). TS cell lines are considered *bona fide* stem cell lines because they can either self-renew in the presence of fibroblast growth factor 4 (FGF4) and Activin or TGF β (Erlebacher, Price, & Glimcher, 2004; Kubaczka et al., 2014), or differentiate on withdrawal of self-renewal factors (Tanaka et al., 1998). The establishment of human TS cell lines would provide a valuable research tool for studying trophoblast development and differentiation, but efforts to derive human TS cell lines from human blastocysts have not been successful (Roberts & Fisher, 2011; Rossant, 2015). Therefore, genetic studies in

mouse TS cells have, and will continue, to serve as the preeminent model for identifying genetic mechanisms that define TS cell identity.

While ES cells are pluripotent, and can give rise to any cell type in the body, it has long been appreciated that ES cells lack the intrinsic potential to efficiently produce cell types of the trophoblast lineage (Beddington & Robertson, 1989). Thus, even though ES and TS cells can be derived from the embryo at the same stage, these two cell lines are complementary in terms of developmental potential. This observation is consistent with the hypothesis that progenitors of ES and TS cell lines are specified in parallel during development, as opposed to one lineage deriving from the other. Additionally, this observation is consistent with the hypothesis that a genetic barrier must exist within ES and TS cells, which prevents these two distinct cell fates from interconverting. Exciting studies in the last two decades have tested these hypotheses by identifying genes that are involved in establishing and maintaining the ES/TS lineage barrier.

One of the first studies to identify factors limiting conversion of ES cells to trophoblast was the pluripotency-promoting transcription factor OCT4 (encoded by *Pou5f1*) (Niwa, Miyazaki, & Smith, 2000; Niwa et al., 2005). Conditional inactivation of *Oct4* in ES cells led to the expression of TS cell genes such as *Cdx2* and *Eomesodermin* (*Eomes*) and to acquisition of trophoblast cell morphologies. Supplementation of *Oct4*-inactivated ES cells with FGF4 and fibroblast-conditioned medium, which support TS cell self-renewal (Tanaka et al., 1998), enabled *Oct4*-depleted ES cell lines to proliferate as TS-like cells (Niwa et al., 2000). These observations suggested that the pluripotency pathway overrides trophoblast fate in ES cells. Consistent with this interpretation, OCT4 can override trophoblast fate and induce pluripotency in TS cells on its own (Wuetal.,2011), or in the presence of other pluripotency-promoting factors (Kuckenberget al., 2011). Therefore, OCT4 is both necessary and sufficient to define pluripotency in stem cell lines.

The requirement for other pluripotency factors in repressing TS cell fate in ES cells has also been investigated. The pluripotency gene *Sox2* has also been shown to be essential for repressing acquisition of TS cell fate in ES cells (Ivanova et al., 2006; Li et al., 2007; Masui et al., 2007). However, SOX2 has been shown to be essential for maintaining expression of *Oct4* in ES cells, arguing that SOX2 represses TS cell fate indirectly, via OCT4. By contrast, the pluripotency gene *Nanog* plays no role in repressing TS cell gene expression or cell fate (Chambers et al., 2007). Therefore, it is likely that only a subset of pluripotency genes represses trophoblast cell fate, especially those that act to stabilize expression of OCT4. For example, several *Cyclin* genes have been shown to limit acquisition of TS cell fate in ES cells, likely acting through OCT4 (Liu et al., 2017). Interestingly, this study also showed that Cyclins could phosphorylate OCT4, limiting OCT4 ubiquitination, and stabilizing OCT4 expression in ES cells.

The observation that *Cdx2* and *Eomes* are both upregulated in *Oct4*-inactivated ES cells led to the investigation of the effects of overexpression of each of these genes in ES cell lines. While overexpression of either *Cdx2* or *Eomes* induced formation of TS-like cells, only *Cdx2*-expressing cells were able to contribute to placenta development in chimeras (Niwa et al., 2005). Therefore, CDX2 is more instructive than EOMES in the conversion of ES to TS-

