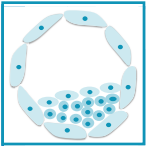


# EX UNO PLURES: MOLECULAR DESIGNS FOR EMBRYONIC PLURIPOTENCY

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**Loh KM, Lim B, Ang LT.** Ex Uno Plures: Molecular Designs for Embryonic Pluripotency. *Physiol Rev* 95: 245–295, 2015; doi:10.1152/physrev.00001.2014.—Pluripotent cells in embryos are situated near the apex of the hierarchy of developmental potential. They are capable of generating all cell types of the mammalian body proper. Therefore, they are the exemplar of stem cells. In vivo, pluripotent cells exist transiently and

become expended within a few days of their establishment. Yet, when explanted into artificial culture conditions, they can be indefinitely propagated in vitro as pluripotent stem cell lines. A host of transcription factors and regulatory genes are now known to underpin the pluripotent state. Nonetheless, how pluripotent cells are equipped with their vast multilineage differentiation potential remains elusive. Consensus holds that pluripotency transcription factors prevent differentiation by inhibiting the expression of differentiation genes. However, this does not explain the developmental potential of pluripotent cells. We have presented another emergent perspective, namely, that pluripotency factors function as lineage specifiers that enable pluripotent cells to differentiate into specific lineages, therefore endowing pluripotent cells with their multilineage potential. Here we provide a comprehensive overview of the developmental biology, transcription factors, and extrinsic signaling associated with pluripotent cells, and their accompanying subtypes, in vitro heterogeneity and chromatin states. Although much has been learned since the appreciation of mammalian pluripotency in the 1950s and the derivation of embryonic stem cell lines in 1981, we will specifically emphasize what currently remains unclear. However, the view that pluripotency factors capacitate differentiation, recently corroborated by experimental evidence, might perhaps address the long-standing question of how pluripotent cells are endowed with their multilineage differentiation potential.

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hundreds of diverse cell types of the body proper. This capacity is known as pluripotency. Such expansive multilineage potential is unparalleled by any other somatic cell type (32, 363, 371).

Mammalian pluripotency was initially appreciated over six decades ago. In the 1950–1970s, the epiblast was first recognized as the seat of embryonic pluripotency in mammalian development (120) (reviewed in sect. I). In 1981, pluripotent embryonic stem cell (ESC) lines were established from epiblast tissue explants (sects. I and II). Subsequently, transcription factors (sect. III), extrinsic signals (sect. IV), and chromatin configurations (sect. VI) associated with pluripotency have come into view.

## I. THE FOUNDATION OF MAMMALIAN DEVELOPMENT

### A. Pluripotent Epiblast Cells Are the Foundation of Mammalian Development

The cells of the epiblast/primitive ectoderm (326) are positioned near the peak of the hierarchy of mammalian embryonic development. They are capable of generating all the

The question how pluripotent cells are equipped with their developmental competence (362) is a long-standing one. Yet, over the past several decades, it has remained cryptic how such a diverse range of developmental potentials are made accessible to pluripotent cells.

During embryogenesis, epiblast cells temporarily inhabit an undifferentiated state but are on the precipice of imminent differentiation along multiple lineage pathways. The prevailing model of pluripotency suggests that transcription factors (TFs) expressed in pluripotent cells restrict differentiation and thereby entrap pluripotent cells in a “self-renewing,” undifferentiated state (153, 305, 398). However, this may be inconsistent with the definition of pluripotency itself, because it incongruously suggests that pluripotency regulators limit developmental competence by prohibiting differentiation.

We propose an alternate hypothesis that pluripotency factors are lineage specifiers that drive lineage commitment (203). In this capacity, pluripotency factors bestow pluripotent cells with their multilineage differentiation potential by capacitating downstream differentiation (203), possibly explaining the prodigious developmental competence of pluripotent cells.

## B. The Formation of Pluripotent Cells In Vivo

Pluripotent cells are born in the earliest events of embryonic development, and they are the culminating point from which all fetal germ layers and organs emerge, eventually forming all somatic cell-types (FIGURE 1A).

Mammalian embryogenesis initially begins with the totipotent unicellular zygote, which can form both fetal and extraembryonic lineages. While fetal cells constitute the body proper, extraembryonic tissues (including the hypoblast/primitive endoderm and trophoctoderm) nourish the fetus and provide patterning signals that direct embryogenesis (301, 334). These fetal and extraembryonic lineages progressively form over the course of eight cleavage divisions (292, 293), during which the zygote develops into blastomeres, morulae, and finally the 64-cell preimplantation early blastocyst by  $\sim$ E3.5 in mouse embryogenesis (6). The early blastocyst comprises the inner cell mass (ICM) and the extraembryonic trophoctoderm (FIGURE 1B): the ICM is the bipotent progenitor to the pluripotent epiblast and the extraembryonic hypoblast. After another cell division, the 128-cell peri-implantation expanded blastocyst is formed ( $\sim$ E4.5) (6, 86) (FIGURE 2A). At this stage, the ICM has become segregated into distinct epiblast and hypoblast fates (6, 14, 109, 111), resolving in the generation of a dozen or so pluripotent epiblast cells. As the epiblast is configured for pluripotency, it also generally irreversibly loses the capacity to generate extraembryonic cell types (trophoctoderm or hypoblast) and vice versa (109, 111) (FIGURE 4A).

At  $\sim$ E4.5, the expanded blastocyst implants into the uterine wall, and epiblast cells become preconditioned for imminent differentiation. The blastocyst undergoes a dramatic morphological alteration, extensively expanding 40-fold (332), losing its approximately spherical appearance and

forming instead a more elongated structure known as the “egg cylinder” (FIGURE 2B) (6, 307). The epiblast also undergoes a significant molecular and morphological remodeling: the dozen epiblast cells in the  $\sim$ E4.5 blastocyst furiously divide to form a pseudostratified epithelium of  $\sim$ 120 pluripotent cells (315) on the inward surface of the  $\sim$ E5.5 egg cylinder.

The pluripotency of the epiblast begins to be expended at E6.5 as uncommitted epiblast cells undergo lineage specification during gastrulation. This is the developmental process through which the three fetal germ layers (definitive endoderm, mesoderm, and definitive ectoderm) are born (14, 334) (FIGURE 1A). During gastrulation, epiblast cells at the posterior-proximal region of the egg cylinder ingress to form a structure known as the primitive streak, which harbors the precursors to definitive endoderm and mesoderm (183, 331). In contrast, the anterior epiblast produces the definitive ectoderm (183, 330) (FIGURE 2C). Through these intricate cell-fate decisions and morphogenetic movements that culminate in germ-layer formation, the pluripotency of the epiblast becomes fully expended by E7.75–E8.0 (264) as these cells differentiate into the plurality of lineages that comprise the fetus. Indeed, when E8.0 epiblast derivatives were heterotopically grafted in the 1970s, Beddington (19, 22) found they were largely restricted in their germ-layer commitment, indicating epiblast pluripotency had become fully extinguished by this stage.

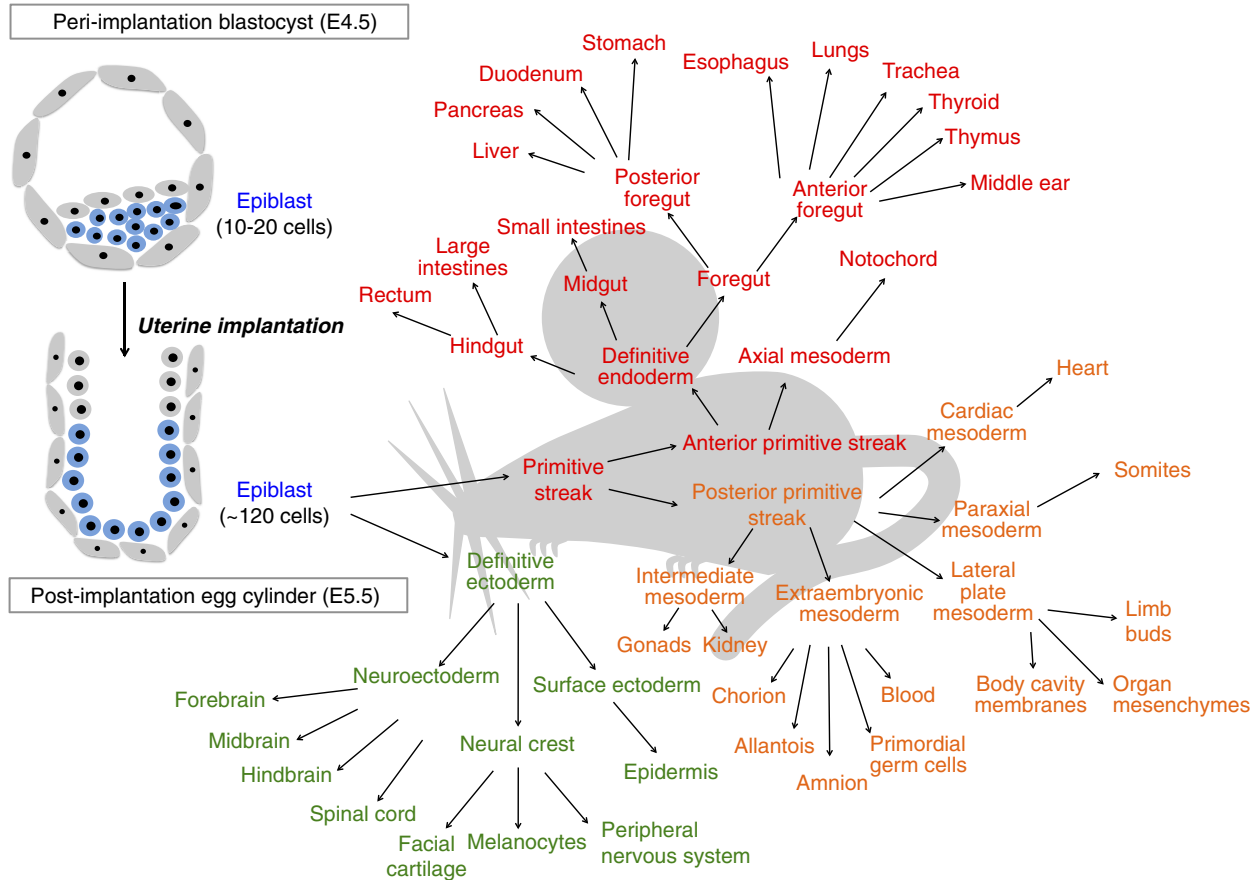
## C. Explantation of Pluripotent Cells Generates Self-Renewing Pluripotent Cell Lines In Vitro

Pluripotency exists only transiently within the early conceptus. It is established within the peri-implantation blastocyst at E4.5 and expires shortly after E7.5 as pluripotent cells commence lineage commitment. Nevertheless, it is possible to stably capture the ephemeral condition of pluripotency by explanting pluripotent cells into specific culture conditions that restrain differentiation. At least three such types of cells can be cultivated as pluripotent stem cell lines.

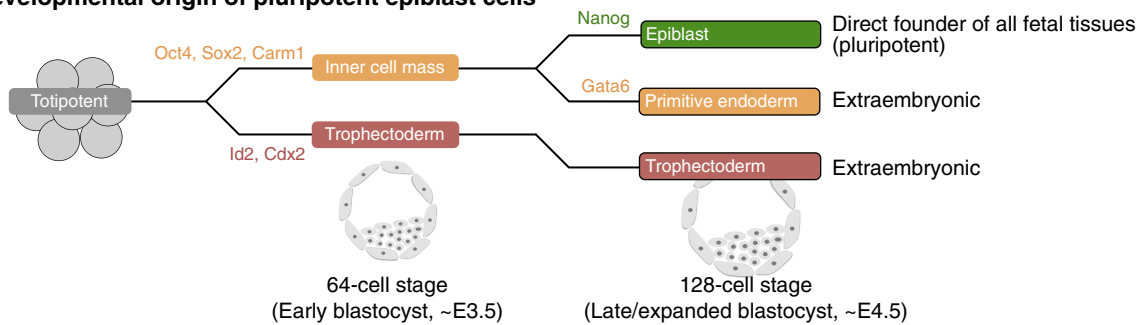
Explantation of mouse peri-implantation epiblast cells (35, 42) at E4.5 yields pluripotent ESC lines (97, 215), which stably retain their pluripotentiality and unrestricted multilineage differentiation ability in vitro (235) (FIGURE 2A, and discussed below).

Explantation of postimplantation epiblast cells from the mouse egg cylinder (E5.5–E7.5) into distinct culture conditions also yields pluripotent cell lines known as epiblast stem cells (EpiSCs) (41, 264, 345). However, these EpiSCs, derived from the postimplantation egg cylinder only 1 day later than ESCs, are molecularly and functionally distinct from ESCs (17, 41, 345) (FIGURE 2B). These differences will be a focus of discussion in section II.

**A The foundation of mammalian development**



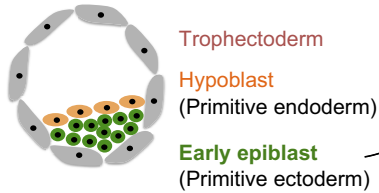
**B Developmental origin of pluripotent epiblast cells**



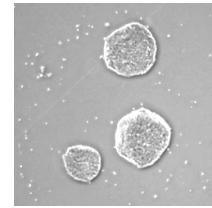
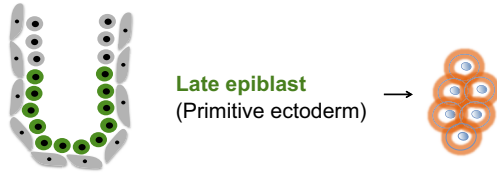
**FIGURE 1.** Pluripotent epiblast cells are the foundation of mammalian development. *A*: the peri-implantation mouse blastocyst at ~E4.5 contains 10–20 epiblast cells [246], which after uterine implantation develop into ~120 epiblast cells by ~E5.5 [315]. During gastrulation, postimplantation epiblast gives rise to the definitive ectoderm (which forms the skin as well as central and peripheral nervous systems) and the primitive streak (which generates the definitive endoderm and mesoderm germ layers). Specification of various body parts and organ cell types from pluripotent cells occurs sequentially through the intermediacy of multiple embryonic progenitors. *B*: hierarchy of lineage segregations in preimplantation development. During the morula stage, totipotent cells become progressively committed to either the trophectoderm or the inner cell mass [293], which become definitively resolved in the ~E3.5 early blastocyst. After another cleavage division, the inner cell mass is segregated into the epiblast/primitive ectoderm and the hypoblast/primitive endoderm within the ~E4.5 late blastocyst.

A third type of pluripotent cell line may be cultivated from a later embryonic stage. During gastrulation, the epiblast differentiates into primordial germ cells (PGCs), proliferative stem cells which are the progenitors to gametes and, therefore, the germline (113) (FIGURE 2C). Although PGCs

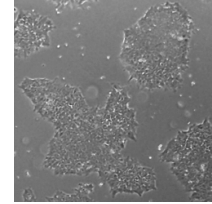
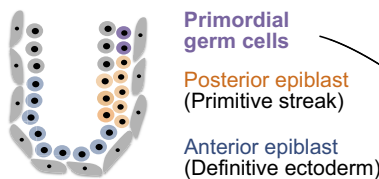
are fated to produce only gametes in vivo, they harbor transcriptional and epigenetic characteristics evocative of pluripotent epiblast (142). Therefore, when explanted in vitro into culture conditions similar to those used to capture ESC lines, PGCs produce pluripotent embryonic germ cell

**A Peri-implantation blastocyst (E4.5)****Mouse embryonic stem cells (mESCs)**

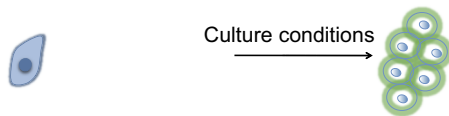
- Express Oct4, Sox2, and Nanog
- Can differentiate into all three germ layers
- Can contribute to pre-implantation embryos
- X chromosome activation (in females)
- LIF/Stat, Wnt, Bmp signaling and FGF/MAPK inhibition for self-renewal

**B Post-implantation egg cylinder (E5.5-beyond)****Epiblast stem cells (EpiSCs)**

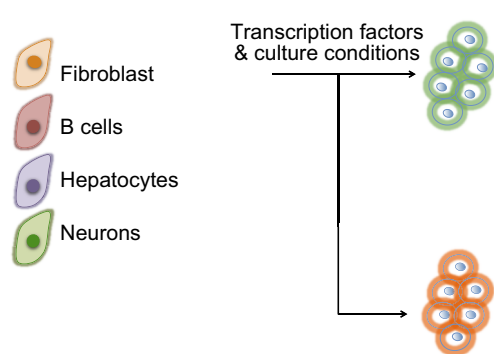
- Express Oct4, Sox2, and lower Nanog
- Can differentiate into all three germ layers
- Can contribute to post-implantation (but not pre-implantation) embryos
- X chromosome inactivation (in females)
- TGF $\beta$  and FGF for self-renewal

**C Gastrulation-stage egg cylinder (E6.5-beyond)****Embryonic germ cells (EGCs)**

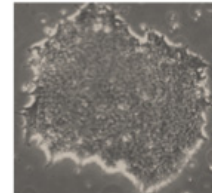
- Express Oct4, Sox2, and Nanog
- Can differentiate into all three germ layers
- Can contribute to pre-implantation embryos
- X chromosome activation (in females)
- LIF/Stat, Wnt, Bmp signaling and FGF/MAPK inhibition for self-renewal

**D Spermatogonial stem cells****Germline stem cells (GSCs)**

- Express Oct4, Sox2, and Nanog
- Can differentiate into all three germ layers
- Can contribute to pre-implantation embryos
- X chromosome status undetermined
- LIF/Stat and Bmp? for self-renewal (other signals undefined)

**E Terminally differentiated cells****Induced pluripotent stem cells (iPSCs)**

- Express Oct4, Sox2, and Nanog
- Can differentiate into all three germ layers
- Can contribute to pre-implantation embryos\*
- X chromosome activation (in female cells)
- LIF/Stat, Wnt, Bmp signaling and FGF/MAPK inhibition for self-renewal

**Induced epiblast stem cells (iEpiSCs)**

- Express Oct4, Sox2, and lower Nanog
- Can differentiate into all three germ layers
- Embryonic contribution undetermined
- X chromosome inactivation (in female cells)
- TGF $\beta$  and FGF for self-renewal

**FIGURE 2.** The formation of pluripotent cells *in vivo* and the provenance of pluripotent stem cell lines. The origins of various pluripotent stem cell lines are shown on the *left*; on the *right*, their derivative pluripotent stem cell lines are detailed. *A*: the mouse peri-implantation epiblast within the E4.5 blastocyst and its *in vitro* counterpart, mouse ESCs. *B*: the mouse postimplantation epiblast within the E5.5 egg cylinder and its *in vitro* derivatives, mouse EpiSCs (though EpiSCs can also be derived at later stages, until at least E7.5). *C*: primordial germ cells within the postimplantation egg cylinder at E6.75, from which embryonic germ cells (EGCs) can be derived with maximum efficiency at E8.5, although EGCs can also be derived from E7.5 until E12.5 (180, 188, 221). EGCs may carry unbalanced imprints, unless derived from early PGCs prior to the initiation of imprint erasure. *D*: adult spermatogonial stem cells are a source of germline stem cells. *E*: a variety of terminally differentiated lineages can be converted into induced pluripotent stem cells (iPSCs) or induced epiblast stem cells, although the developmental potency of iPSC lines has been reported to differ somewhat.