like cells. The mechanism by which CDX2 drives trophoblast cell fate in ES cells is proposed to be by binding to OCT4 and preventing OCT4 from reinforcing its own expression (Niwa et al., 2005). These observations support the idea that CDX2 is a master regulator of trophoblast fate, which is consistent with the requirement for *Cdx2* in the embryo during establishment of TS cell progenitors (Strumpf et al., 2005). Initially, it was unclear whether the only role for CDX2 in TS cells is to repress OCT4, or if CDX2 could have additional essential functions. To test this, *Cdx2* and *Oct4* were simultaneously deleted in ES cell lines, with the expectation that, if the only role of CDX2 is to repress OCT4, then *Cdx2* should not be needed for cells to acquire or maintain TS cell fate in *Oct4* null ES cells. Interestingly, *Cdx2*; *Oct4* double knockout ES cell lines adopted trophoblast fate, but could not self-renew in the presence of FGF4 (Niwa et al., 2005). Therefore, CDX2 is thought to play two main roles in TS cells: repressing OCT4 and maintaining stem cell proliferation downstream of FGF4.

Given the central importance of OCT4/CDX2 in maintaining the ES/TS cell lineage barrier, much interest has focused on identifying factors that initially activate expression of *Cdx2* when the trophoblast lineage is first specified in the embryo. This led to the identification of TEAD4, which is essential for the initial expression of *Cdx2* in early embryos (Nishioka et al., 2008; Yagi et al., 2007). In addition, TEAD4 is sufficient to drive conversion of ES cell lines to TS-like cells, when fused to the transactivation domain of the viral protein VP16 (Nishioka et al., 2009). Therefore, genetic networks at play in the early embryo are also functional within ES cell lines. However, these studies were unable to provide insight into how similar converted ES cells are to embryo-derived TS cell lines. There are many ways to compare embryo- and ES cell-derived TS cell lines, including morphology, functional assays, transcriptome, and chromatin modifications. However, it is not necessarily obvious which of these readouts would provide the best measure of the authentic TS cell properties, nor what degree of difference would indicate biologically meaningful variation. Nevertheless, differences in all these readouts have been noted among ES cell-derived TS cell lines (Cambuli et al., 2014).

Whether there are other factors capable of inducing TS cell fate in ES cells downstream of TEAD4 is supported by analysis of the role of TEAD4 in vivo. For example, the embryonic *Tead4* null phenotype is more severe than is the embryonic *Cdx2* null phenotype, indicating that TEAD4 promotes expression of multiple trophoblast regulators in parallel, and pointing to the existence of additional genes important for defining the ES/TS cell lineage barrier. One example is *Gata3*, which is regulated by TEAD4 and not CDX2, and is sufficient to induce trophoblast gene expression in ES cell lines (Ralston et al., 2010). While GATA3 was able to induce many trophoblast genes independently of CDX2, GATA3 was not sufficient to induce formation of self-renewing TS cell lines. Therefore, TEAD4 is likely to regulate expression of multiple downstream genes, each with differing roles in promoting trophoblast gene expression, proliferation, and establishment of stem cell progenitors.

Beyond transcription factors, other regulators of TS cell fate have been identified. For example, Wnt and Collagen have also been shown to influence TS-like differentiation of ES cells (He, Pant, Schiffmacher, Meece, & Keefer, 2008; Schenke-Layland et al., 2007). In addition, the evidence that transcription factors help define the ES/TS cell lineage barrier led

researchers to investigate how lineage-specific expression of transcription factors is achieved, and whether chromatin modification plays a role. For instance, ES cells deficient in DNA methylation pathway members such as Dnmt1 acquired TS cell-like properties, indicating that DNA methylation normally represses expression of one or more trophoblast genes in ES cell lines (Ng et al., 2008). Interestingly, DNA methylation does not regulate expression of *Cdx2* or *Eomes* in ES cells. Rather, DNA methylation regulates expression of the trophoblast transcription factor *Elf5*, which is itself capable of inducing expression of *Cdx2* and *Eomes* when overexpressed in ES cell lines (Ng et al., 2008). During development, *Elf5* plays an essential role in maintaining the trophoblast lineage after implantation (Donnison et al., 2005) and is therefore later-acting than CDX2 and EOMES. Based on these observations, ELF5 is proposed to act as a gatekeeper of trophoblast fate, acting downstream of DNA methylation, which represses expression of trophoblast genes in ES cell lines.