(EGC) lines that harbor pluripotency in vitro, akin to ESCs (FIGURE 2C) (221).

#### D. Artificially Engineering Pluripotent Cells From Nonpluripotent Lineages In Vitro

While explantation of pluripotent cells extant within the embryo generates pluripotent stem cell lines including ESCs, EpiSCs, and EGCs, it is possible to convert many types of terminally differentiated cells into induced pluripotent stem cells (iPSCs) (328) that are similar to ESCs or induced epiblast stem cells (iEpiSCs) that are similar to EpiSCs (128) via the ectopic expression of specific pluripotency-affiliated transcription factors (FIGURE 2E). The differentiated cells from which iPSCs are derived do not have any pluripotent characteristics akin to embryonic epiblast. Instead, they are artificially coerced to attain pluripotent identity, as summarized in recent reviews (131, 149, 275).

Pluripotent cells known as germline stem cells (GSCs) can also be generated by explanting adult spermatogonial stem cells into specific pluripotency-inducing culture conditions (169) (FIGURE 2D). Reprogramming of spermatogonial stem cells into GSCs only requires culture condition manipulations and does not entail transcription factor overexpression unlike iPSC generation.

Having described the dramatis personae of pluripotent stem cells that may be derived from the early embryo (ESCs, EpiSCs, EGCs) as well as pluripotent cells that are obtained via the reprogramming of differentiated cells (iPSCs, iEpiSCs, and GSCs), we now highlight and describe specifically ESCs, which will be a main focus of this review.

#### E. Salient Properties of the ESC

We define three salient characteristics of ESCs: they 1) stably retain the pluripotential capability to generate every single cell type in the fetus even after extended in vitro culture (236), 2) proliferate indefinitely, and 3) can remain in an undifferentiated and uncommitted state yet can flexibly embark upon commitment to any fetal lineage. The latter two capabilities, the ability to proliferate whilst remaining undifferentiated, are sometimes amalgamated into a single characteristic, “self-renewal” (308). However, we opine that self-renewal qualifies as a cell culture artifact rather than a genuine property of pluripotent cells, because pluripotency is strictly transitive in vivo (as discussed above) and can only be artificially stabilized in vitro under specific extrinsic signaling conditions (see sect. IV).

The fundamental definition of pluripotency harkens from a rich history of embryological research. Embryonic pluripotency in mammals was first appreciated by Grobstein, Solter, Švajger, and coworkers in the 1950–1970s when

they transplanted rodent epiblast tissue into heterotopic locations (under the kidney capsule or into the eye chamber) (120, 189, 318). The results were striking. Epiblast spontaneously generated a menagerie of unrelated cell types from all across the body, ranging from gut to contractile muscle to hair (120, 189, 216, 217, 318), thereby providing evidence that epiblast was the seat of embryonic pluripotency. Subsequently orthotopic transfer of genetically labeled epiblast cells into recipient blastocysts by Gardner in the 1960–1970s firmly established that donor epiblast correctly differentiated into a diversity of fetal lineages during normal embryogenesis to form live animals (108), and furthermore, it was restricted to fetal (as opposed to extraembryonic) lineages (111).

Despite early indications that mouse ESCs cultured in vitro could differentiate into a number of lineages in culture (89) or after embryo injection (38), formal demonstration of their pluripotency remained lacking. In pivotal experiments, Nagy et al. (235) rigorously assessed their developmental potential using tetraploid complementation. In tetraploid embryos, epiblast cells cannot produce persisting fetal progeny, thereby leading to early embryonic lethality although extraembryonic tissues remain relatively intact (316, 317, 338). Therefore, transplantation of cells into tetraploid embryos (207) assesses whether the donor cells can substitute for the absent epiblast and generate a viable fetus in their stead. Remarkably, following transplantation to tetraploid embryos (235), mouse ESCs were fully capable of rescuing these embryos and generating live mice, unequivocally demonstrating that they are capable of differentiating into every cell type within the mammalian body (236). Within such chimeras, virtually all adult tissues are ESC derived (92, 115, 236) as any residual tetraploid recipient cells are typically completely outcompeted by ESC-derived diploid progeny.

Altogether such capabilities indicate that despite explantation and prolonged in vitro propagation, ESCs are bona fide pluripotent cells that have stably inherited the pluripotency of the epiblast and maintain the faculty to differentiate into all fetal cell types.

## II. FAMILY RELATIONSHIPS BETWEEN CLASSES OF PLURIPOTENT STEM CELLS

It is now evident that “pluripotency” does not monolithically refer to a singular developmental state; in contrast, pluripotent epiblast cells in vivo traverse a number of developmental phases and in so doing they progressively acquire distinct characteristics. Analogously, in vitro while mouse ESCs, human ESCs, and mouse EpiSCs are all nominally “pluripotent,” there are striking functional and molecular differences between these classes of pluripotent stem cell. These differences are attributable to the disparate develop-

mental origins of these cell lines and possibly the manner with which they are derived or propagated. However, inconsistencies between these in vitro cell lines and their presumed in vivo counterparts continue to incite controversy.

### A. Common and Dissimilar Characteristics of Mouse ESCs and EpiSCs: Naive and Primed Pluripotent States

Originating from distinct embryonic precursors (the E4.5 peri-implantation blastocyst and E5.5–E7.5 egg cylinder, respectively), mouse ESCs and EpiSCs exhibit both common and divergent characteristics. Both cell types qualify as genuinely pluripotent: they can form all three germ layers in vivo and in vitro and ubiquitously express pluripotency TFs Oct4 and Sox2 among others (FIGURE 2, A AND B, TABLE 1). Likewise in vivo, classical developmental fate tracing and heterotopic transplantation studies have found both peri-implantation (111) and postimplantation epiblast cells (19, 21, 333) may contribute to all fetal germ layers.

Nevertheless, postimplantation epiblast is chronologically 1 day closer to germ-layer differentiation than its peri-implantation predecessor. This developmental proximity is reflected in molecular and functional changes in anticipation of differentiation (below). Accordingly, Nichols and Smith (247) have described mouse EpiSCs as “primed” pluripotent cells (“primed” for germ-layer differentiation) and have termed mouse ESCs as “naive” (more primitive) pluripotent cells (FIGURE 3A). Akin to how peri-implantation epiblast precedes postimplantation epiblast in vivo, mouse

ESCs transit through an EpiSC-like intermediate during fetal lineage specification in vitro (141, 407). Therefore, EpiSCs represent transiently arrested postimplantation epiblast en route to fetal differentiation, and they are one step closer to lineage commitment than mouse ESCs (247, 291).

In line with this notion, mouse ESCs highly express markers of E3.5 preimplantation ICM/E4.5 peri-implantation epiblast including *Rex1* (289), *Stella/Dppa3* (295), *Tbx3* (60), *Dax1/Nr0b1* (70), *Nr5a2* (121), *Tfcp2l1/Ctrr-1* (271), and *CD31/Pecam1* (288) (FIGURE 3A, TABLE 1). In contrast, EpiSCs minimally express these blastocyst-stage epiblast markers (17, 41, 345) and instead are distinguished by E5.5 postimplantation epiblast markers *Fgf5* (137), *Nodal* (74), and *Otx2* (3) (FIGURE 3A, TABLE 1). Naive markers *Rex1* and *Stella* are suppressed in EpiSCs by substantial promoter methylation (17, 140). Consistent with a “primed” identity, EpiSCs may also express germ-layer determinants including *Brachyury*, *Eomes*, *Sox17*, *Foxa2*, and *Gata6* to some extent (27). However, it remains unclear whether these lineage specifiers are expressed by all EpiSCs or are selectively expressed in certain subsets of EpiSCs perhaps reflecting sporadic differentiation (27), a question to which we return below.

Mouse ESCs and EpiSCs are further distinguished at the level of chromatin: female EpiSCs display X chromosome inactivation (17, 124), an epigenetic marker of differentiation in vivo (230), while mouse ESCs do not (see APPENDIX). Additionally, EpiSCs seemingly harbor globally increased DNA methylation due to enhanced *Dnmt3b* expression (125, 141). In summary, EpiSCs are on the cusp of differentiation akin to their postimplantation epiblast anteced-

**Table 1.** Diagnostic characteristics of mouse embryonic stem cells, mouse epiblast stem cells, and human embryonic stem cells

	Mouse ESCs	Mouse EpiSCs	Human ESCs (Primed)	Human ESCs (Naive-like)
Embryological origin	Peri-implantation epiblast (E4.5)	Postimplantation epiblast (E5.5–E7.5)	Human blastocyst (exact origin unclear)	Human blastocyst
Morphology	Domed	Flattened	Flattened	Domed
Cell surface profile	SSEA-1 <sup>+</sup> , CD31 <sup>+</sup>	CD31 negative	SSEA-3 <sup>+</sup> , SSEA-4 <sup>+</sup> , TRA-1-60 <sup>+</sup> , and TRA-1-81 <sup>+</sup>	SSEA-4 <sup>+</sup> , TRA-1-60 <sup>+</sup> , TRA-1-81 <sup>+</sup> (SSEA-1 negative)
Alkaline phosphatase activity	Positive (stronger)	Positive (weaker)	Positive	N.D.
Upregulated transcription factors	<i>Dax1</i> , <i>Esrrb</i> , <i>Rex1</i> , <i>Tbx3</i> , <i>Stella</i> , <i>Klf4</i> , <i>Prdm14</i> , <i>Tfcp2l1</i> , <i>Nr5a2</i>	<i>Otx2</i>	<i>REX1</i> , <i>PRDM14</i> , <i>OTX2</i>	<i>REX1</i> , <i>PRDM14</i> , <i>STELLA</i> , <i>TBX3</i> , <i>KLF4</i> (59, 105)
X chromosome status (female cells)	X <sub>a</sub> X <sub>a</sub>	X <sub>a</sub> X <sub>i</sub>	Some lines X <sub>a</sub> X <sub>a</sub> , others X <sub>a</sub> X <sub>i</sub>	X <sub>a</sub> X <sub>a</sub> ?
Oct4 enhancer utilized	Preferentially distal enhancer	Preferentially proximal enhancer	Preferentially proximal enhancer	Preferentially distal enhancer
Common transcription factors	Oct4, Sox2, higher Nanog	Oct4, Sox2, lower Nanog	Oct4, Sox2, lower Nanog	Oct4, Sox2, higher Nanog

ESCs, embryonic stem cells; EpiSCs, epiblast stem cells; N.D.; not determined.

ents. They are molecularly primed for germ-layer differentiation and are developmentally “closer” to gastrulation as opposed to their naive mouse ESC counterparts (291). One pragmatic corollary is that EpiSCs should therefore be more rapidly differentiated *in vitro* into various fetal lineages than naive mouse ESCs, although this remains to be formally demonstrated.

Mouse ESCs and EpiSCs also diverge in their ability for embryonic complementation. While EpiSCs can generate all three fetal germ layers *in vitro*, when they are reintroduced into the preimplantation embryo, they cannot integrate nor contribute to fetal lineages (41, 345). Initially this lack of *in vivo* contribution, combined with elevated expression of germ-layer markers, was inferred to mean that EpiSCs represent an illegitimate quasipotent cell type. However, it is notable that cells from E6.0 postimplantation epiblast are also incapable of blastocyst complementation (110). Their inability to engraft into the preimplantation embryo may result from their epithelialization into a pseudostratified columnar epithelium (315). This could alter their cell adhesive properties and prevent them from adjoining with preimplantation cells. Indeed, *E-cadherin* overexpression re-enables EpiSCs to engraft in the preimplantation embryo and to subsequently form chimeras (261). Finally, orthotopic transfer of EpiSCs into postimplantation egg cylinder also results in their integration and proper contribution (147), indicating earlier failures to attain EpiSC engraftment to preimplantation embryos could be attributed to developmental asynchrony, not some form of developmental incompetence.

The extrinsic stimuli needed to maintain undifferentiated mouse ESCs and EpiSCs are also contradistinct (see sect. IV). While EpiSCs require both transforming growth factor (TGF)- $\beta$  and fibroblast growth factor (FGF) signaling for their undifferentiated propagation (40, 345), these factors instead drive naive mouse ESCs to differentiate. In contrast, mouse ESC propagation is driven by several factors including leukemia inhibitory factor (LIF) (311, 377), Wnt/ $\beta$ -catenin signals, and FGF/MAPK inhibition, which are instead innocuous or prodifferentiation cues to EpiSCs (see sect. IV).

In summary, it is clear that despite their mutual retention of pluripotent multilineage differentiation ability, mouse ESCs and EpiSCs diverge in their transcription factor expression, chromatin state, and extrinsic signaling dependencies. These differences reflect their differential embryological origins; mouse ESCs and EpiSCs derive from earlier and later stages on the concourse of development, respectively.

However, the developmental boundary between mouse ESCs and EpiSCs has become recently contested by the unexpected demonstration that each may be derived from unexpectedly early embryonic stages. For instance, EpiSCs have been derived from preimplantation blastocysts (237).

However, this does not indicate that EpiSCs occupy a naive epiblast-like state (cf. Ref. 237). In a parallel situation, mouse ESCs have also been seemingly derived from the zygote (344). This does not signify that ESC *per se* are totipotent nor that ESCs originate directly from the zygote, as the zygote lacks various ESC markers including *Nanog*, *Klf5*, and *Utf1* (123). Rather, the zygote likely develops in culture into blastocyst-stage epiblast from which ESCs may be derived (344). An analogous explanation suffices for why EpiSCs may be apparently derived from mouse blastocysts (237). We surmise that during the lengthy 2-wk EpiSC derivation period in these studies, the blastocyst naturally progressed to an egg cylinder-like intermediate that was the direct provenance of EpiSCs.

## B. Human and Mouse ESCs Occupy Distinct Developmental States

As with mouse ESCs (mESCs), human ESCs (hESCs) originate from the human blastocyst (284, 348), presumably from the epiblast. Nevertheless, hESCs are rather contradistinct to mESCs morphologically, transcriptionally, and in terms of requisite signaling requirements (TABLE 4). Strikingly, despite their apparent provenance from the preimplantation blastocyst, human ESCs share many (but not all) defining characteristics with mouse postimplantation epiblast-like EpiSCs, perhaps defining a shared “primed” pluripotent state cooccupied by hESCs and mEpiSCs.

Most outstandingly, hESCs are bereft of *TBX3*, *STELLA/DPPA3*, and *NROB1/DAX1*, which are markers of preimplantation ICM/peri-implantation epiblast in the mouse embryo (60, 70, 295). Instead, hESCs express postimplantation epiblast marker *OTX2* (118) and may express germ layer differentiation markers to some extent (291, 345), therefore paralleling the situation of mouse EpiSCs. Further accentuating the difference between mESCs and hESCs, they differentially express more than 2,000 genes and only commonly express some 200 genes (297).

Morphology and cell-surface marker profiles also serve to distinguish mouse versus human ESCs. mESCs form domed and refractive colonies, whereas hESCs assemble as flat and planar colonies very similar to mEpiSCs. While hESCs express cell surface markers *SSEA3*, *SSEA4*, *TRA-1-60*, and *TRA-1-81* (143, 348), mESCs lack all these markers and instead express *SSEA1* (215) (TABLE 1).

Human and mouse ESCs also harbor distinct chromatin states. For example, hESCs have lost the repressive H3K27me3 histone modification at developmental gene loci such as *OTX2* (345), potentially accounting for higher expression of lineage determinants in hESCs versus mESCs. Furthermore, some female hESC lines display X chromosome inactivation (44, 306) in contrast to mESCs (247). However, as discussed in the APPENDIX, X chromosome inactivation is not

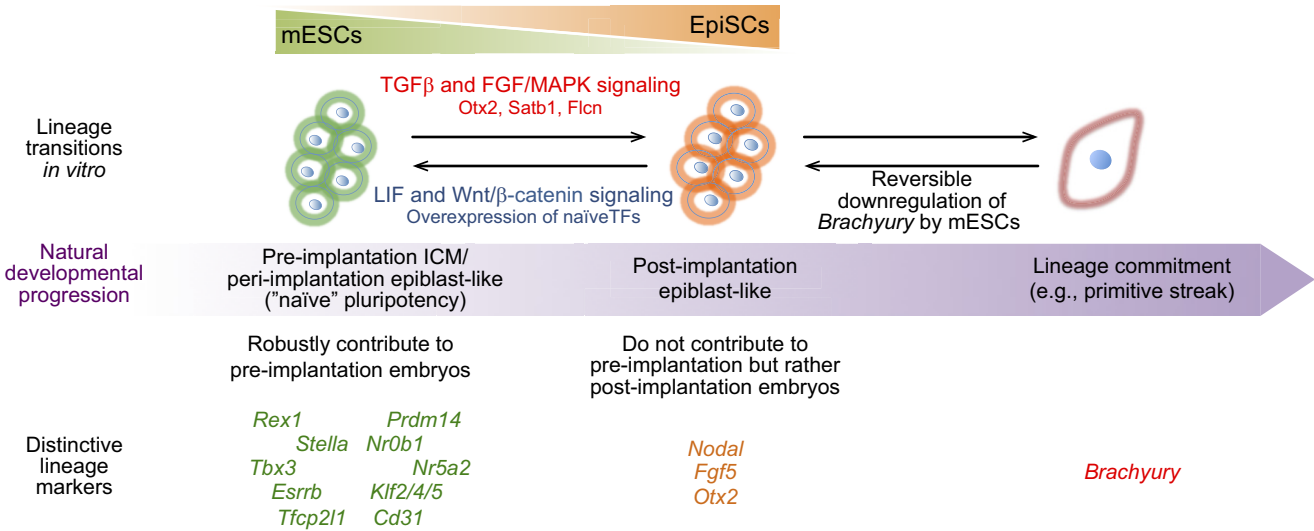
manifest in all female hESC lines (44, 306), and therefore, this attribute may not stringently distinguish the two types of ESCs. Nevertheless, taken as a whole, the molecular differences between hESCs and mESCs are substantial.

### C. Do Human ESCs Correspond to Postimplantation Epiblast?

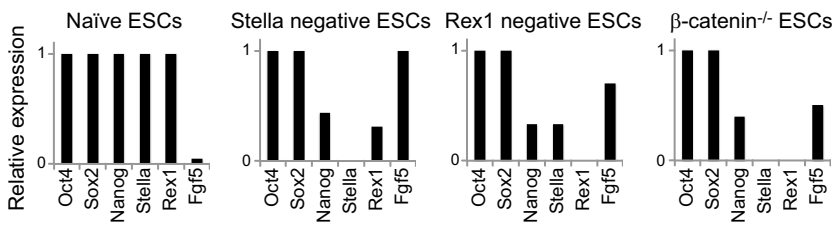
The various similarities between hESCs and mouse EpiSCs (TABLE 1, FIGURE 4, D AND E) may herald that hESCs functionally correspond to the human postimplantation epiblast (131, 247, 291).

Why do hESCs resemble the postimplantation epiblast when they are derived from the preimplantation blastocyst (284, 348)? The situation here appears to parallel the mouse situation wherein primed mEpiSCs can also apparently be derived from the blastocyst (237) (see sect. IIA); it is likely that that blastocyst-stage human naive epiblast matures into postimplantation epiblast in culture if the correct conditions to stably immortalize pre/peri-implantation human epiblast cells are not provided. Such differentiated postimplantation epiblast might be the actual cells from which hESCs are derived. Supporting this notion, human peri-implantation epiblast actually expresses naive markers

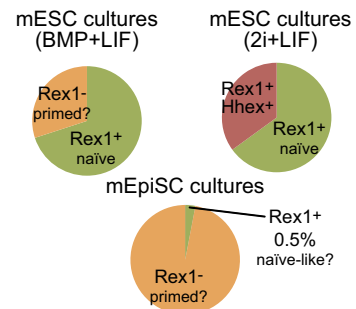
#### A Naïve mESCs and primed mEpiSCs: two distinct states that can spontaneously interconvert



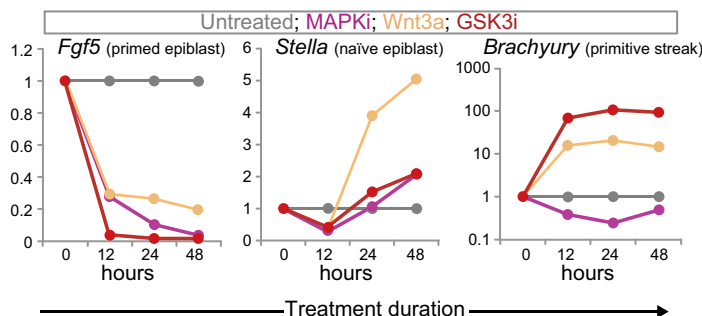
#### B Gene expression of naïve mESCs and their primed progeny



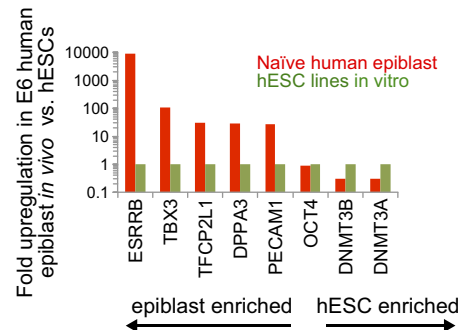
#### C Heterogeneous culture compositions



#### D Wnt signaling and MAPK blockade sustain naïve mouse pluripotency



#### E Loss of human naïvety upon typical hESC derivation



*ESRRB*, *STELLA*, *TFCP2L1*, *TBX3*, and *CD31/PECAM1* in vivo but then typically relinquishes them upon explantation (391) (FIGURE 3E). It then matures into an ambiguous intermediate with some characteristics of post-implantation epiblast prior to the emergence of fully fledged hESC lines (258).

However, whether hESCs and mouse EpiSCs are truly analogous has been challenged. There are several often-overlooked similarities between hESCs and mESCs. For example, akin to mESCs, hESCs express naive pluripotency TFs *REX1* and *PRDM14* (1, 66, 350); in the mouse, *Rex1* and *Prdm14* are specific markers of E3.5–E4.5 “naive” ICM/peri-implantation epiblast (289, 390) that are absent from “primed” EpiSCs (40, 345). Furthermore, some female hESC lines apparently harbor two active X chromosomes (44, 306), similar to the situation of female mESCs (see APPENDIX).

Given that hESCs display hallmarks of naive pluripotency to some extent, is it possible that hESCs are the genuine ex vivo counterparts of human peri-implantation epiblast, but that species differences between human and mouse account for the observed differences between hESCs and mESCs? Indeed, there are marked differences in human versus mouse blastocyst development (71, 241, 290) likely attributable to genuine evolutionary differences (178). For instance, the mouse embryo reaches the expanded blastocyst stage at ~E4.5, which is the time for optimal mESC derivation (42). However, the human conceptus matures to the expanded blastocyst stage by ~E6.0 (71), which is when hESCs are most efficiently established (62). A further differ-

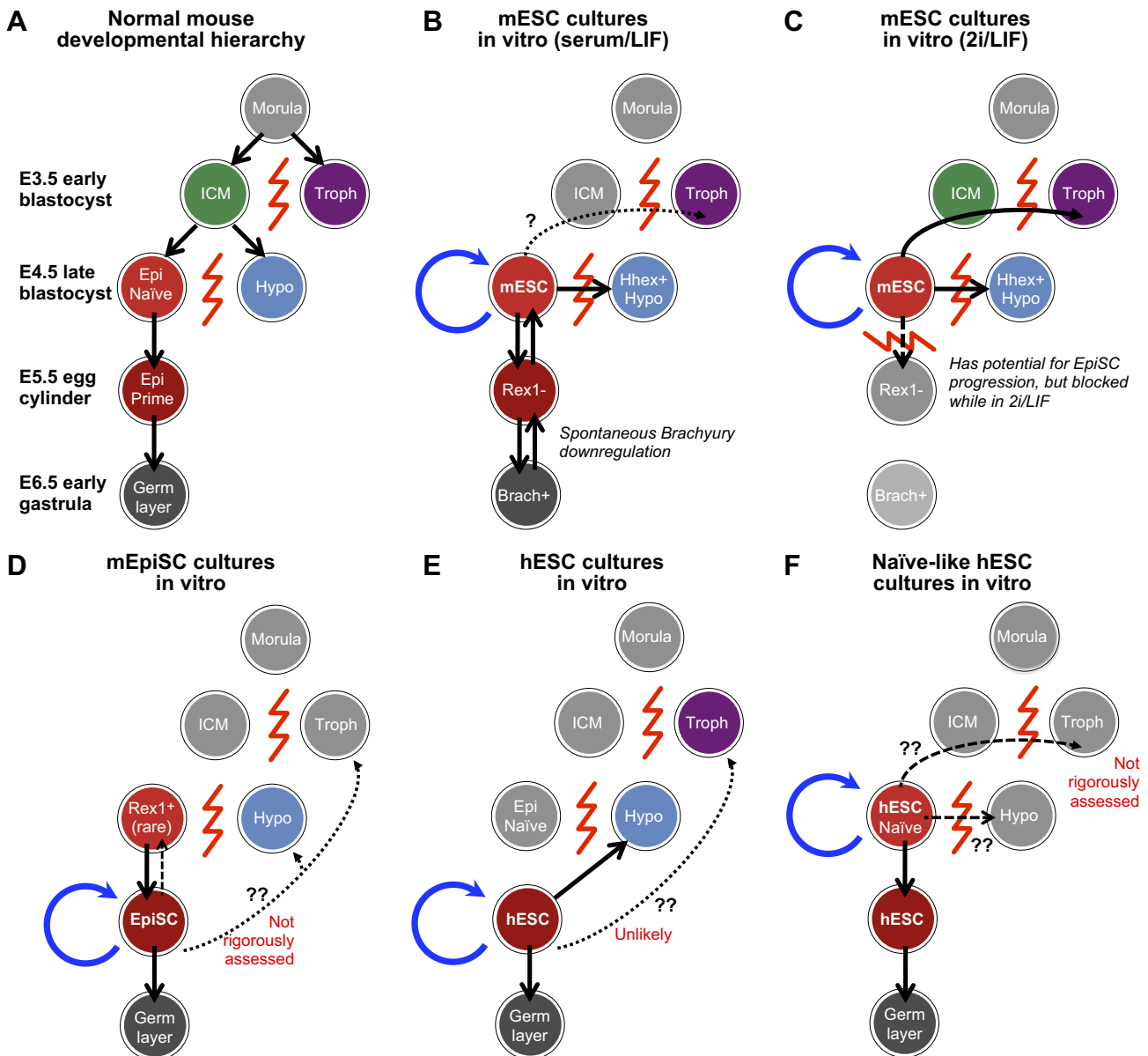
ence is that the mouse ICM expresses mESC marker SSEA-1, which instead marks trophoblast in human blastocysts (143). hESCs instead share with the human ICM expression of SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 (143), which are absent from mESCs. Hence, some assert that hESCs resemble a pre/peri-implantation naive epiblast state, given that hESCs are not fully equatable to mouse EpiSCs.

However, whether conventional hESCs truly resemble naive epiblast seems less likely given the recent findings that particular conditions can cause hESCs to surrender their primed characteristics and instead gain certain features of naive epiblast (59, 105). Because a regression to a naive-like state is possible (explained below), by inference conventional hESCs likely occupy a postimplantation epiblast state.

## D. At Last: Rejuvenating Human ESCs to a Naivelike State

The long-awaited derivation of naivelike pluripotent stem cells from human blastocysts (105) has finally filled the vacant position of a human naive pluripotent lineage in the family tree of pluripotent stem cells (FIGURE 4F, TABLE 1). These naivelike hESCs may be directly obtained from human blastocysts and can also be generated by manipulating conventional primed-type hESCs to induce regression to a more primitive state (59, 105, 346). Naivelike hESCs are distinguished by increased expression of human peri-implantation epiblast (391) markers *STELLA* and *TBX3*

**FIGURE 3.** Continual flux between naive and primed states. *A*: a schematic representation of “naive” and “primed” pluripotent cells. Naive cells (e.g., mESCs) are characterized by a number of “naive”-specific transcription factors and can robustly contribute to preimplantation embryos. In contrast, primed cells (e.g., mEpiSCs) are distinguished by several “primed”-specific regulatory genes and normally only can engraft into postimplantation embryos, not preimplantation embryos. Naive pluripotent cells are more developmentally primitive, whereas primed pluripotent cells are on the precipice of germ-layer differentiation. Though developmental progression occurs unidirectionally during embryogenesis as naive pluripotency segues into primed pluripotency, there can be continual flux between naive and primed states in vitro in certain culture conditions. TGF- $\beta$  and FGF/MAPK signaling drive naive cells towards the primed epiblast state, potentially through regulators *Otx2* (3, 45), *Satb1* (298), and *Fln* (29) that are important in establishing the primed state. In contrast, LIF/STAT3 and Wnt/ $\beta$ -catenin signaling (in conjunction with other factors, including overexpression of naive pluripotency TFs; see sect. VB) can reversibly rejuvenate primed EpiSCs towards a naive state once more. *B*: different transcriptional programs underpin distinct pluripotent states. Naive ESCs display high levels of expression of *Nanog*, *Stella*, and *Rex1* and minimally express postimplantation epiblast marker *Fgf5*. mESCs that spontaneously downregulate *Stella* or *Rex1* (see sect. V) express naive markers at reduced levels and instead have elevated expression of *Fgf5*, reflecting primed-like identity (140, 349). Meanwhile,  $\beta$ -catenin<sup>-/-</sup> ESCs (cultivated in standard culture conditions) do not express *Rex1* and show upregulation of *Fgf5*, demonstrating Wnt/ $\beta$ -catenin signaling is important for the naive state (10). These data are qualitative representations of other groups’ respective data (10, 140, 349) and are not meant to be exact. *C*: mouse pluripotent stem cell cultures are markedly heterogeneous and continually fluctuate between different lineages. BMP- and LIF-grown mESC cultures contain a 30% *Rex1*<sup>-</sup> EpiSC-like subpopulation (349). 2i- and LIF-grown mESC cultures are uniformly *Rex1*<sup>+</sup> and *CD31*<sup>+</sup> (379, 380), yet ~65% of these cells coexpress hypoblast marker *Hhex* and have extraembryonic lineage potential (232). mEpiSC populations are largely primed but may contain a small (~0.5%) *Rex1*<sup>+</sup> naive-like subfraction (129). Therefore, lineage interconversions are constantly ongoing in most pluripotent stem cell cultures. *D*: E14 mESCs in feeder-free conditions were treated with varying naive stimuli, a MAPK/ERK inhibitor (PDO325901), Wnt3a, or a GSK3 inhibitor (CHIR99021), for 12–48 h [K. M. Loh and S. W. S. Lim, unpublished data]. All three stimuli suppress primed epiblast marker *Fgf5* and induce naive epiblast marker *Stella*. However, Wnt/ $\beta$ -catenin agonism (Wnt3a or CHIR99021) also concomitantly upregulates primitive streak marker *Brachyury*, which is instead repressed by MAPK/ERK blockade. *E*: analysis of single-cell RNA-seq data of single naive epiblast cells from human E6.0 late blastocysts or single hESCs (391). The naive epiblast highly expresses *ESRRB*, *TBX3*, *TFCP2L1*, *STELLA/DPPA3*, and *CD31/PECAM1*, yet these naive markers are downregulated upon conventional hESC derivation. Instead, markers of the primed state including *DNMT3A* and *DNMT3B* are increased, culminating in conventional primed-type hESC lines. Therefore, the normal hESC derivation procedure does not capture the naive state present in vivo.



**FIGURE 4.** In vivo lineage restriction impositions and the expanded multilineage potential displayed by cultured pluripotent stem cells in vitro. *A:* ICM and trophoblast are distinctly segregated from totipotent morulae, and cannot interconvert after a given stage (lineage barrier represented by red thunderbolt). Subsequently, the ICM irreversibly bifurcates into the hypoblast and the naive epiblast, and a stringent lineage restriction is imposed between the two states. Naive epiblast unidirectionally matures into primed epiblast, which then forms various germ layers. *B:* mESCs cultured in conventional serum conditions largely self-renew in a naive epiblast state but are capable of breaching a developmental barrier, as they readily form hypoblast. They may also have weak trophoblast potential. Furthermore, they spontaneously differentiate into Rex1<sup>-</sup> EpiSC-like cells (349) and even Brachyury<sup>+</sup> primitive streak-like cells (324) yet seemingly can reversibly regain naive status. *C:* mESCs cultured in 2i/LIF environs largely self-renew in a naive epiblast state but unexpectedly can access both hypoblast and trophoblast (232), therefore subverting two developmental lineage restrictions. However, they do not spontaneously mature into Rex1<sup>-</sup> EpiSC-like cells, because EpiSC differentiation is strongly inhibited in 2i/LIF. *D:* mEpiSC cultures largely consist of self-renewing primed epiblast cells, but these infrequently interconvert with a Rex1<sup>+</sup> naive mESC-like state (which comprises ~0.5% of the population). There are claims that mEpiSCs can differentiate into hypoblast and trophoblast, but these have yet to be rigorously assessed. *E:* conventional hESC cultures largely consist of self-renewing primed epiblast cells. Yet a number of reports suggest they can apparently form hypoblast, which represents another developmental “transdifferentiation.” Claims that hESCs can differentiate into trophoblast have been contested. *F:* naive-type hESC cultures contain self-renewing naive-like epiblast cells, although these populations apparently still contain a degree of heterogeneity. Their capacity to form hypoblast and trophoblast has not yet been interrogated.

among others (59, 105, 346). They also harbor several additional trademarks of naive epiblast identity seen in the mouse, including preferential utilization of the preimplantation-specific proximal *Oct4* enhancer, dependence on LIF/Stat3 signaling for their propagation, global DNA hypomethylation, and in female cell lines, X chromosome reactivation (59, 105).