Besides DNA methylation, additional epigenetic mechanisms have been identified that reinforce the ES/TS cell lineage barrier. For instance, TS cell-expressed microRNAs (miRNAs) were identified that induced formation of TS cell-like colonies, when transiently overexpressed in ES cell lines in the presence of the histone deacetylase inhibitor valproic acid (VPA) (Nosi, Lanner, Huang, & Cox, 2017). Curiously, however, miRNA-induced TS-like cells were not able to differentiate in vitro. When introduced into embryos, miRNA-induced TS-like cell lines contributed mainly to the mural trophectoderm, which is located distal to the ICM in the blastocyst and destined to produce Reichert's Membrane. Taken together, these observations suggest that the transient overexpression of trophoblast miRNAs leads ES cells to acquire a blastocyst-like trophoblast phenotype. The targets of the TS-inducing miRNAs are predicted to include known ES cell maintenance genes *Sall1*, *Sall4*, *Ccnd1*, *Ccnd2*, and *Lin28* (Nosi et al., 2017). However, it is unclear whether loss of one or more of these targets from ES cell lines would also produce TS-like cells with Reichert's Membrane-oriented developmental potential.

3. REPROGRAMMING AND THE TS CELL LINEAGE BARRIER

The discovery that factors such as CDX2 are sufficient to convert ES cells to TS-like cells did not address whether ES cells are uniquely responsive to TS cell-inducing factors, or whether TS cell-inducing factors are more broadly capable of inducing TS cell fate in other cell types. The ability of CDX2 to induce TS cell fate in other cell types has been investigated. One study focused on the ability of CDX2 to override pluripotency and induce trophoblast fate in pluripotent cells such as EC cells and iPS cells. Notably, CDX2 was able to induce TS-like cells in EC and iPS cells, arguing that EC and iPS cells are equivalent in their ability to respond to ectopic CDX2 (Blij, Parenti, Tabatabai-Yazdi, & Ralston, 2015). However, CDX2 was unable to induce TS cell fate in EpiSCs (Blij et al., 2015). Rather, CDX2 is thought to induce mesoderm formation in EpiSCs (Bernardo et al., 2011). These observations indicate that multiple pluripotent states exist, which can be defined functionally in terms of the response to overexpressed *Cdx2*.

Two subsequent studies investigated the abilities of trophoblast factors, including CDX2, to induce TS cell fate in differentiated somatic cells. The idea of identifying transcription

factors capable of converting somatic cells to stem cells was first realized by screening a library of transcription factors to identify factors capable of reversing differentiation in somatic cells to produce induced pluripotent stem (iPS) cells (Takahashi & Yamanaka, 2006). Similarly, by screening a library of candidate trophoblast genes, four factors, *Eomes*, *Gata3*, *Tfap2c*, and *Ets2*, were identified as being sufficient to induce formation of TS-like cells (iTS cells) in mouse embryonic and adult tail tip fibroblasts (Benchetrit et al., 2015; Kubaczka et al., 2015). Interestingly, these same four factors were not able to convert ES cells to iTS cells (Kubaczka et al., 2015), indicating that the cellular context in which factors are overexpressed matters and can influence transcription factor activity. Similarly, *Cdx2* and *Elf5* were not able to induce TS cell fate in somatic cells (Benchetrit et al., 2015). One way to make sense of this observation is to consider the mechanisms by which ELF5 and CDX2 induce TS cell fate in ES cell lines. For example, CDX2 is thought mainly to induce TS cell fate in ES cells by repressing expression of OCT4 (Niwa et al., 2005). Similarly, ELF5 promotes expression of CDX2 in ES cells (Ng et al., 2008), which in turn represses OCT4. Since OCT4 is not present in fibroblasts, CDX2 and ELF5 lack TS cell-inducing ability in fibroblasts.