However, the human and mouse naive pluripotent states should not be conflated together, just as a human is not a mouse (218) (TABLE 1). Naivelike hESCs express human ICM markers (143) SSEA-4, TRA-1-60, and TRA-1-81 (which are absent from mESCs), yet they do not express mouse naive marker SSEA-1 (59, 105). Therefore, human and mouse differences in epiblast cell-surface marker expression in vivo (143) are likewise respected in vitro by their respective naive ESCs. These species divergences also apparently extend to requisite extrinsic signaling inputs for naive pluripotency.

Surprisingly, the signaling environment required to sustain naivelike hESCs is a hybrid of the conditions used to maintain both naive and primed mouse cells. Namely, naivelike hESCs require provision of LIF, activation of Wnt/ $\beta$ -catenin signaling, and suppression of mitogen-activated protein kinase (MAPK)/ERK (59, 105), as expected of naive mouse cells (discussed in sect. IV). Yet, they also contemporaneously require TGF- $\beta$  and FGF signaling (59, 105): two signals critical for mouse primed EpiSCs that instead drive naive mESC differentiation (see sect. IV). A priori, this unorthodox marriage of mouse naive and primed conditions was not expected to sustain human naive pluripotency. Yet this signaling regime apparently suffices to capture naive-type hESC lines de novo from human blastocysts (105), attesting to its robustness. The naive human state may also be consolidated by a number of additional signaling manipulations, including BMP/AMPK inhibition (59), p38 MAPK and JNK/MAPK blockade, protein kinase C (PKC) antagonism (105), Src inhibition and B-Raf inhibition in the context of feeder coculture (346), and potentially, transient histone deacetylase inhibition (369). The paradox of why both mouse naive and primed stimuli are used to maintain naive human cells could be resolved by examining whether these signals are endogenously active within the human blastocyst-stage epiblast in vivo. If so, this would provide a developmental rationale for why these signals can sustain human naive pluripotency in vitro.

However, several ambiguities regarding these naive-like hESCs remain to be resolved. First, although these cells critically depend on FGF signaling, their production also relies on simultaneous inhibition of MAPK/ERK, p38 MAPK, and JNK/MAPK signaling (59, 105), which constitute the main signaling cascades subordinate to FGF. It remains unclear why FGF must be activated yet why most of its downstream effectors must also be silenced to main-

tain naive human pluripotency. However, FGF also activates PI3K/Akt signaling, which is crucial for mESC self-renewal (266): presumably PI3K/Akt signaling is a crucial effector in this situation. A second issue regards the in vivo contribution of naive-type hESCs. Incredibly, naivelike hESCs can integrate into preimplantation mouse embryos and contribute to E10.5 postimplantation embryos, although the specific lineage(s) to which these cells contribute remain to be determined (105). Such contribution has been interpreted as prima facie evidence of the naive identity of these cells, as conventional primed-type hESCs rarely if ever contribute to preimplantation mouse embryos (155). Nevertheless, this must be interpreted tentatively, as even primed mEpiSCs can similarly contribute to preimplantation embryos if E-cadherin is simply overexpressed (261) and indeed this gene is upregulated in naivelike hESCs (105). Finally, at a clonal level, naivelike hESC cultures are heterogeneous, with only a subset expressing *TBX3* (59). The implications of this heterogeneity remain as-of-yet unclear (see sect. V).

In summary, a naivelike epiblast state clearly exists in human blastocysts demarcated by *TBX3* and *STELLA* (391) (FIGURE 3E). However, upon explantation into primed-type culture conditions in which the naive state is not stabilized, these naive markers are subdued (FIGURE 3E), and instead, postimplantation epiblast-like hESCs arise, as evinced by the original derivation of conventional hESCs in 1998 by Thomson (348). It was 15 years later that the human naive epiblast state could be stabilized and immortalized in vitro, yielding naivelike hESCs (59, 105). This was achieved by painstakingly deconvoluting signals essential for naive pluripotency. Therefore, in retrospect, the initial derivation of naive mESCs from mouse blastocysts by Evans, Kaufman, and Martin in 1981 was propitious (97, 215). In the 1980s, little was known about epiblast self-renewal cues. Yet based on preexisting protocols shaped around teratocarcinoma-derived cell lines (217), feeder layers and feeder-conditioned media were employed and serendipitously managed to capture naive epiblast as mESC lines (97, 215). Only three decades later could naive human pluripotency be similarly captured from human blastocysts.

### E. Mouse ESCs Possess Expanded Lineage Capabilities Compared With Peri-implantation Epiblast

Although mESCs resemble peri-implantation epiblast in their capacity to generate fetal cell types in vivo (111, 236), they seemingly possess expanded multilineage potential in vitro (TABLE 2, FIGURE 4, B AND C). While peri-implantation epiblast has largely lost the capacity to generate extra-embryonic trophoectoderm and hypoblast in vivo (109, 111) (FIGURE 4A), in contrast, mESCs can readily differentiate in culture into hypoblast (89, 234, 243, 277) (FIGURE 4, B AND C). This is unprecedented because by E4.5, extra-

**Table 2.** A comparison of the multilineage differentiation potential of pluripotent cells and related cells during development and in vitro culture

	Definitive Endoderm	Mesoderm	Definitive Ectoderm	Hypoblast (Primitive Endoderm)	Trophectoderm
mESCs	+	+	+	+	Uncertain
mEpiSCs	+	+	+	Uncertain	Uncertain
hESCs	+	+	+	Uncertain	Uncertain
ICM	+	+	+	+	Early ICM only (245)
Peri-implantation epiblast (E4.5)	+	+	+	— (111)	—
Postimplantation epiblast (E5.5)	+	+	+	—	—

A positive sign marks that the cell type in question is capable of readily differentiating towards the relevant lineage without exogenous transcription factor overexpression or downregulation/deletion. A negative sign indicates lack of competence to naturally differentiate towards the relevant lineage.

embryonic hypoblast and the pluripotent epiblast have irreversibly segregated (109, 111) from the ICM (**FIGURE 1B**). The ability of mESCs to “cross lineages” and concomitantly generate both hypoblast as well as fetal cell types is evocative of the early ICM as opposed to the epiblast (**TABLE 2**). To this end, it is noteworthy that Beddington and Robertson (20) asserted many years ago that “it would appear that ESCs resemble early (ICM) cells in their developmental potential,” which contrasts with more recent proposals that mESCs are most closely related to the peri-implantation epiblast (247).

Do mESC truly resemble early ICM? In our view, evidence for this thesis remains limited. In vivo, the early blastomeres/morulae coexpress epiblast marker *Nanog* and hypoblast marker *Gata6* [signifying ICM-like bipotentiality (292)], yet mESC largely express only *Nanog*, consistent with an epiblast identity. Furthermore, mESCs can spontaneously differentiate into *Sox17*<sup>+</sup> hypoblast (242), perhaps contributing to reports of hypoblast lineage potential/ marker expression formerly attributed to mESCs themselves. In vivo analysis of lineage potential also argues against a putative ICM identity. When Beddington and Robertson (20) injected small numbers (10–15) of mESCs into mouse blastocysts, there was typically robust fetal contribution but infrequently, extraembryonic hypoblast or trophoectoderm outcomes were noted (20). Nevertheless, when single mESCs were rigorously transplanted, fetal lineages were exclusively formed but extraembryonic cell-types never emerged (20), therefore clonally demonstrating that mESC are mostly constrained to a pluripotent (that is, fetal-restricted) fate. Finally, direct transcriptional comparison of mESCs with their counterparts within the early mouse embryo indicates that mESCs are most similar to E4.5 epiblast, not ICM (35).

Hence, one surprise in recent years is that mESCs may also have some faculty to differentiate into trophoectoderm (210, 232, 299) (**FIGURE 4C**). Although it remains controversial, the latest reports have validated the identity of mESC-derived trophoectoderm via morula aggregation (210, 232).

Such “totipotent” capacity appears restricted to small subpopulations of mESC cultures (210, 232), perhaps explaining why trophoectoderm potential was formerly overlooked when the potential of single mESCs was analyzed (20). Why some mESCs are capable of trophoectodermal differentiation is particularly unclear, as from a developmental perspective, this is clearly an illegitimate fate choice for epiblast cells (**FIGURE 4A**). Although these data are difficult to reconcile, this may suggest that after their explantation and in vitro propagation, rare subsets of mESCs might spontaneously “regress” to an ICM-like stage, as early ICM is still transiently capable of committing to trophoectoderm (245) (**TABLE 2**). Pluripotent stem cell lines propagated in vitro may acquire different capacities from their embryological antecedents when withdrawn from their native embryonic constraints in vivo. Altogether, this debate fundamentally challenges our knowledge of the origin and developmental competence of cultured pluripotent cell lines as we attempt to assign their position in the known rigid hierarchy of embryonic lineage restrictions in vivo (202).

## F. Expanded Multilineage Potential of Human ESCs and Mouse EpiSCs?

Further challenging the notion of established lineage hierarchies, it was suggested that mouse EpiSCs and hESCs are also capable of generating trophoectoderm (9, 40, 385, 399) (**TABLE 2, FIGURE 4, D AND E**). This phenomenon again belies an easy developmental explanation (291) as postimplantation epiblast is incapable of forming trophoectoderm in vivo. Some have interpreted the ascribed trophoectoderm potential of hESCs to mean that hESCs originate from or are related to the ICM (291), which as aforementioned is transiently capable of forming trophoectoderm (at least in the mouse embryo) (245). Nevertheless, the notion that hESCs/mEpiSCs can differentiate into trophoblast has been challenged on the grounds that these differentiated cells may in fact resemble mesoderm (26), which is an expected developmental outcome of epiblast. Therefore, the jury is still out on whether hESCs and mEpiSCs truly harbor trophoblast potential, or

whether they occupy a fetal-restricted, postimplantation epiblast state (202) (FIGURE 4, D AND E).

That being said, there are numerous reports that hESCs can differentiate into hypoblast (73, 272), which should also be a developmentally inaccessible extraembryonic fate for postimplantation epiblast *in vivo*. Therefore, the extraembryonic competence of hESCs and mEpiSCs *in vitro* remains to be reconciled with the *in vivo* situation (FIGURE 4, D AND E). In summary, while pluripotent stem cells are a close approximation of their *in vivo* counterparts, they seem to possess expanded multilineage differentiation potential and can breach various lineage barriers that are typically imposed *in vivo* (FIGURE 4, TABLE 2).

### G. Similarities Between Primed Pluripotent Cells and the Primitive Streak

Adding to the uncertainty of where hESCs/mEpiSCs precisely reside in the developmental lineage hierarchy, although mEpiSCs were originally derived from and thought to correspond to the E5.5–E5.75 postimplantation pregastrulating epiblast (41, 345), they may instead resemble a more developmentally advanced fate, the primitive streak. At the onset of gastrulation (~E6.5) in mouse embryogenesis, the posterior epiblast differentiates to form the primitive streak (91), which contains progenitors to both the definitive endoderm and mesoderm germ layers (183, 331). Contrary to initial expectations that primed pluripotent cells correspond to pregastrulating (~E5.5–E5.75) epiblast, a subpopulation of mEpiSCs expresses primitive streak TF Brachyury (27) and likewise hESCs also express primitive streak marker *WNT3* to some extent (161). Furthermore, mEpiSCs can be derived from not only pregastrulating epiblast, but also from gastrulating embryos until expectedly late developmental timepoints: E7.5–E8.0 (175, 264). Finally, the signaling pathways that maintain hESCs/mEpiSCs, namely, TGF- $\beta$  and FGF (summarized in sect. IV), mirror the signaling requirements for primitive streak induction *in vivo* (74, 148) and *in vitro* (26, 201, 399) (TABLE 4).

However, hESCs/mEpiSCs may not fully resemble a primitive streaklike intermediate en route to differentiation. *In vivo* (50) and *ex vivo* (49), PS progenitors undergo an overt epithelial-to-mesenchymal transition as they relinquish E-cadherin, decant from the epithelium of the pluripotent epiblast and acquire migratory capacities. In contrast, hESCs/mEpiSCs exist as epithelialized colonies *in vitro* (17, 41, 345), much like primary explants of mouse postimplantation epiblast (49). Finally, though  $\beta$ -catenin is crucial for primitive streak specification (148),  $\beta$ -catenin<sup>-/-</sup> mEpiSCs readily self-renew in culture (321), again implying that mEpiSCs/hESCs may be distinct from primitive streak. Instead of corresponding to ~E6.5 primitive streak, we instead suggest that primed pluripotent stem cells (or a subset of them) may instead approximate ~E6.0–E6.25 prestreak

posterior epiblast, an intermediate transition state wherein *Brachyury* and *Wnt3* are expressed (286) in anticipation of primitive streak formation, yet wherein *Oct4* and *Sox2* continue to be expressed and ectoderm potential is still retained (144, 192). However, the exact developmental correspondence between primed pluripotency and the posterior epiblast/primitive streak state remains to be fully clarified.

Further complicating the matter, “primed pluripotency” seems to span a range of related lineages, as individual hESC and mEpiSC lines vary in their molecular status. For instance, *WNT3* levels differ between individual hESC lines (161). Given that hESC derivation is a protracted, ill-defined process, we speculate that line-to-line variability in primed pluripotent stem cell lines might reflect derivation from slightly earlier or later developmental stages for each individual line (before or after the onset of primitive streak marker expression in the epiblast) or differing anterior-posterior position of the original donor cell within the primed epiblast. Even within a given primed pluripotent stem cell line, Brachyury can be heterogeneously expressed (27), indicating that ~E5.5 *Brachyury*<sup>-</sup> epiblast-like and ~E6.0–E6.25 *Brachyury*<sup>+</sup> posterior epiblast-like progenitors might coexist in primed mEpiSC cultures. The relative proportions of these subpopulations might vary between individual hESC/mEpiSC lines, further contributing to line-to-line variability.

In sum, the original binary classification of “naive” versus “primed” pluripotency (247) is a useful conceptual framework to broadly assign pluripotent subtypes, yet in reality it seems that pluripotent cells in culture can occupy a complex diversity of graded states along a developmental continuum (218). For instance, some “naive” peri-implantation epiblast-like mESCs tend towards an even earlier ICM-like state with extraembryonic potential (see sects. IIE and V), while some “primed” postimplantation epiblast-like mEpiSCs gravitate towards a more primitive streaklike fate, implying a spectrum (as opposed to a duality) of developmental states.

## III. PLURIPOTENCY FACTORS POTENTIATE DIFFERENTIATION

### A. Individual Pluripotency Factors Inhibit Lineage-Specific Differentiation

Pluripotency is initiated and maintained by a regime of TFs, chromatin regulators, and other regulatory genes (overviewed in TABLE 3). Although pivotal for pluripotency, how these pluripotency factors mechanistically instill ESCs with their multilineage potential remains uncertain.

The past 16 years have seen a deluge of genetic loss-of-function studies, either by genetic deletion or RNA interference, to identify genes essential for pluripotency *in vitro* or

**Table 3.** *Transcriptional regulators controlling pluripotency*

Gene	Expression	Knockdown/knockout phenotype	Overexpression phenotype
Oct4 (Pou5f1)*†‡	Naive and primed	<p><i>Core pluripotency factors (naive and primed)</i></p> <p>KD/KO: Trophoctoderm differentiation (in mESCs) (139, 255)</p> <p>KD: Trophectoderm or ectoderm differentiation (in hESCs) (139, 366)</p> <p>KO: Defective epiblast and hypoblast development; gradual respecification of ICM to the trophoctoderm in vivo—early embryonic lethality (in <i>Oct4</i><sup>-/-</sup> mice) (103, 249)</p> <p>KO: Ectoderm development potentially unimpaired, but primitive streak formation apparently abrogated in vivo (upon conditional <i>Oct4</i> deletion at E6.0–E6.5 in <i>ROSA26-CreER</i>; <i>Oct4</i><sup>fl/fl</sup> mice) (84)</p> <p>KD: Crippled postimplantation mesoderm development—embryonic lethality or developmental delay (upon <i>Oct4</i> siRNA injection into E3.5 blastocysts and blastocyst transfer to pseudopregnant recipients) (403)</p> <p>Partial KD: Enhanced self-renewal; self-renewal in LIF alone (<i>Oct4</i><sup>+/-</sup> mESCs) (171) or self-renewal in basal media alone (in mESCs with 15–20% of wild-type <i>Oct4</i> levels) (279)</p>	<p>OE: Mesoderm, ectoderm, and primitive endoderm differentiation (in mESCs) (255, 302); but also seen when nonDNA-binding Oct4 mutant is overexpressed (254)</p> <p>OE: Enhanced primitive streak differentiation and reduced ectoderm differentiation (in hESCs) (366)</p>
Sox2*†‡	Naive and primed	<p>KO: Trophectoderm differentiation (in mESCs) (219)</p> <p>KD: Primitive streak differentiation (in hESCs, upon double <i>SOX2/SOX3</i> knockdown) (366)</p> <p>KO: Defective epiblast development—early embryonic lethality (in <i>Sox2</i><sup>-/-</sup> mice); also some trophoblast defects (15)</p>	<p>OE: Ectoderm differentiation (in mESCs) (177)</p> <p>OE: Enhanced ectoderm differentiation and inhibition of primitive streak differentiation (in hESCs) (366)</p>
Nanog*†‡	Naive and reduced in primed	<p>KD: Trophectoderm, mesoderm and ectoderm differentiation (in mESCs) (150)</p> <p>KD: Ectoderm differentiation (in hESCs) (343, 353)</p> <p>KO: Defective epiblast development and elevated ICM apoptosis—early embryonic lethality (in <i>Nanog</i><sup>-/-</sup> mice) (228, 304)</p> <p>KO: Reduced self-renewal and increased spontaneous hypoblast differentiation, though undifferentiated cells can still be maintained (in <i>Nanog</i><sup>-/-</sup> mESCs) (57)</p>	<p>OE: Resistance to differentiation and LIF-independent self-renewal (in mESCs) (56, 228)</p> <p>OE: Enhanced primitive streak differentiation and inhibition of ectoderm differentiation (in hESCs) (343, 353, 366)</p>
Sall4*	Naive and primed	<p>KD: Trophectoderm differentiation (in mESCs) (405)</p> <p>KO: Reduced proliferation (in <i>Sall4</i><sup>-/-</sup> mESCs) (294)</p> <p>KO: Postimplantation lethality at ~E6.5, though unclear whether epiblast specification is markedly impaired (294) (in <i>Sall4</i><sup>-/-</sup> mice)</p>	
Foxd3*†‡	Naive and primed	<p>KO: Primitive streak differentiation (in <i>CAG-CreER</i>; <i>Foxd3</i><sup>fl/fl</sup> mESCs, upon <i>Foxd3</i> excision) (199)</p>	<p>OE: Mesoderm differentiation (in hESCs) (11)</p>

Continued

Table 3.—Continued

Gene	Expression	Knockdown/knockout phenotype	Overexpression phenotype
		KO: Pluripotent epiblast largely absent in postimplantation embryo (in <i>Foxd3</i> <sup>-/-</sup> mice) (132)	
<i>Zic3</i> *	Naive and primed	KD: Endoderm differentiation (in hESCs) (11) KD: Endoderm differentiation (in mESCs) (198)	OE: Mesoderm and ectoderm differentiation (mESCs) (unpublished observations)
<i>c-Myc</i> *	Naive and primed	KO: Hypoblast differentiation (in <i>c-Myc</i> <sup>-/-</sup> ; <i>n-Myc</i> <sup>-/-</sup> mESCs) (314, 359)	OE: Temporarily-improved LIF-independent self-renewal (55)
<i>n-Myc</i> *	Naive and primed?	KO: Hypoblast differentiation (in <i>c-Myc</i> <sup>-/-</sup> ; <i>n-Myc</i> <sup>-/-</sup> mESCs) (314, 359)	
		<i>Naive-specific pluripotency factors</i>	
<i>Esrrb</i> *	Naive-specific	KD: Ectoderm and endoderm differentiation (in mESCs) (100, 150)  KD: Partial abrogation of self-renewal (LIF-grown mESCs); complete abrogation of self-renewal (2i-grown mESCs) (214)  KD: Reduced naive marker expression but undifferentiated status maintained (LIF-grown <i>Esrrb</i> <sup>-/-</sup> mESCs); abrogation of self-renewal (Wnt agonist-grown <i>Esrrb</i> <sup>-/-</sup> mESCs) (214)	OE: Endoderm differentiation and inhibition of mesoderm or ectoderm differentiation (in mESC-derived EBs) (150)  OE: LIF-independent self-renewal (408) (214) (in mESCs)
<i>Tbx3</i> *†	Naive-specific	KD: Trophectoderm or neuroectoderm differentiation (in mESCs) (150, 206)	OE: Hypoblast differentiation (in mESCs) (206)  OE: LIF-independent self-renewal (in mESCs) (256), presumably level-dependent effect
<i>Klf2</i> *	Naive-specific	KD: Apoptosis and abrogated self-renewal (in 2i-grown mESCs) (393)  KD: Decreased naive gene expression despite no morphological phenotype (in serum-grown mESCs) (393)	OE: LIF-independent self-renewal (in mESCs) (126)
<i>Klf4</i> *	Naive-specific	KD: No apparent phenotype (upon <i>Klf4</i> knockdown alone in mESCs) (159)  KD: Differentiation to a variety of lineages (upon combined <i>Klf2/Klf4/Klf5</i> knockdown in mESCs) (159)  KD: Enhanced endoderm differentiation (during mESC embryoid body differentiation) (8)	OE: LIF-independent self-renewal (in mESCs) (126, 256)
<i>Klf5</i> *‡	Naive-specific	KD: Primitive streak differentiation (269) or no apparent phenotype (159) (in mESCs)  KD: Upregulation of differentiation markers and decreased proliferation (in <i>Klf5</i> <sup>-/-</sup> mESCs)  KD: Peri-implantation lethality—defective blastocyst implantation in vivo and inability to derive mESC lines from the epiblast (from <i>Klf5</i> <sup>-/-</sup> mice) (93), potentially trophectoderm defects also  KD: Enhanced mesoderm differentiation (during mESC embryoid body differentiation) (8)	OE: LIF-independent self-renewal (in mESCs) (93)
<i>Nr5a2</i> (Lrh1)‡	Naive-specific	KO: Postimplantation lethality— <i>Oct4</i> expression unaltered in the ICM of the E3.5–E4.0 preimplantation blastocyst, but near-complete loss of <i>Oct4</i> expression in the epiblast of the E6.5–E7.0 gastrulating egg cylinder (in <i>Nr5a2</i> <sup>-/-</sup> mice) (121)	

Continued

Table 3.—Continued

Gene	Expression	Knockdown/knockout phenotype	Overexpression phenotype
Dax1 (NrOb1)* <sup>†</sup>	Naive-specific	KD: Hypoblast differentiation from mESCs (175) KD: Failure to successfully generate <i>Dax1</i> <sup>-/-</sup> mESCs by gene targeting in conventional culture conditions (175)	OE: Trophoblast differentiation (in mESCs) (322)
Prdm14*	Naive-specific	KD: Extraembryonic endoderm differentiation (in mESCs) (209) KD: Differentiation and abrogation of self-renewal (for serum-grown <i>Prdm14</i> <sup>-/-</sup> mESCs) (116, 390) KD: Morphologically unaffected, though differentiation genes upregulated (for 2i-grown <i>Prdm14</i> <sup>-/-</sup> mESCs) (116, 390) KD: Primitive streak differentiation (in hESCs) (66, 350)	OE: Increased resistance to spontaneous differentiation triggered by LIF withdrawal (116) OE: Impaired embryoid body differentiation (in hESCs) (350)
Tfcp2l1 (Crtr-1)*	Naive-specific	KD: Differentiation to a variety of lineages, including primitive streak (for LIF-grown mESCs) (213, 392) KD: Decreased proliferation but undifferentiated state intact (for 2i-grown mESCs) (213)	OE: LIF-independent self-renewal (for mESCs) and can enable self-renewal of <i>Stat3</i> <sup>-/-</sup> mESCs (213, 392)
<i>Signaling effectors for naive-specific extrinsic self-renewal stimuli</i>			
Stat3* <sup>‡</sup>	Naive-specific self-renewal activity	Primary transcriptional effector of LIF signaling in mESCs, phosphorylated by LIF-activated Jak1 kinase KD: Abrogation of undifferentiated self-renewal in the presence of LIF (for <i>Stat3</i> <sup>-/-</sup> mESCs) (283) DN: Abrogation of undifferentiated self-renewal in the presence of LIF (for mESCs) (253) KD: Undifferentiated self-renewal maintained in alternate culture conditions (for 2i-grown <i>Stat3</i> <sup>-/-</sup> mESCs) (395) KD: Early embryonic lethality upon combined maternal and zygotic <i>Stat3</i> deletion; epiblast severely compromised in E4.5 blastocyst (in <i>Stat3</i> <sup>-/-</sup> mice) (87)	OE: LIF-independent self-renewal (for mESCs overexpressing conditionally-activated STAT3ER) (220)
$\beta$ -Catenin* <sup>†</sup>	Naive-specific self-renewal activity (induces differentiation in primed cells)	Primary transcriptional effector of Wnt signaling in mESCs KD: Collapse of the naive state and transition to the primed state (for LIF-grown $\beta$ -catenin <sup>-/-</sup> mESCs) (10, 379) KD: Undifferentiated self-renewal maintained (for LIF/PDO325901-grown $\beta$ -catenin <sup>-/-</sup> mESCs) (379)	OE: Delayed differentiation following LIF withdrawal (329)
Tcf1*	Naive-specific self-renewal activity	Transcriptional effector of Wnt signaling in mESCs KD: Decreased self-renewal (for Wnt-grown mESCs) (394)	
Id1, Id2, and Id3*	Precise expression unclear	Primary transcriptional targets of BMP4 signaling in mESCs (396), though loss-of-function phenotype(s) not yet established	OE: BMP4-independent self-renewal in the presence of LIF (for mESCs) (396)

Continued

Table 3.—Continued

Gene	Expression	Knockdown/knockout phenotype	Overexpression phenotype
<i>Primed-enriched pluripotency factors</i>			
Otx2 <sup>†</sup>	Preferential expression in the primed state	KD: Enhanced naive pluripotency gene expression; refractory to differentiate into postimplantation epiblast (for <i>Otx2</i> <sup>-/-</sup> mESCs) (3, 45)	OE: Precocious exit from the naive state and acquisition of primed-associated characteristics (for mESCs) (3, 45, 150)
<i>Pluripotency factors of unclear naive versus primed specificity</i>			
Ncoa3*	Precise expression unclear	KD: Differentiation to postimplantation epiblast and primitive streak fates (273)	OE: Temporarily enhanced, but only transient, LIF-independent self-renewal (273)
Zfx*	Precise expression unclear	KD: Slower growth and spontaneous apoptosis (in mESCs) (107) KD: Decreased proliferation (in hESCs) (134)	OE: LIF-independent self-renewal and increased resistance to differentiation (in mESCs) (107) OE: Enhanced proliferation (in hESCs) (134)
Esrrg*	Precise expression unclear	KD: Differentiation (to unspecified lineages) (2) (in mESCs)	
Rhox5 (Pem)*	Precise expression unclear		OE: Blockade in mESC differentiation (especially to the endodermal lineage), LIF-independent self-renewal, and improper differentiation during teratoma formation (68, 98)
Ronin* <sup>‡</sup>	Precise expression unclear	KD: Peri-implantation lethality—inability to derive mESC lines from <i>Ronin</i> <sup>-/-</sup> blastocysts presumably due to extensive apoptosis and abrogated self-renewal (81)	OE: LIF-independent self-renewal (81)
Zfp143*	Precise expression unclear	KD: Differentiation to unspecified lineages, possibly trophectoderm (in mESCs) (64)	
Zfp206*	Precise expression unclear	KD: No morphologically apparent defect, though primed pluripotency marker <i>Fgf5</i> increased (in mESCs) (367)	OE: Enhanced self-renewal and improved resistance to retinoic-acid induced differentiation (in mESCs) (367)
Rif1*	Precise expression unclear	KD: Impaired proliferation (attributable to telomere defects, Ref. 79) as well as spontaneous differentiation, potentially to trophectoderm (in mESCs) (205)	
<i>Intrinsic transcriptional repressors of naive pluripotency</i>			
Utf1 <sup>†</sup>	Naive and primed	KD: Increased expression of pluripotency genes and perturbed in differentiation (in mESCs) (176) KD: Slower proliferation in undifferentiated state; perturbed in differentiation (in <i>Utf1</i> <sup>-/-</sup> mESCs) (158)	
Mbd3 <sup>†</sup>	Naive and primed	KD: Naive-specific pluripotency genes increased (285) and resistant to differentiation; in fact LIF-independent self-renewal possible (167) (in <i>Mbd3</i> <sup>-/-</sup> mESCs), though illegitimate trophoblast potential may be resuscitated	
Tcf3 <sup>†</sup>	Naive and primed	KD/KD: Pluripotency gene expression increased and mESCs become refractory to differentiation (in <i>Tcf3</i> -knockdown mESCs and <i>Tcf3</i> <sup>-/-</sup> mESCs) (72, 276, 335)	OE: Abrogates self-renewal (394)
Zfp281* <sup>†</sup>	Precise expression unclear	Role in driving self-renewal versus differentiation remains equivocal based on KD versus KO studies KD: Differentiation to primitive endoderm and potentially primitive streak (in mESCs) (365, 368), though	OE: LIF-independent self-renewal? (368)

Continued

Table 3.—Continued

Gene	Expression	Knockdown/knockout phenotype	Overexpression phenotype
		<i>Nanog</i> is purportedly upregulated (101)	
		KD: Self-renewal enhanced, pluripotency genes modestly increased (in <i>Zfp281</i> <sup>-/-</sup> mESCs) (102)	

A nonexhaustive summary of various transcriptional regulators that operate in the establishment and perpetuation of pluripotency, in vivo and/or in vitro. Preferential expression of certain pluripotency transcriptional regulators either in “naive” or “primed” pluripotent cells was based on in vivo expression patterns or expression in naive versus primed mouse pluripotent stem cell lines in vitro (some expression patterns differ in human pluripotent stem cells). In vivo knockout phenotypes that are not pertinent to pluripotency have been omitted. OE, overexpression; KD, knockdown; KO, knockout; DN, dominant negative. \*Known differentiation-inhibiting effects; †known pro-differentiation effects; ‡known in vivo pluripotency phenotype.

in vivo. Early defining studies from 1998 and onwards demonstrated that if TFs such as *Oct4* (248), *Sox2* (15), *Foxd3* (132), or *Nanog* (228) are genetically deleted in mouse embryos, the epiblast is compromised and consequently embryonic lethality results (TABLE 3). Such findings have since led to the acclaim of a “trinity” of key TFs (*Oct4*, *Sox2*, and *Nanog*) among others that are critical for pluripotency (305). Loss of these imperative pluripotency TFs within ESCs often provokes precocious differentiation into specific lineages or cell death (FIGURE 5A). For example, upon loss of *Oct4*, *Sox2*, or *Sall4*, mESCs exit self-renewal and specifically differentiate towards trophoblast (248, 255, 405), whereas knockdown of *Prdm14* or *Dax1* elicits hypoblast differentiation (209, 240). In hESCs, disabling either *OCT4* or *NANOG* begets ectoderm formation (353, 366), whereas suppression of *SOX2* leads to primitive streak differentiation (5, 343, 366).

Therefore, the prevailing “ground state” model of pluripotency asserts that pluripotency factors maintain pluripotency by practicing lineage-specific blockades on differentiation (153, 305, 398). Each pluripotency TF prohibits ESC differentiation towards a particular lineage such that coexpression of diverse pluripotency TFs suppresses differentiation to all available lineage outcomes. Therefore, in the ground state model, the pluripotent state is headed by an oppressive regime of TFs that unilaterally repress differentiation to maintain undifferentiated self-renewal (153, 305, 398). Such a transcriptional regime appears to be self-reinforcing and thus indefinitely perpetuating, as pluripotency TFs may recursively upregulate their own expression and that of other pluripotency factors, redundantly blocking downstream differentiation (153, 305, 398).

## B. Pluripotency Factors as Lineage Specifiers That Enable Differentiation

If ESCs are dominated by a monolithic regime of pluripotency factors that cooperatively inhibit differentiation to all prospective lineages, this compellingly explains the mecha-

nism of undifferentiated self-renewal. However, this model does not explain the most quintessential capability of pluripotent cells: their expansive multilineage differentiation potential. If pluripotency TFs curtail developmental potential, how could ESCs possess the capability to develop into all bodily lineages? We shall review a number of heretofore-contradictory observations over the past decade or so that depart from the established model.

If pluripotency factors solely inhibit differentiation, then overexpression of pluripotency TFs should render ESCs refractory to differentiation. Indeed, overexpression of specific pluripotency factors including *Prdm14* (116), *Tfcp2l1* (213, 392), *Ronin* (81), and *Zfx* (107) enables mESCs to resist differentiation to varying extents (TABLE 3). Nevertheless, these represent the exception rather than the rule.

Strikingly, modest overexpression of cardinal pluripotency factor *Oct4* in mESCs does not reinforce undifferentiated self-renewal. Instead, it induces mesoderm and hypoblast differentiation (255). Furthermore, overexpression of *Sox2* specifies neuroectoderm (177); overexpression of *Sall4*, *Tbx3*, or *Esrrb* elicits endoderm differentiation (150, 206, 405); and *Dax1* overexpression instructs trophoblast differentiation (FIGURE 5A, TABLE 3) (323). Other pluripotency factors also induce mESC differentiation when overexpressed (250). Similar effects have been observed in hESCs; *NANOG* overexpression induces supernumerary primitive streak differentiation (343, 399).

The prevailing dogma that pluripotency factors solely inhibit differentiation (153, 305, 398) does not explain why lineage-specific differentiation occurs when these factors are upregulated. Nevertheless, overexpression studies must be tentatively interpreted because extreme pluripotency factor induction may beget nonphysiological effects (127, 254). This therefore raises the question whether pluripotency factors genuinely specify lineage commitment under physiological conditions: if pluripotency TF overexpression artificially induces differentia-

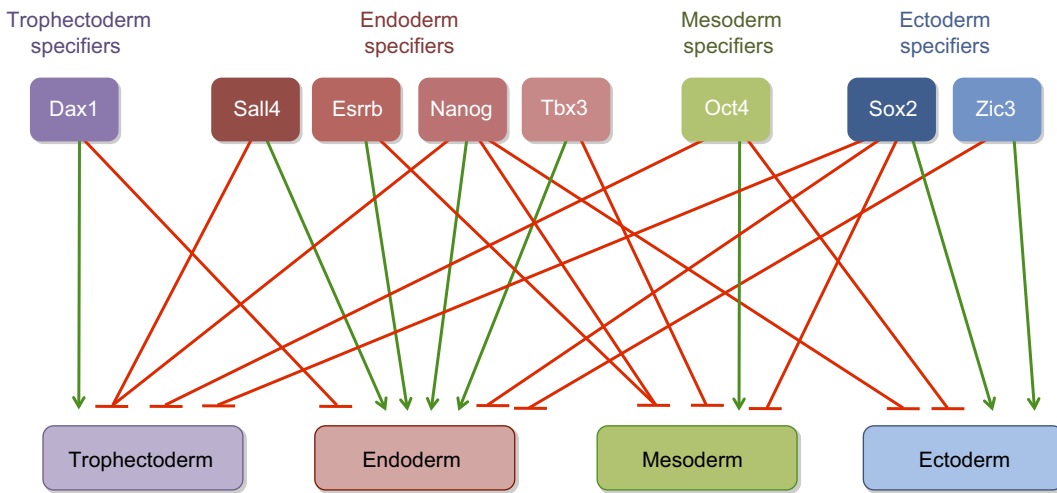
tion towards specific lineages, are pluripotency factors endogenously required for normal commitment to these same lineages?