4. MECHANISMS REPRESSING XEN CELL FATE IN ES CELLS

During embryo development, extraembryonic endoderm is the second lineage to be specified and follows specification of the trophoblast lineage (Fig. 2A). Mature cells of the extraembryonic endoderm lineage play myriad roles in development, including nourishing the developing embryo, patterning the developing epiblast as visceral endoderm, inducing primitive hematopoiesis, contributing to the yolk sac, and even the definitive endoderm (Moerkamp et al., 2013). XEN cells are derived from the primitive endoderm in the blastocyst stage (Kunath et al., 2005) (Fig. 2B) or from the postimplantation extraembryonic endoderm (Lin et al., 2016), and are a useful in vitro tool for studying the differentiation of and inductive roles of the extraembryonic lineage (Artus et al., 2012; Brown et al., 2010; Paca et al., 2012). XEN cells self-renew when grown in the presence of serum, without need for additional growth factors. XEN cell self-renewal is thought to be dependent on PDGFRA-dependent activation of ERK (Artus, Panthier, & Hadjantonakis, 2010), while differentiation of XEN cells is guided by alternative pathways involving bone morphogenetic protein 2 (BMP2) (Artus et al., 2012; Paca et al., 2012).

While ES cells are extremely limited in their ability to produce TS cells in the absence of genetic manipulation, ES cells give rise more readily to primitive endoderm-like cells in response to appropriate extrinsic cues. The first evidence that pluripotent ES cells could give rise to extraembryonic endodermal cell types was through experiments with embryoid bodies, which result when ES cells are grown in suspension culture in the absence of the self-renewal factor leukemia inhibitory factor (LIF) (Brickman & Serup, 2017; Martin & Evans, 1975). As the embryoid body grows in size, the inner cells of the embryoid body express markers of the pluripotent epiblast lineage, whereas the outside cells epithelialize and express markers of the primitive endoderm lineage. While these outer cells express markers of extraembryonic endoderm cells, they fail to give rise to stable, self-renewing XEN cell lines (Coucouvanis & Martin, 1995).

Evidence that ES cells can spontaneously give rise to XEN-like cells has been provided by careful analyses of ES cell cultures, which showed that endodermal markers such as SOX17, GATA6, and HEX are expressed in subsets of ES cells (Canham, Sharov, Ko, & Brickman, 2010; Hamilton & Brickman, 2014; Niakan et al., 2010). Moreover, upon injection into blastocysts, these endoderm gene-expressing cells found in ES cell cultures contribute to the extraembryonic endoderm lineage (Canham et al., 2010; Niakan et al., 2010). Notably, LIF can enhance formation of XEN cell progenitors in vivo (Morgani et al., 2013). However, exogenous LIF is not needed for maintaining XEN cells in vitro, underscoring differences in pathways regulating the establishment of stem cell progenitors and the maintenance of bona fide stem cells.

Knowledge that XEN cells can spontaneously arise from ES cells, either in culture or through embryoid body formation points to the existence of specific signals that can direct ES cells to differentiate to XEN cells. Consistent with this proposal, expression of endodermal genes is repressed in ES cell cultures by interfering with FGF/ERK signaling (Canham et al., 2010; Hamilton & Brickman, 2014), consistent with the role of FGF/ERK signaling in promoting primitive endoderm development in the blastocyst (Chazaud, Yamanaka, Pawson, & Rossant, 2006; Kang, Garg, & Hadjantonakis, 2017; Kang, Piliszek, Artus, & Hadjantonakis, 2013; Molotkov, Mazot, Brewer, Cinalli, & Soriano, 2017; Nichols, Silva, Roode, & Smith, 2009; Yamanaka, Lanner, & Rossant, 2010).

While repressing acquisition of XEN cell fate in ES cell cultures can be advantageous, efforts have also focused on identifying ways to enrich XEN cell fate in ES cell cultures. Lessons from classical studies of EC differentiation pointed to a possible role for retinoic acid (RA) in extraembryonic endoderm differentiation. For example, F9 EC cells treated with RA in monolayer culture differentiate to primitive endoderm, whereas treatment with RA and dibutyl cyclic AMP results in parietal endoderm differentiation (Strickland, Smith, & Marotti, 1980). Additionally, F9 EC cells grown as embryoid bodies in the presence of RA adopt to visceral endoderm cell fate (Hogan, Taylor, & Adamson, 1981). While F9 EC cells treated with RA exhibit morphological and molecular characteristics similar to embryo-derived XEN cells, it is important to note that as a result of their malignant origin, F9 EC cells often exhibit aneuploidy and do not reliably contribute to development in chimeric embryos (Cronmiller & Mintz, 1978; Papaioannou, Evans, Gardner, & Graham, 1979). Thus, the developmental potential of EC-derived XEN cells in vivo has not been tested.

Similar to its role in EC cells, RA can, in the presence of Activin, increase the proportion of ES cells that convert to XEN cells, a cell typed named cXEN (converted XEN) (Cho et al., 2012). These cXEN cells not only morphologically resemble embryo-derived XEN cells but also are molecularly indistinguishable from embryo-derived XEN cells and are able to differentiate to visceral endoderm upon BMP-induced differentiation, as well as contribute to the parietal endoderm when injected into blastocysts. These results suggested that ES cells, or a subpopulation of ES cells, can be prompted to differentiate to XEN cells upon receiving the right exogenous cues, in this case Activin/RA.

Just as FGF4/ERK signaling is essential for the spontaneous formation of XEN-like cells in ES cell cultures, FGF4/ERK signaling is also required for the Activin/RA-driven conversion

of ES cells to XEN-like cells. Addition of Activin/RA was not sufficient to induce conversion of *FGF4* null ES cells to XEN cells nor were Activin/RA sufficient to induce conversion of ES cells to XEN cells in the presence of a MEK inhibitor (Cho et al., 2012). However, the role of FGF4/ERK signaling may be relatively late in the conversion process (Schroter, Ru€e, Mackenzie, & Martinez Arias, 2015). Thus, multiple extrinsic factors regulate the conversion of ES cells to XEN-like cells.

While Activin/RA induce XEN cell fate in ES cells, XEN cells cannot be derived from EpiSCs in the same way (Cho et al., 2012). This observation is consistent with differing developmental properties of ES cells and EpiSCs in vivo (Brons et al., 2007; Tesar et al., 2007), and in response trophoblast-inducing signals (Bernardo et al., 2011; Blij et al., 2015). Thus, neither TS nor XEN-like cells have been derived from EpiSCs. However, TS- and XEN-like cells have been derived from differentiated somatic cells (Benchetrit et al., 2015; Kubaczka et al., 2015; Parenti, Halbisen, Wang, Latham, & Ralston, 2016; Wamaitha et al., 2015), suggesting that the derivation of extraembryonic endoderm stem cell lines from EpiSCs should be possible, but may require identification of a unique set of extrinsic or intrinsic factors that are able to override primed pluripotency and induce extraembryonic pathways in EpiSCs. Identification of such factors could facilitate discovery of protocols for deriving human extraembryonic stem cell lines from human ES cells, since mouse EpiSCs are hypothesized to be more similar to human ES cells than are other mouse stem cell lines.

5. CELL-INTRINSIC REPRESSION OF XEN CELL FATE IN PLURIPOTENT CELLS

Evidence that XEN cells can be coaxed out of a population of otherwise pluripotent ES or EC cells by altering the signaling environment points to the existence of cell-autonomous regulators of XEN cell-specific gene expression. Similar to studies aimed at identifying transcription factors sufficient to convert ES cells to TS cells, studies have aimed at identifying transcription factors sufficient to convert ES cells to XEN cells. One of the first such studies showed that overexpression of GATA factors *Gata4* or *Gata6* could induce primitive endoderm gene expression (Fujikura et al., 2002; Shimosato, Shiki, & Niwa, 2007). When introduced into blastocysts, these XEN-like cells contributed to the parietal endoderm (Shimosato et al., 2007), indicating that not only do these ES cell-derived XEN-like cells express primitive endoderm markers, but they exhibit functional extraembryonic endoderm activity in vivo.