Indeed, pluripotency TFs are endogenously required to enable the differentiation of pluripotent cells into specific lineage pathways both in vivo and in vitro. If they are ablated, differentiation towards these lineages is abrogated. For example, whereas *Oct4* overexpression ectopically drives mesoderm and hypoblast, acute *Oct4* knockdown deprives ESCs of the ability to differentiate into mesoderm (403) (FIGURE 5B). Likewise, in vivo ablation of *Oct4* either by siRNA injection into mouse blastocysts (403) or conditional *Oct4* deletion immediately preceding gastrulation (~E6.0–E6.5) (84) specifically cripples mesoderm devel-

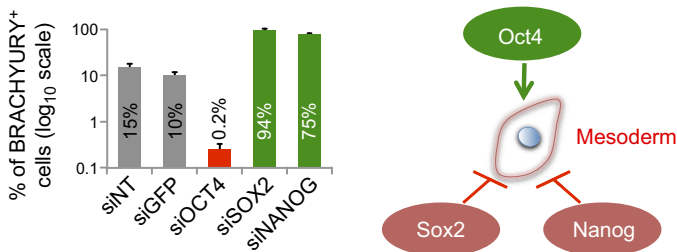
opment, leading to embryonic lethality. If *Oct4* is genetically ablated at earlier stages, hypoblast fails to form in the blastocyst (103). This therefore affirms the overexpression studies (255), showing that *Oct4* dually capacitates both hypoblast and mesoderm potential in mESCs. Similarly, whereas *Tbx3* induction elicits hypoblast induction, its knockdown reciprocally annuls the ability of mESCs to differentiate towards hypoblast (150, 206), therefore indicating *Tbx3* is natively required for hypoblast potential. Thus pluripotency factors endow naive mESCs with the competence to subsequently generate various developmental lineages (FIGURE 5C, TABLE 3).

Pluripotency factors are equally critical to bequeath primed hESCs with the capacity for germ-layer formation. *OCT4*

**A The transcription factor regime overseeing pluripotency**



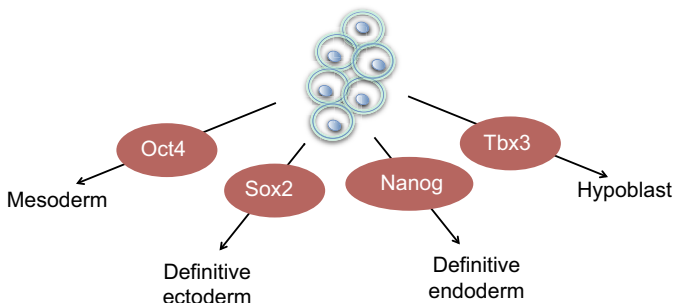
**B OCT4 potentiates, whereas SOX2 and NANOG blocks, mesoderm**



**D Lineage-specifying pluripotency factors duel in ESCs**



**C Pluripotency factors endow ESCs with multilineage potential**



expression endows hESCs with the capacity to form mesoderm, whereas *SOX2* is essential for ectoderm differentiation and *NANOG* is imperative for definitive endoderm generation (343, 366). These differentiation-deficient phenotypes do not constitute a total loss of “pluripotency” after pluripotency TF ablation because in each of these instances, only differentiation to a specific lineage is abrogated. For example, *SOX2* suppression in hESCs selectively disrupts ectoderm development, whereas endoderm differentiation remains unimpaired or even enhanced (366).

Therefore, we propose that some pluripotency factors are lineage specifiers that drive ESC differentiation towards a specific lineage of interest while suppressing “mutually exclusive” fates (FIGURE 5A). For example, *SOX2* induces ectoderm from hESCs and simultaneously represses endoderm and mesoderm, blocking alternate options to solely induce ectoderm (343, 366). In contrast, *NANOG* instigates definitive endoderm from hESCs by concomitantly inhibiting mesoderm and ectoderm (343, 366). This reconciles the widely known behavior of pluripotency factors inhibiting specific lineages (153, 305, 398). In fact, lineage-specifying pluripotency factors may suppress differentiation towards mutually exclusive lineages to unidirectionally direct ESCs to their specific lineage of interest, akin to classical lineage specifiers in other developmental systems (117).

Altogether, we surmise that certain pluripotency factors are bona fide lineage specifiers, each of which confers ESCs with the innate ability to differentiate into a specific embryonic lineage (203) (FIGURE 5C). Coexpression of diverse lineage-specifying pluripotency TFs (e.g., Oct4, Sox2, and Tbx3) therefore enables ESCs to access all available lineage options (203) (FIGURE 5C). This potentially explains the multilineage potential of pluripotent cells (203). Even in the uncommitted state, pluripotency TFs preconfigure ESCs for

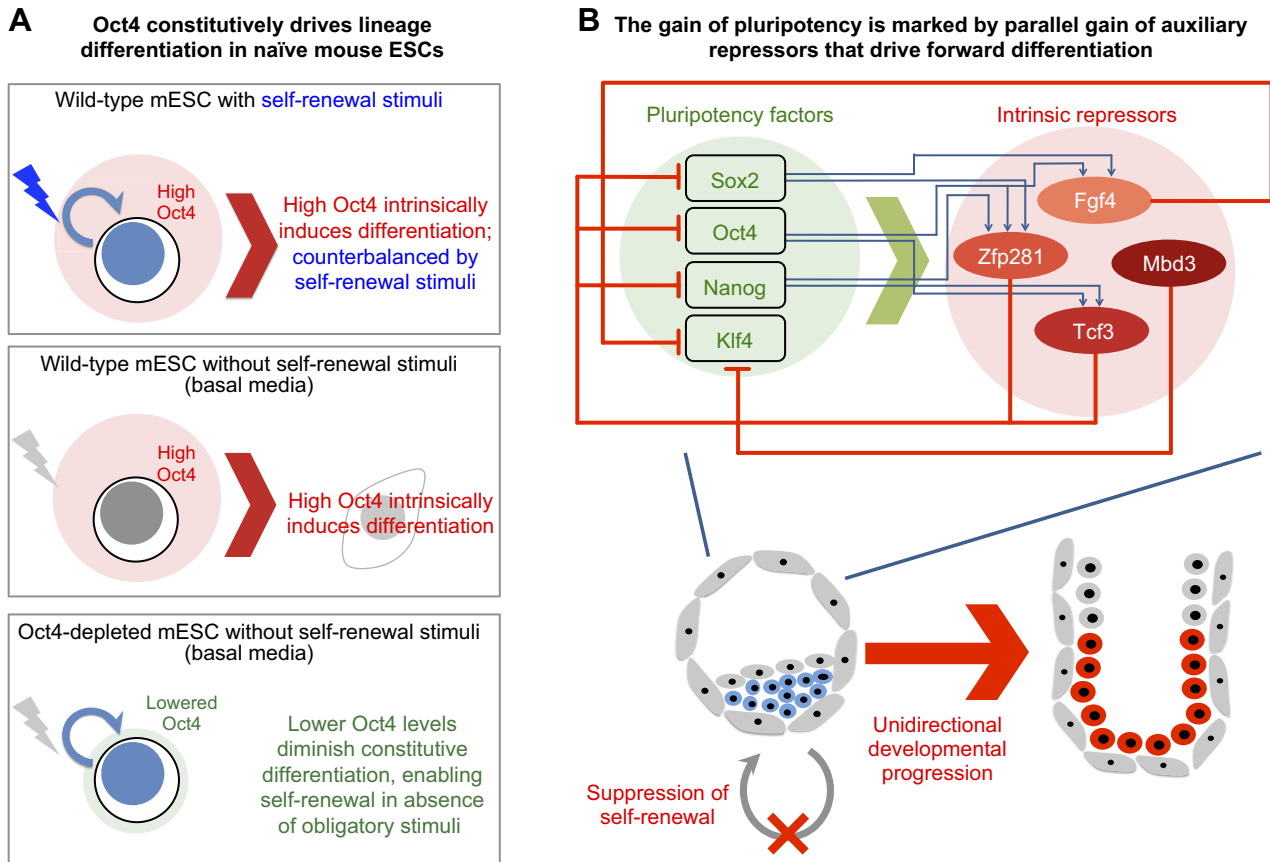
differentiation, preparing them for the next developmental step by installing prospective lineage potential.

Consistent with this notion, specific pluripotency factors endow blastocyst-stage naive epiblast (mESCs) and postimplantation primed epiblast (hESCs/mEpiSCs) with differing lineage options, as befits these tissues’ different developmental potentials. In the naive state, Nanog exclusively induces self-renewal by blocking the progression of mESCs to a postimplantation epiblast fate (56), yet after implantation, *NANOG* reprises a distinct role in “primed” hESCs, instead driving definitive endoderm differentiation (343, 366) in anticipation of gastrulation. Naive-specific pluripotency factor *Tbx3* is present only in mESCs, where it capacitates formation of hypoblast (206), which is still a proximal developmental fate for blastocyst-stage epiblast. However, *TBX3* is absent from primed hESCs, likely because hypoblast is no longer a legitimate lineage choice for postimplantation epiblast.

### C. The Precarious Balance of Pluripotency

How can undifferentiated self-renewal be stably perpetuated if pluripotency factors are truly lineage specifiers that induce differentiation? We suggest self-renewal is maintained because competing lineage-specifying pluripotency TFs are coexpressed in ESCs, each inducing a distinct developmental fate while cross-repressing differentiation to one another’s respective lineages (FIGURE 5A). As a consequence, no individual factor prevails and an undifferentiated state is maintained, albeit precariously. For instance, while *SOX2* directs ectodermal differentiation in hESCs, such ectodermal proclivities are countermanded by mesoderm-specifying *OCT4* and endoderm-specifying *NANOG* (343, 366), and vice versa. Therefore, *OCT4*, *SOX2*, and *NANOG* counterbalance one another, generating a situa-

**FIGURE 5.** Pluripotency factors as lineage specifiers. *A*: pluripotency appears to be governed by a regime of transcription factors (TFs) in which many drive differentiation towards a specific lineage option while repressing other fate choices. These TFs are categorized as trophectodermal, ectodermal, endodermal, or mesodermal specifiers. Green arrow indicates lineage induction; red arrow indicates lineage repression. For example, *Dax1* elicits trophectoderm but represses endoderm; *Sall4* induces endoderm but represses trophectoderm; *Esrrb* activates endoderm genes, but represses mesoderm; *Nanog* specifies definitive endoderm but represses both trophectoderm and mesoderm lineages; *Tbx3* activates endoderm genes, but represses mesoderm; *Oct4* begets mesoderm but represses trophectoderm; and *Sox2* generates ectoderm but represses endoderm and trophectoderm. In undifferentiated ESCs, each lineage option is being concomitantly induced and suppressed by various pluripotency factors. This therefore culminates in a transcription factor stalemate, in which no factor dominates in proclaiming a single lineage in the pluripotent regime. For the purposes of simplicity, the effects of pluripotency factors in both naive and primed cells have been summarized together here. *B*: pluripotency factors compete in hESCs to induce or suppress mesoderm differentiation. H1 hESCs were differentiated towards BRACHYURY<sup>+</sup> primitive streak/mesoderm over 3 days in a substandard protocol including FGF2 and LY294002, which yields ~10–15% BRACHYURY<sup>+</sup> cells (L. T. Ang and K. M. Loh, unpublished observations). However, *OCT4* is imperative to drive mesoderm commitment, because when hESCs were acutely transfected with *OCT4* siRNA 12–24 h prior to differentiation, they could no longer differentiate into mesoderm (~0.2% efficiency). In contrast, *SOX2* and *NANOG* block mesoderm formation, because when they were analogously downregulated, mesoderm differentiation efficiencies were vastly increased (to 75–94% efficiency). Therefore, even in suboptimal differentiation conditions, *SOX2* knockdown or *NANOG* knockdown alone is sufficient to maximize differentiation efficiencies. *C*: coexpression of multiple lineage-specifying pluripotency factors endows ESCs with the capacity to access all downstream lineage options. This could potentially explain their multilineage differentiation potential. *D*: a cartoon depicting the dueling of pluripotency factors in specifying particular cell fates in the precarious balance model of pluripotency.



**FIGURE 6.** Intrinsic repressors expressed within pluripotent cells constitutively drive forward lineage progression. *A*: Oct4 continuously intrinsically drives naïve mESCs to differentiate towards the postimplantation primed epiblast fate, such that when mESCs are cultivated in minimal culture conditions (lacking extrinsic self-renewal stimuli) they differentiate. However, reducing *Oct4* levels averts differentiation, enabling naïve mESCs to self-renew in minimal culture conditions. *B*: pluripotency transcription factors directly bind to and upregulate the *Fgf4*, *Mbd3*, *Tcf3*, and *Zfp281* genes, leading to their robust expression in both naïve epiblast and mESCs. However, these intrinsic repressors act recursively to suppress pluripotency genes. High expression of these intrinsic repressors within the naïve epiblast and ESCs constitutively destabilize self-renewal, ensuring that pluripotency is a transient state *in vivo* and powerfully driving progression to a postimplantation epiblast fate immediately upon gain of naïve pluripotency within the newly formed epiblast.

tion in which there is no net commitment to any fetal lineage.

We therefore propose that pluripotency is a “precarious balance” (203) of competing lineage-specifying forces (**FIGURE 5D**). The undifferentiated state appears to be a contentious transcriptional equilibrium wherein pluripotency factors are expressed at comparable levels and suppress one another’s lineage-specifying activities (203). However, this is an intrinsically unstable configuration. Even a modest twofold upregulation or diminution in *Oct4* expression elicits mESC differentiation (255, 279). Since the undifferentiated equilibrium is maintained by opposing pluripotency factors, slight upregulation or downregulation of a given pluripotency factor could disrupt the equilibrium and drive differentiation. In this scenario, the proposed inherent instability of pluripotency owes to the lineage-specifying activities of pluripotency factors (203). Because pluripotency can be overthrown by minor fluctua-

tions in pluripotency factor expression, we propose that to capture and maintain self-renewing ESCs, extrinsic signaling conditions must be applied to maintain expression of rival pluripotency factors at comparable levels (see sect. IV).

Do pluripotency factors constitutively drive differentiation even in the uncommitted state, or do they simply support lineage commitment at the onset of differentiation only in specialized contexts (280)? If critical self-renewal stimuli including LIF are withdrawn, mESCs invariably differentiate (**FIGURE 6A**). However, if *Oct4* levels are concomitantly reduced by heterozygous deletion (171) or other genetic means (279), differentiation is averted and such *Oct4*-depleted mESCs can be retained in an uncommitted state even in minimal basal media conditions in the total absence of exogenous self-renewal stimuli (279) (**FIGURE 6A**). This therefore signifies that Oct4 continuously exerts a potent pro-differentiative influence on ESCs and constitutively in-

duces lineage specification even in the undifferentiated state (cf. Ref. 280).

Further reifying that pluripotency is an insecure condition, mESCs express a battery of auxiliary factors that are exclusively dedicated to driving differentiation (FIGURE 6B, TABLE 3). These ancillary factors are not “pluripotency factors” per se because they are totally dispensable for epiblast specification in vivo and ESC self-renewal in vitro. Nonetheless, they are highly expressed in both epiblast and ESCs and subserve the sole function of propelling forward developmental progression. Chief among these factors are the transcriptional repressors *Tcf3* (72, 276, 335) and *Mbd3* (167). Both repressors are highly expressed by peri-implantation epiblast and mESCs yet are superfluous for epiblast induction (168, 222) or mESC self-renewal (72, 167, 276, 335). On the contrary, *Tcf3* and *Mbd3* appear necessary for differentiation: if either is disabled, mESCs become recalcitrant to lineage specification (72, 167, 276, 335). Indeed, *Tcf3* promotes differentiation by directly engaging and suppressing the *Oct4* and *Nanog* promoters (72, 276, 335). Likewise, *Mbd3* similarly appears to oppress the *Klf4*, *Rex1*, and *Tbx3* promoters (285), and the transcriptional repressor *Zfp281* may function similarly (101).

Therefore, both in vivo and in vitro, the acquisition of naive pluripotency is coupled to gain of intrinsic repressors that limit “self-renewal” and promote lineage progression towards the next step (FIGURE 6B). Indeed, pluripotency factors themselves strongly induce these intrinsic repressors: despite the pro-differentiation role fulfilled by *Tcf3*, it is directly upregulated by both *Oct4* and *Nanog*, and its expression closely tracks both of these pluripotency factors during blastocyst development (335) (FIGURE 6B). This is further exemplified by signaling ligand *Fgf4* which itself is upregulated by both *Oct4* and *Sox2* (401) and is expressed in peri-implantation epiblast (251). *Fgf4* is abundantly produced by mESCs and signals in autocrine fashion via FGFR/ERK to induce progression to postimplantation epiblast (179, 375). It is therefore an intrinsically produced differentiation stimulus. Indeed, the prodifferentiation effects of *Fgf4* and *Mbd3* are so powerful that if either is inhibited, mESCs can apparently self-renew in the absence of crucial self-renewal signal LIF (167, 395).

In summary, pluripotency is intimately and inextricably connected with differentiation. Once epiblast cells gain pluripotency in the blastocyst, they are endowed with pluripotency transcription factors (e.g., *Oct4*) and auxiliary transcriptional suppressors (e.g., *Mbd3* and *Tcf3*) that immediately destabilize self-renewal and drive onwards differentiation (FIGURE 6B). These factors constitutively drive lineage progression to the next developmental step (postimplantation epiblast), ensuring forward developmental progress. This ultimately explains the highly transitive nature of pluripotency in vivo.