In addition to being sufficient to induce XEN fate in ES cells, *Gata6* is also necessary for the induction of XEN fate in ES cell lines. *Gata6* null ES cells cannot be differentiated to XEN cells in Activin/RA cXEN conditions (Cho et al., 2012). These cells fail to upregulate the primitive endoderm marker *Sox7*, and low levels of the pluripotency marker *Nanog* persist throughout several passages in XEN media, indicating that *Gata6* is necessary to override pluripotency and induce XEN gene expression (Cho et al., 2012). Notably, GATA factors are considered pioneer factors, capable of accessing and opening highly condensed chromatin to activate gene expression (Cirillo et al., 2002; Zaret & Carroll, 2011). Consistent with this, *Gata6* overexpression induced XEN (iXEN) cell fate in differentiated cells, whose chromatin

is likely less open than ES cell chromatin (Wamaitha et al., 2015). These observations suggested that GATA6 regulates endodermal gene expression directly, and this was confirmed by genome-wide analysis of GATA6 binding sites in ES cells overexpressing *Gata6*. In parallel, GATA6 binds to regulatory regions of pluripotency genes, repressing their expression, and indicating that GATA6 induces XEN cell fate by two mechanisms: by direct activation of endodermal gene expression and by direct repression of pluripotent gene expression.

While studies have shown that GATA4/6 most potently and quickly induce XEN cell fate when overexpressed in ES cells, several other primitive endoderm transcription factors also force pluripotent cells to adopt a XEN cell fate. For example, overexpression of *Sox17* in ES cells results in down-regulation of pluripotency markers and upregulation of primitive endoderm-specific genes (McDonald, Biechele, Rossant, & Stanford, 2014; Nikan et al., 2010), although SOX17 induces XEN cell fate much more slowly than do GATA4/6 (Wamaitha et al., 2015). SOX17 binding sites have been identified in EC cells treated with Activin/RA (Aksoy et al., 2013). Interestingly, SOX17 binding sites overlap with many OCT4 binding sites, suggesting that SOX17 and OCT4 promote endodermal fate together. While this idea is at odds with the role of OCT4 as an exclusive inducer of pluripotent cell fate, it is consistent with the discovery that OCT4 promotes primitive endoderm cell fate in parallel to epiblast fate in the blastocyst (Frum et al., 2013; Le Bin et al., 2014). By contrast, the ability of GATA6 to induce XEN cell fate in ES cells is independent of *Oct4* (Wamaitha et al., 2015). Therefore, SOX17 and OCT4 may induce XEN cell fate in parallel to GATA6 in ES cell lines.

6. REPROGRAMMING SOMATIC CELLS TO XEN-LIKE CELLS

Somatic cell reprogramming enables the derivation of colonies of iPS cells from mature, differentiated cells, such as fibroblasts following overexpression of the transcription factors *Oct4*, *Sox2*, *Klf4*, and *Myc* (OSKM) (Takahashi & Yamanaka, 2006). While reprogramming is remarkable, it is inefficient, for reasons that are not yet resolved. In addition to low reprogramming efficiency, colonies with mixed or non-iPS cell phenotypes (Tonge et al., 2014), and cells that exist in a partially reprogrammed state (Meissner, Wernig, & Jaenisch, 2007; Mikkelsen et al., 2008; Silva et al., 2009; Sridharan et al., 2009) have been reported. Distinct from these, iXEN cell colonies have also been observed following OSKM reprogramming (Parenti et al., 2016) (Fig. 3A).

While OSKM are widely appreciated as pluripotency factors, OSKM have also been shown to promote primitive endoderm development in a variety of cellular contexts (Aksoy et al., 2013; Frum et al., 2013; Le Bin et al., 2014; Morgani & Brickman, 2015; Neri et al., 2012; Smith, Singh, & Dalton, 2010; Wicklow et al., 2014). In addition, expression of primitive endoderm genes has been observed during OSKM reprogramming (Serrano et al., 2013). These observations raised the possibility that OSKM could induce extraembryonic endoderm cell fate during reprogramming.

Consistent with this prediction, OSKM have been shown to induce formation of iXEN cells during reprogramming (Parenti et al., 2016). Colonies of iXEN cells appeared larger and

flatter than iPS cell colonies, and outnumbered iPS cell colonies by a factor of three. Individually, iXEN cells exhibited morphologies comparable to embryo-derived XEN cells, they homogeneously expressed endodermal markers such as GATA6, GATA4, SOX17, SOX7, and PDGFRA, and exhibited few significant differences in global transcriptional profile compared to embryo-derived XEN cell lines. Functionally, iXEN cells are multipotent, evidenced by their ability to differentiate into visceral endoderm in an in vitro differentiation assay, and to parietal endoderm in chimeric embryos. Finally, the majority of iXEN cell colonies isolated could be expanded and maintained their iXEN phenotype in culture, demonstrating self-renewal capacity, during continued passage.

In addition to murine iXEN cells, it was recently reported that canine embryonic fibroblasts are also capable of forming iXEN cells during OSKM reprogramming (Nishimura et al., 2017). Like murine iXEN cells, canine iXEN cells are multipotent, self-renewing cells that express XEN cell markers. Therefore, formation of both iXEN and iPS cell types during reprogramming is not limited to mice and indicates that OSKM promote primitive endoderm cell fate in multiple mammalian species.

In addition to transcription factors, small molecules have been shown to facilitate the conversion of somatic cells to pluripotency (Takahashi & Yamanaka, 2016). Interestingly, endodermal gene expression has also been observed during chemical reprogramming of somatic mouse cells (Hou et al., 2013) (Fig. 3). During chemical reprogramming, somatic cells are treated during three stages to produce iPS cell colonies. For the first 20 days, cells are treated with VPA, CHIR99021, 616452, tranylcypromine, and forskolin. During the next 20 days, cells are treated with DZNep. For the final 2 weeks, cells are treated with CHIR99021 and PD0325901, also known as 2i medium, which supports the self-renewal of naïve pluripotent stem cells (Marks et al., 2012; Ying et al., 2008). During chemical reprogramming, endodermal genes are thought to be expressed in the cells as they transition from somatic to pluripotent stem cell states (Zhao et al., 2015). This is at odds with evidence that iXEN and iPS cells arise via distinct lineages during OSKM reprogramming. For example, single-cell analysis of cells undergoing reprogramming revealed that markers of iXEN and iPS cell fate are detected in distinct populations throughout OSKM reprogramming (Parenti et al., 2016). Moreover, lineage tracing showed that iXEN cells are not derived from iPS cell colonies, nor are iPS cells derived from an endodermal population (Parenti et al., 2016). Therefore, intrinsic differences in the mechanisms of OSKM and chemical reprogramming may explain the differing uses of the XEN gene expression in reprogramming (Fig. 3).

In support of the view that chemical reprogramming and OSKM reprogramming utilize the XEN pathway in different ways, chemical reprogramming is much slower than OSKM reprogramming, taking 48–60 days to complete, whereas OSKM reprogramming is complete within 3 weeks or fewer. Moreover, stable, self-renewing iXEN cell lines could not be derived during chemical reprogramming (Zhao et al., 2015), in contrast with OSKM reprogramming, which gave rise to stable, self-renewing iXEN cell lines (Parenti et al., 2016). Understanding the mechanisms by which small molecules regulate cell fate could shed light on this issue. Components of the small molecule reprogramming cocktail could be replaced by overexpression of *SALL4* with either *GATA4* or *GATA6*, pointing to potential

targets of some of the components of the chemical reprogramming cocktail. Further investigation is necessary to reveal the molecular targets and mechanisms of chemical reprogramming.

7. IN SEARCH OF A TOTIPOTENT CELL LINE

Thus far, we have discussed evidence that ES cells give rise relatively inefficiently to extraembryonic cell types. Thus, ES cell lines are considered pluripotent, but not totipotent. While methods exist for diverting ES cells to trophoblast or extraembryonic endoderm cell fates, it is also interesting to consider whether a totipotent cell line could be created from ES cells or derived from embryos. So far, experimental conditions that would capture cells of the embryo in the totipotent state have not been identified. However, several studies have attempted to expand the potential of ES cells, enabling them to contribute to both embryonic and extraembryonic cell fate simultaneously (Morgani, Nichols, & Hadjantonakis, 2017). For example, cells with properties of the two-cell (2C) embryo, including totipotency and gene expression profile, have been detected within ES cell populations (Macfarlan et al., 2012). Subsequent studies have identified pathways that repress the 2C state in ES cells. These studies pointed to a role for miRNAs in repressing the 2C state in ES cells by limiting activity of transposable elements of the MERVL family (Choi et al., 2017; Hendrickson et al., 2017; Morgani et al., 2017). While these studies have led to the identification of novel pathways limiting the potential of ES cell lines to differentiate to extraembryonic cell fates, 2C cells cannot be stably maintained as a homogenous population.