## IV. EXTRINSIC SIGNALS ARE REQUIRED TO MAINTAIN PLURIPOTENCY

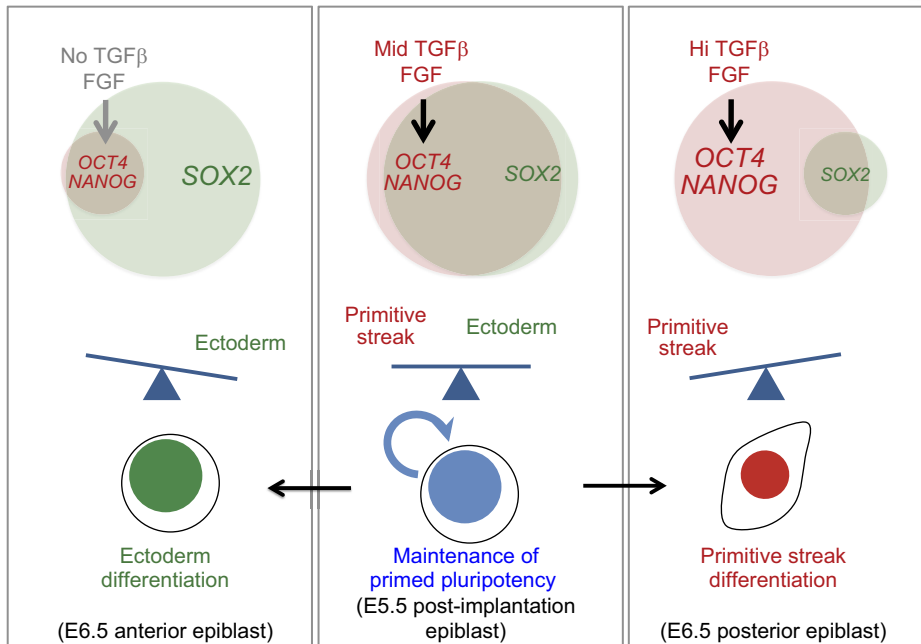
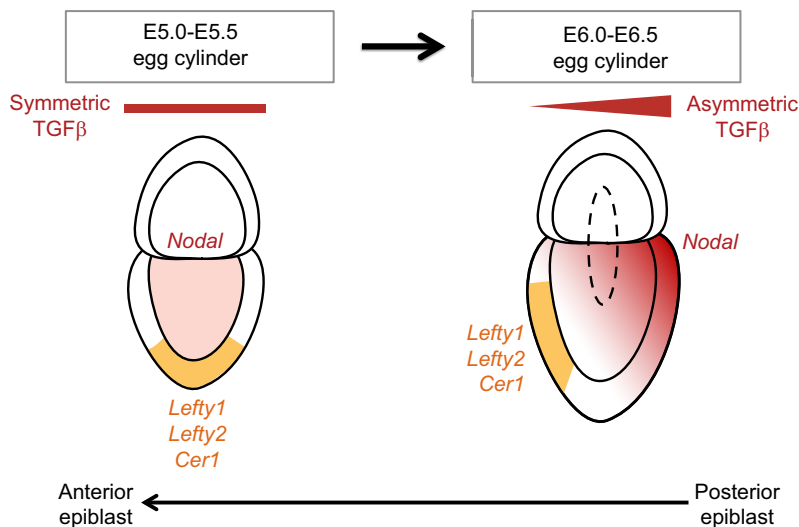
Pluripotency is an ephemeral state in vivo and therefore highly specific extrinsic signals must be applied to capture self-renewing ESC lines in vitro. Due to the intrinsic transcriptional instability of pluripotency, undifferentiated ESCs cannot be maintained without extrinsic intervention. Extrinsic signals stimulate the expression of pluripotency TFs to sustain self-renewal. We suggest extrinsic signals upregulate opposing pluripotency TFs to comparable levels to maintain the fragile balance, which explains why self-renewal signals must often be employed within narrow dose ranges.

### A. Propagation of Undifferentiated Postimplantation Epiblast

Propagation of postimplantation epiblast cells, either human ESCs or rodent EpiSCs, depends on two factors: joint TGF- $\beta$  and FGF signaling (FIGURE 7A). Combinations of TGF- $\beta$  and FGF effectively maintain hESCs/EpiSCs in fully defined conditions (41, 63, 352) and inhibiting either pathway induces differentiation (352) (TABLE 4). These culture environs are reminiscent of what postimplantation epiblast experiences in vivo, as TGF- $\beta$  signaling is endogenously active in postimplantation epiblast.

The E5.0–E5.5 mouse postimplantation epiblast homogeneously expresses the TGF- $\beta$  ligand *Nodal* (74, 224) (FIGURE 7B), leading to phospho-Smad2 signaling throughout the epiblast (358). TGF- $\beta$  signals specifically upregulate *Oct4* and *Nanog* (FIGURE 7A) by deploying TGF- $\beta$  transcriptional executor Smad2/3 to the promoters of both these genes (185, 387). In accord, E5.0–E5.5 *Nodal*<sup>-/-</sup> epiblasts are devoid of *Oct4* and *Nanog* yet robustly express *Sox2* (39, 224). As aforementioned, *Oct4* and *Nanog* drive primitive streak differentiation and suppress the mutually exclusive ectoderm fate, whereas *Sox2* conversely specifies ectoderm (see sect. III) (343, 366). Therefore, with *Sox2* unopposed (by *Oct4* and *Nanog*) in TGF- $\beta$ -deficient conditions, *Nodal*<sup>-/-</sup> mouse epiblasts undergo precocious neural differentiation (39, 52, 224) and likewise hESCs differentiate into ectoderm upon TGF- $\beta$  blockade (58, 313, 354). In summary, TGF $\beta$  signaling in postimplantation epiblast upregulates *Oct4* and *Nanog*, therefore suppressing *Sox2*-driven ectoderm differentiation and maintaining the delicate balance (FIGURE 7A).

*Fgf5* is also expressed by E5.5 mouse postimplantation epiblast (137), and FGF is a critical self-renewal factor for hESCs and rodent EpiSCs in vitro (41, 345, 352, 381). How FGF mechanistically sustains primed pluripotency remains unresolved (182), but one role is to suppress ectoderm differentiation possibly by upregulating *Oct4* and *Nanog* (118). However, FGF does not induce

**A Maintaining and unbalancing primed pluripotency by TGF $\beta$  & FGF signaling****B Endogenous TGF $\beta$  signaling fields in the post-implantation epiblast**

*Sox2* expression (118), and therefore, its effects appear reminiscent of TGF- $\beta$  signaling although the two pathways are not redundant, as revealed by epistatic experiments (352). The *in vivo* relevance of FGF signaling in epiblast pluripotency is less clear, however. Despite the expression of *Fgf5* ligand (137), phospho-ERK activity (an expected downstream consequence of FGF signaling) has not been robustly detected in E5.5–E6.5 postimplantation epiblast (75). Furthermore, genetic ablation of *Fgfr1* shows FGF is required for extraembryonic commitment in the blastocyst (12) but is likely dispensable for postimplantation epiblast pluripotency, only driving primitive streak differentiation at later stages (82, 389).

Nevertheless, it is possible that an epiblast phenotype has been overlooked, as was previously the case for TGF- $\beta$  signaling (224).

While TGF- $\beta$  and FGF sustain postimplantation pluripotent epiblast, both BMP and Wnt signaling are dispensable and instead drive forward progression into primitive streak fates (TABLE 4). Wnt was initially advocated as a self-renewal factor for hESCs (296). However,  $\beta$ -catenin<sup>-/-</sup> mEpiSCs robustly self-renew (321), and recent evidence confirms Wnt does not maintain hESCs but instead specifies the primitive streak (33, 80, 201), though a modest pro-survival role has been noted (33). Therefore, inhibition of BMP

**FIGURE 7.** Extrinsic supervision of the primed pluripotent regime. *A*: postimplantation pluripotent epiblast can directly access one of two fates: primitive streak (induced by Oct4 and Nanog) versus ectoderm (induced by Sox2). TGF- $\beta$  signaling upregulates *Oct4* and *Nanog*, and therefore, moderate levels of TGF- $\beta$  signaling lead to the coexpression of Oct4, Sox2, and Nanog thereby maintaining an equilibrium. Higher TGF- $\beta$  activity (as seen in the ~E6.5 posterior epiblast/prospective primitive streak) upregulates *Oct4* and *Nanog* expression, thereby overwhelming *Sox2* and leading to primitive streak specification. In contrast, inhibiting TGF- $\beta$  signaling (as seen in the ~E6.5 anterior epiblast/prospective ectoderm) leads to the expression of *Sox2* in the absence of *Oct4* and *Nanog*, triggering ectoderm differentiation. *B*: shortly after implantation, TGF- $\beta$  signaling ligand *Nodal* is uniformly expressed by the E5.0–E5.5 mouse postimplantation epiblast (74, 224). However, by E6.5, TGF- $\beta$  signaling becomes asymmetric along the anterior-posterior axis of the epiblast. Diffusible TGF- $\beta$  antagonists *Lefty1* and *Cer1* are expressed in the anterior extra-embryonic endoderm abutting the anterior epiblast (274), repressing TGF- $\beta$  signaling at the anterior end. In contrast, TGF- $\beta$  signaling becomes elevated in the posterior epiblast due to the restriction of *Nodal* expression to the posterior epiblast by E6.0 (360) as well as complex embryonic-extraembryonic reciprocal interactions (18) that amplify *Nodal* signaling in the posterior domain.

**Table 4.** Extrinsic signaling pathways operating to direct the lineage specification or self-renewal of human and mouse embryonic stem cells

	Mouse Embryonic Stem Cells ("Naive" Pluripotency)	Human Embryonic Stem Cells and Mouse Epiblast Stem Cells ("Primed Pluripotency")
LIF	Directs undifferentiated self-renewal (specifically inhibits endoderm/mesoderm differentiation)	No effect (in typical culture conditions) Reversion to naive state (only when in combination with other factors)
Activin/Nodal/TGF- $\beta$	Drives progression to postimplantation epiblast (though a role in undifferentiated self-renewal has been proposed)	High TGF- $\beta$ : primitive streak differentiation  Medium TGF- $\beta$ : undifferentiated self-renewal (together with FGF) TGF- $\beta$ inhibition: ectoderm differentiation
BMP	Directs mesodermal specification (when alone) Directs undifferentiated self-renewal (in combination with LIF; by inhibiting neuroectodermal specification)	Directs primitive streak differentiation
FGF	Drives progression to postimplantation epiblast; also drives extraembryonic and primitive streak differentiation	Directs undifferentiated self-renewal (in combination with medium TGF- $\beta$ )  Supports primitive streak differentiation (in combination with high TGF- $\beta$ )
Wnt	Consolidates undifferentiated self-renewal (when together with LIF or FGFR/MAPK blockade) Directs primitive streak differentiation	Directs primitive streak differentiation  Reversion to naive state (only when in combination with other factors)

LIF, leukemia inhibitory factor; TGF- $\beta$ , transforming growth factor- $\beta$ ; FGF, fibroblast growth factor; MAPK, mitogen-activated protein kinase.

(114, 386) and Wnt (80, 321) signaling avails hESC and mEpiSC self-renewal by suppressing supernumerary primitive streak differentiation. This is further supported by the fact that E5.5 postimplantation epiblast does not significantly transduce either BMP or Wnt signaling, as assayed by phospho-Smad1/5/8 (85) and *Axin2-LacZ* activity (341, 342), respectively. Therefore, both pathways are dispensable for postimplantation epiblast pluripotency in vivo and in vitro and instead herald lineage commitment.

## B. Unbalancing the Pluripotency Equilibrium: Initiating Differentiation

Although the uncommitted state is underpinned by a balanced equilibrium of opposing pluripotency TFs, differentiation can ensue from this stalemate when particular lineage-specifying pluripotency TFs become upregulated over others, thus "unbalancing" the equilibrium. This initial asymmetry occurs when the extrinsic signals that control pluripotency factor expression are elevated or depressed beyond a crucial threshold. This mechanism accounts for how the E5.5 postimplantation pluripotent epiblast becomes segregated into two distinct fates by E6.5: anterior epiblast (fated to become ectoderm) versus posterior epiblast (prospective primitive streak) (183, 331) (FIGURE 7A).

The unbalancing of the Oct4/Sox2/Nanog equilibrium is developmentally imposed by changes in TGF- $\beta$  signaling levels, among other factors. Although *Nodal* is uniformly and moderately expressed throughout the E5.0–E5.5 epiblast (safeguarding the pluripotent equilibrium in postimplantation epiblast; see above), TGF- $\beta$  signals soon become spatially asymmetric by E6.0 (FIGURE 7B). TGF- $\beta$  signaling is amplified in posterior epiblast (18, 360), whereas TGF- $\beta$  signaling becomes repressed in anterior epiblast by downregulation of anterior *Nodal* expression (18) combined with diffusion of soluble TGF- $\beta$  antagonists Cer1 and Lefty1 from the adjacent anterior visceral endoderm (274) (FIGURE 7B).

High TGF- $\beta$  levels in posterior epiblast destabilize the pluripotent equilibrium, upregulating *Oct4* and *Nanog* while excluding *Sox2* by ~E6.5 (135, 144) (FIGURE 7A). Consequently, high Oct4 and Nanog specify the primitive streak by directly inducing primitive streak regulator *Eomes* (343, 366) (see sect. VI) and therefore prevail over the (now-absent) ectoderm-specifying pluripotency factor *Sox2*. In this incipient transition between pluripotency and differentiation, *Oct4* and *Nanog* become transiently coexpressed with primitive streak genes in the same single cells (233, 343). Downregulation of *Sox2* in posterior epiblast is likely crucial for primitive streak formation, as *Sox2* conversely induces ectoderm at the expense of the primitive streak (see

sect. IVA) and ectopic *SOX2* can abort primitive streak differentiation from hESCs even in primitive streak-inducing culture conditions (366).

The reciprocal situation unfolds in ~E6.5 anterior epiblast, wherein due to decreased TGF- $\beta$  levels, *Sox2* is maintained whereas *Nanog* (which is TGF- $\beta$  dependent) is relinquished (FIGURE 7A) (135, 144, 224). Therefore, in the anterior epiblast, *Sox2* is free to enact ectoderm differentiation (52) as it is no longer opposed by rival primitive streak-specifying TF *Nanog*; if *NANOG* is constitutively expressed in vitro in hESCs, the pluripotency equilibrium cannot be properly disrupted even in neuralizing conditions and therefore ectoderm differentiation is abrogated (353, 366). FGF deprivation accelerates ectoderm specification from hESCs by further decreasing *OCT4* and *NANOG* expression (118).

In summary, it is evident that TGF- $\beta$  levels must be providently maintained to sustain postimplantation pluripotent epiblast (52) as well as self-renewing mEpiSCs/hESCs. At a moderate dosage, *Oct4*, *Sox2*, and *Nanog* are all comparably maintained and none prevail. Raising TGF- $\beta$  levels triggers an end to the standoff, enabling primitive streak formation to rapidly ensue (FIGURE 7A). In contrast, TGF- $\beta$  inhibition enables *Sox2* to predominate, instead culminating in ectoderm formation (FIGURE 7A). This divergence in the expression of individual pluripotency factors is also evinced in the earliest steps of hESC differentiation during the selection of mutually exclusive primitive streak or ectoderm fates. Even within 24 h of hESC differentiation, prospective primitive streak (induced by high TGF- $\beta$  levels) is designated by and specified by the expression of *OCT4* and *NANOG* (77, 201, 343), whereas prospective ectoderm (specified by TGF- $\beta$  blockade) is instead marked by and induced by *SOX2* in the absence of the other two regulators (118). This asymmetry in pluripotency factor expression and bifurcation in anterior versus posterior epiblast identities therefore heralds the transition from primed pluripotency forward unto gastrulation, both in vivo and in vitro.

### C. Propagation of Undifferentiated Peri-implantation Epiblast

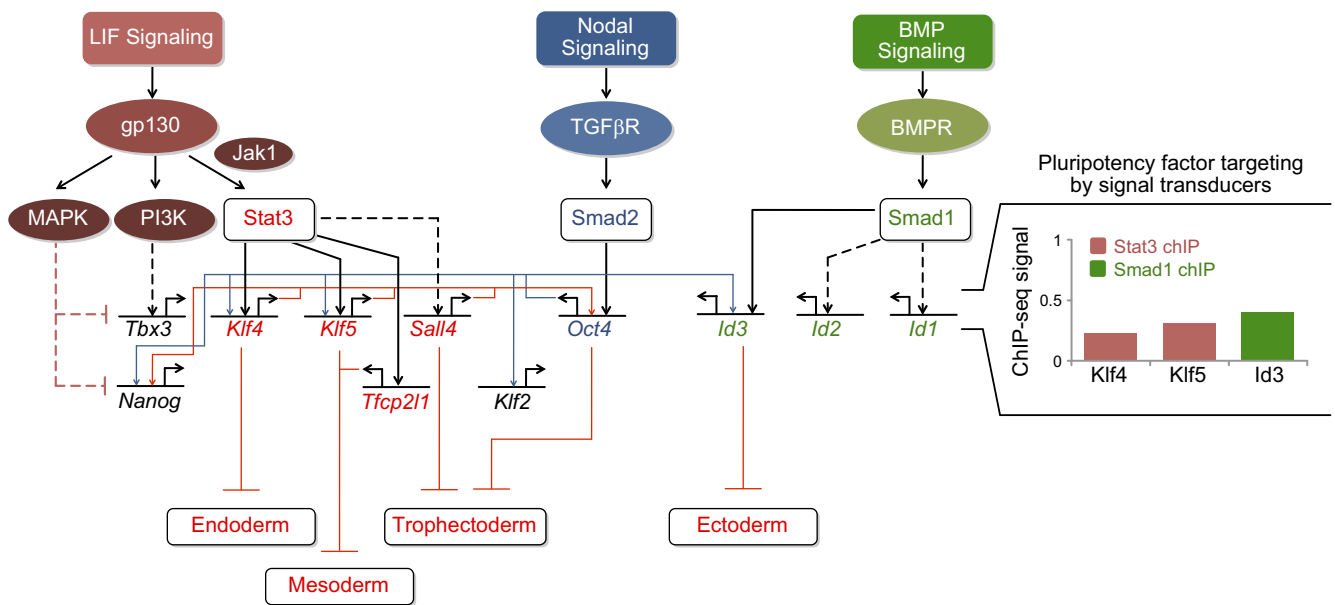
While TGF- $\beta$  and FGF sustain primed hESCs/mEpiSCs (above), the propagation of naive mESCs requires completely distinct signals, reflecting the differing developmental states occupied by peri-implantation and postimplantation epiblast. Historically, mESCs were derived and propagated in complex conditions including stromal feeder coculture, conditioned medium, and/or animal serum (97, 215, 312). Capitalizing on early studies (311, 377), pivotal work by the Smith group since 2003 fully deconvoluted these culture environs (395, 396) and delineated the minimal signals essential for mESC self-renewal.