By contrast, small molecules that produce a self-renewing cell line with expanded developmental potential have recently been reported (Yang, Liu, et al., 2017; Yang, Ryan, et al., 2017). These studies, from two separate groups, identified two distinct chemical cocktails that reportedly enabled single cells to contribute to both fetal and extraembryonic lineages more efficiently than ES cells normally do. One group used inhibitors of JNK, p38, Src kinase, Axin1, in 2i+LIF medium, while the other group used only one of the 2i inhibitors (CHIR99021)+LIF, in the presence of a GPCR and a PARP1 inhibitor. It is not yet clear to what extent cells of expanded potential state resemble each other, and to what extent they resemble 2C cells.

The discovery of stem cells that, either as a population, or individually, exhibit expanded developmental potential provides several directions for future research. First, this discovery provides the opportunity to identify new pathways that define lineage barriers. Currently, the targets of small molecules are unknown in this context, so the mechanism by which these cocktails lead to expanded developmental potential are poorly defined. Second, the discovery of stem cells with expanded potential could be an important first step toward producing homogenous populations of human extraembryonic stem cell lines. As mentioned earlier, human blastocyst-derived extraembryonic stem cell lines would be a valuable resource, but do not currently exist. However, this goal is still in its infancy since protocols to produce mouse stem cells with expanded potential have failed to identify means for directing their differentiation down single, specific lineages. Finally, expanded potential stem cells provide an opportunity to discover what, if any, resemblance these cells bear to

living embryos, and what these cells could teach us that we could not learn from studying embryos. The answers to these exciting unknowns await our continued investigation.

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Fig. 1.

Origins and regulation of ES and TS cell lines. The first lineage decision in development occurs prior to blastocyst stage and results in trophoblast and inner cell mass (ICM). In mice, trophoblast stem (TS) cells can be derived from the trophoblast lineage as early as the blastocyst stage. The trophoblast is destined to produce extraembryonic tissues including the placenta. TS cells can self-renew in the presence of cues such as Fibroblast Growth Factor 4 (FGF4) and Activin. TS cells retain the capacity to differentiate in a lineage-appropriate manner on withdrawal of FGF4 or when introduced into host blastocysts. Embryonic stem (ES) cells are derived from a subset of cells present in the ICM, which are destined to produce the fetus, the umbilical cord, and portions of the surrounding fetal membranes. ES cells can self-renew in the presence of factors such as leukemia inhibitory factor (LIF) and WNT (Nichols et al., 1998; Willert et al., 2003; Williams et al., 1988; Ying et al., 2008), or to differentiate on withdrawal of these factors or when introduced into host blastocysts. Genes and pathways that reinforce the lineage-specific developmental potential of TS and ES cell lines include TEAD4, CDX2, ELF5, and OCT4.

Fig. 2.

Origins and regulation of ES and XEN cell lines. (A) Overview of extraembryonic endoderm development. Extraembryonic endoderm is first specified prior to implantation, in the blastocyst ICM. Following embryo implantation into the uterus, the primitive endoderm differentiates into parietal endoderm, which comprises Reichert's membrane, and visceral endoderm, which contacts and patterns the epiblast of the egg cylinder. The visceral endoderm goes on to comprise yolk sac endoderm midgestation. (B) The second lineage decision in development occurs during the blastocyst stage and produces epiblast, the

progenitors of ES cells, and primitive endoderm, progenitors of XEN cells. XEN cells can either self-renew, in the presence of receptor tyrosine kinase (RTK) signaling, or give rise to differentiated cell types of the extraembryonic lineage. While ES cells give rise to extraembryonic endoderm-like cells at low efficiency, ES cells can be coaxed to a XEN-like state by factors that function intrinsically or extrinsically.

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Fig. 3.

XEN cell fate during somatic cell reprogramming. Either retroviral or transgenic overexpression of the reprogramming factors OSKM leads to parallel formation of iPS cells and iXEN cells. By contrast, chemical reprogramming is reported to produce a XEN-like state, which cannot be stabilized as a self-renewing population, but is proposed to serve as an intermediate between the fibroblast and iPS cell states.