A combination of LIF and BMP4 was initially found to sustain mESC propagation (311, 377, 396) (TABLE 4). LIF and BMP upregulate distinct subsets of pluripotency factors within the “pluripotency network” (FIGURE 8A). Collectively, these factors block differentiation to endoderm, mesoderm, and ectoderm and thus temporarily sustain undifferentiated self-renewal (FIGURES 5A AND 8A). LIF precludes both endodermal and mesodermal differentiation by upregulating pluripotency factors *Klf4*, *Klf5*, *Tbx3*, and *Tfcp2l1*. LIF engages the gp130 receptor, activating the Jak/STAT, PI3K/Akt, and Ras/MAPK/ERK cascades (96, 253, 266). Jak1 kinase activation subsequently leads to phosphorylation and nuclear translocation of TF Stat3 which binds to the promoters of the *Klf4*, *Klf5*, and *Tfcp2l1* genes and directly upregulates their expression (36, 65, 126, 195, 213, 256, 392) (FIGURE 8, A AND B). *Klf4* curtails endoderm while *Klf5* and *Tfcp2l1* limit mesodermal commitment (8, 36, 392) (TABLE 3). Concomitantly, through an undefined transcriptional mechanism, LIF-activated PI3K-Akt signaling specifically upregulates *Tbx3* (256), which may inhibit ectoderm to some extent (150, 206).

Whereas LIF primarily inhibits endoderm and mesoderm specification, BMP4 represses neuroectoderm (396). It does so by activating TF Smad1 (the transcriptional effector of BMP signaling), which directly targets and upregulates the *Id3* gene, a potent inhibitor of neural differentiation (65, 396) (FIGURE 8, A AND B). In this manner, LIF and BMP prohibit mESC differentiation to all possible lineages and therefore sustain self-renewal. However, it is crucial to note that these effects are intrinsically combinatorial: if BMP is supplied alone, mesoderm commitment ensues (396). Parallel addition of LIF is required to suppress ancillary mesoderm induction and maintain pluripotency (396).

Wnt and FGF signaling also influence mESC pluripotency, although they yield disparate outcomes (TABLE 4). Whereas Wnt provides a potent self-renewal stimulus, FGF signaling prompts differentiation. Indeed, FGF has long been known as a prodifferentiation factor: it triggers extraembryonic and primitive streak fates by activating MAPK/ERK signaling (48, 179, 395).

Wnt signaling has historically been ascribed discrepant roles in instructing either mESC self-renewal or primitive streak differentiation (10, 88, 133, 259, 296, 329), effects that likely depend on both dose and context. At moderate levels, Wnt preserves naive mESC self-renewal by blocking progression to “primed” postimplantation epiblast identity (342). However, Wnt also dichotomously drives primitive streak differentiation from primed EpiSCs especially at higher dosages (379, 395) (FIGURE 3C), and exaggerated Wnt signaling in vivo precociously induces primitive streak markers in blastocyst-stage epiblast (61). Therefore, Wnt alone is insufficient to sustain mESCs (259, 395). Nevertheless, concomitant

**A Maintaining naïve pluripotency under duress with extrinsic cytokines****B Signaling effector ChIP-seq**

**FIGURE 8.** Extrinsic supervision of the naive pluripotent regime. **A:** extrinsic signaling pathways such as the LIF, TGF- $\beta$ , and BMP pathways maintain pluripotency by activating the expression of pluripotency regulators. LIF binds to gp130, which acts through Jak1 to phosphorylate Stat3. Phosphorylated Stat3 then binds to *B*, Stat3 ChIP and activates the transcription of *Klf4* and *Klf5*, repressors of endoderm and mesoderm, respectively. Nodal/Activin ligands bind to TGF- $\beta$ R, which then phosphorylates Smad2. Phosphorylated Smad2 activates the expression of *Oct4*, which blocks trophoderm and may potentially activate a host of other pluripotency genes (blue lines). Bmp ligands bind to BMPPR, which phosphorylates Smad1. Smad1 binds to *B*, Smad1 ChIP and increases the expression of *Id3*, which is a repressor of ectoderm differentiation. Solid linkages indicate high-confidence direct interactions in which the transcription factor likely directly upregulates the target gene, as assessed by ChIP, reporter assays, and/or gene perturbation experiments. Dashed linkages indicate indirect regulatory interactions for which direct *cis* regulation has not been formally proven to date. For convenience, the pro-pluripotency effects of LIF and BMP signaling in naive mESCs and the propluripotency effects of TGF- $\beta$  signaling in primed hESCs have been summarized in the same cartoon. **B:** analysis of ChIP-seq data (65) in mESCs showing Stat3 binds the *Klf4* and *Klf5* genes and Smad1 binds the *Id3* locus (65), suggesting these signaling effectors directly upregulate the expression of these pluripotency factors.

FGFR/MAPK inhibition can block Wnt-induced super-numerary primitive streak differentiation (395) (**FIGURE 3C**). Therefore, activating Wnt/ $\beta$ -catenin signaling (often achieved by chemical GSK3 inhibitors) while repressing FGFR/MAPK signaling suffices to maintain naive ESC lines from both mice and rats (46, 194, 395). This signaling condition is colloquially referred to as “2i” (395). However, even in “2i,” it is still necessary to limit Wnt signaling to moderate levels to forestall primitive streak specification (379, 395). In summary, Wnt elicits both self-renewal and differentiation programs in mESCs: concurrent FGFR/MAPK inhibition is necessary to occlude differentiation and to reveal the stabilizing effect of Wnt on naive pluripotency.

Mechanistically, Wnt upregulates naive-specific pluripotency TFs by antagonizing the endogenous transcriptional repressor Tcf3 (see sect. III). In steady-state mESCs, Tcf3 binds to and represses Wnt-dependent naive pluripotency genes (72, 276, 335). However, Wnt stimulation leads to nuclear translocation of  $\beta$ -catenin, which disables the repressive influence of Tcf3 and instead directs the transcriptional activator Tcf1

to upregulate naive pluripotency TFs including *Esrrb*, *Klf2*, *Nr0b1/Dax1*, and *Tfcp2l1* (214, 394).

In conclusion, it is clear that naive pluripotency is an intrinsically transcriptionally unstable state that can be stabilized by extrinsic coercion. For instance, *Oct4* apparently downregulates *Nanog* even in naive mESCs, therefore explaining the requirement for extrinsic signals to continually undo such downregulation and maintain *Nanog* expression (171). The combined effect of multiple signals is necessary to support mESC self-renewal. BMP and Wnt dichotomously promote both self-renewal and mesoderm differentiation. Therefore, parallel provision of FGFR/MAPK inhibitors or LIF is required to divert mESCs away from the mesodermal pathway and stabilize an undifferentiated state. Interestingly, multiple combinations of these self-renewal stimuli can flexibly enable mESC propagation. For example, among the triad of LIF signaling, Wnt agonism, and FGFR/MAPK inhibition, any two of these three factors can adequately maintain mESC self-renewal (380).

## D. Are mESC Self-renewal Stimuli Equally Necessary for Mouse Epiblast Pluripotency In Vivo?

Insight into signals that maintain mESC self-renewal in culture (TABLE 4) has revealed corresponding roles for some signals in specifying peri-implantation pluripotent epiblast in vivo.

The in vitro dependency of mESCs on LIF parallels an analogous role for LIF/STAT signaling in developmental pluripotency in vivo. When blastocyst implantation is temporarily halted in mice, peri-implantation epiblast is entrapped in a pluripotent state for several weeks until fetal development resumes. This phenomenon of extended pluripotency is known as diapause. Initial reports suggested that LIF/gp130 signaling is required for maintenance of pluripotent peri-implantation epiblast specifically during diapause (244) but not during normal embryogenesis (193). This implied the LIF-stabilized unnaturally prolonged pluripotency of mESC lines might thereby approximate a diapause-like state. Yet there are some differences. mESCs are proliferative in culture, yet during diapause, epiblast cells are maintained in relative quiescence during this state of suspended development (372).

However, LIF/STAT signaling also sustains embryonic pluripotency during normal embryogenesis as well. Even in the absence of diapause, phospho-STAT3 is active in peri-implantation epiblast, and both IL-6 and LIF serve as the endogenous upstream gp130 receptor ligands in vivo (87). Fully eliminating *Stat3* both maternally and zygotically demonstrates it is indispensable: the ~E4.5 epiblast fails to develop normally, and consequently peri-implantation lethality results (87). These findings accord a key endogenous role for STAT3 signaling for naive epiblast function, both during normal embryogenesis and diapause.

The prodifferentiation effect of FGF signaling in mESC cultures is also clearly reproduced in vivo. During segregation of epiblast and hypoblast from ICM, *Fgfr2* expression is sharply downregulated in epiblast and instead confined to hypoblast (123). FGFR downregulation shields prospective epiblast from the hypoblast inductive signal FGF4 (282), thereby permitting epiblast specification. Indeed, if FGF signaling is pharmacologically inhibited in developing blastocysts, ICM differentiates almost exclusively into epiblast, whereas hypoblast is virtually eliminated (123, 246). This provides a rationale for why FGFR/MAPK inhibitors stabilize mESC self-renewal.

Nevertheless, while cell culture experiments suggested BMP and Wnt maintain mESC self-renewal, whether this is phenocopied in vivo is disputable. *Bmp4* is highly expressed by peri-implantation epiblast (123), and Wnt signaling is intensely transduced by E3.5 ICM and E4.5 early epiblast

(342), owing to expression of multiple Wnt ligands in the early blastocyst (172). However, genetic abrogation of either BMP (24, 227, 378) or Wnt (148) signaling has not yet demonstrated any functional role in epiblast pluripotency, instead showing these signals are only essential later during gastrulation to drive primitive streak differentiation.

How may the contrary findings from in vitro and in vivo studies be reconciled? One possibility is indicated by the fact that the in vitro function of Wnt is critical to prevent mESC progression to a primed EpiSC state (342). Therefore, because the peri-implantation epiblast state is transitive during normal development, Wnt may be required only for prolonged preservation of this state, that is, during diapause. However, Wnt is equally dispensable for epiblast maintenance during diapause (31).

Pathway redundancy is another possibility. For instance, *β-catenin*<sup>-/-</sup> mESCs differentiate in conventional culture conditions (10) (379), yet can be sustained if two potent self-renewal factors are provided: LIF agonists together with FGFR/MAPK inhibitors (379). This adaptability underscores that pluripotency stimuli act combinatorially. By analogy, Wnt knockout epiblasts may be sustained in vivo (31, 148) due to compensatory pathways. It may therefore be necessary to cripple multiple pathways in vivo to demonstrate a clear dependence for these signals in peri-implantation epiblast.

On a final note, the role of TGF-β in peri-implantation epiblast/mESC pluripotency has yet to be fully resolved. Although genetic ablation of TGF-β signaling affects post-implantation epiblast (see sect. IVA), no earlier blastocyst phenotypes have been noted to date. Despite apparent activation of TGF-β in blastocyst-stage epiblast in vivo (as shown by pSmad2 activity; Ref. 154), various reports have yielded contradictory findings as to whether it is essential for mESC self-renewal in vitro (136, 154, 186, 260, 403). Instead, in some signaling contexts, TGF-β instructs naive mESCs to differentiate towards EpiSCs (124, 141), consistent with the pivotal requirement of TGF-β in primed pluripotency.

## E. Do ESCs Autonomously Self-renew in the Absence of Extrinsic Signals?

Above, we have described how extrinsic intervention is crucial to safeguard ESC self-renewal. However, in contrast, the prevailing ground state model suggests that pluripotency is intrinsically stable and self-maintaining in the absence of extrinsic signals because pluripotency factors continually repress differentiation (380, 395). This is supposedly substantiated by the finding that combined GSK3 and FGFR/MAPK inhibition can maintain undifferentiated mESCs in “2i” media (395). Dual GSK3 and FGFR/MAPK blockade has been construed to instate a signal-less envi-

ronment, therefore exposing the autonomous intrinsic stability of pluripotency as a default “ground state” (395).

However, do “2i” environs truly provide a context in which all extrinsic signals are nullified? While FGFR/MAPK inhibitors suppress differentiation-inducing autocrine FGF signaling, GSK3 inhibition instead activates Wnt/ $\beta$ -catenin signaling and therefore provides a positive self-renewal stimulus in “2i” conditions (342, 380, 395). mESCs fail to self-renew in the presence of a MAPK/ERK inhibitor alone, therefore indicating that a second positive self-renewal signal (provided by Wnt/ $\beta$ -catenin or alternatively, LIF) is necessary to stabilize the uncommitted state (48, 380, 395).

Therefore, in our view, there are limited data to support the thesis that ESCs can autonomously self-maintain in the absence of extrinsic intervention (203). In contrast, it is clear that positive signaling from both FGF and TGF- $\beta$  pathways are essential to maintain undifferentiated hESCs/mEpiSCs by providently managing the expression of OCT4, SOX2, and NANOG to comparable levels (FIGURE 7A). During embryogenesis, pluripotency is a highly transitive state lasting for only a few brief days in the epiblast, and therefore, there is no physiological rationale for why the pluripotency transcriptional network should be indefinitely stable. Thus exogenous signals are pivotal to coherently maintain undifferentiated pluripotent cells in vitro. Therefore, the condition of ESCs has been likened to the situation of Tithonus (204): an immortal intrinsically longing to perish (differentiate) yet artificially retained in a youthful state by external forces.

Finally, a widespread idea in the field is that “2i” culture conditions uniformly effectuate a pluripotent “ground state” in mESCs equivalent to peri-implantation epiblast. Indeed, naive epiblast markers (e.g., CD31 and Rex1) exhibit increasingly homogeneous expression in the 2i culture regimen (379, 380). Furthermore, the “2i” signaling environment closely approximates what the peri-implantation epiblast experiences in vivo [as Wnt signaling is transduced by E4.5 peri-implantation epiblast whereas MAPK/ERK signaling is minimized (4, 406) due to *Fgfr2* downregulation], although the presence and/or absence of heretofore-cryptic signals cannot be discounted. Nonetheless, not all “2i”-grown mESCs harbor an authentic epiblast identity; they are still heterogeneous to some extent (232), as we discuss below.

## V. EMBRYONIC STEM CELL HETEROGENEITY IN VITRO

### A. A Menagerie of Dissimilar Embryonic Stem Cells

ESCs have previously been regarded as a homogeneous and equipotent population of cells, each of which may unre-

strictedly contribute to all embryonic lineages. Yet increasing evidence suggests that ESC cultures may comprise a mélange of dissimilar cell types, each of which differs in its immediate differentiation potential, transcriptional and chromatin status, and capacity for embryonic complementation (FIGURES 3C AND 4, B AND C).

Providing evidence for heterogeneity within ESC cultures, pluripotency factor expression levels often fluctuate between individual ESCs. Cardinal pluripotency TFs *Oct4* and *Sox2*, previously asserted to be homogeneously expressed (305), also vary in expression among individual mESCs (FIGURE 9B) (171, 203, 337). Such mosaicism is even more pronounced in the case of pluripotency TFs *Nanog*, *Stella*, *Rex1*, *Prdm14*, *Esrrb*, *Klf4*, and *Tbx3* (57, 140, 256, 349, 356), which can be totally absent from a fraction of mESCs (FIGURE 9B). This is particularly striking in the case of *Tbx3* (FIGURE 9B), which is a LIF target gene yet typically is only expressed by 15–30% of mESCs despite continual provision of LIF (256). Expression of *Nanog*, *Stella*, *Prdm14*, and *Rex1* is dynamic, as mESCs can cyclically upregulate or downregulate these genes (57, 140, 349, 390).

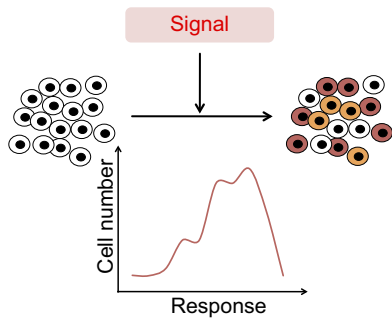
Not only do mESCs mosaically express pluripotency TFs, they also heterogeneously express lineage commitment genes including the primitive streak inducers *Brachyury* (324) and *Hes1* (174) and hypoblast determinants *Hhex* and *Rest* (53, 54, 388) (FIGURE 4B). These lineage determinants appear pervasive even in overtly undifferentiated mESCs; up to ~20% of mESCs express *Brachyury* (324), whereas up to ~60% express *Hhex* (53, 232). Altogether these data clearly show ESC cultures are not uniform but instead might in some cases be regarded as diverse conglomerates of disparate lineages.

### B. Continual Flux Between Naive and Primed Pluripotent States

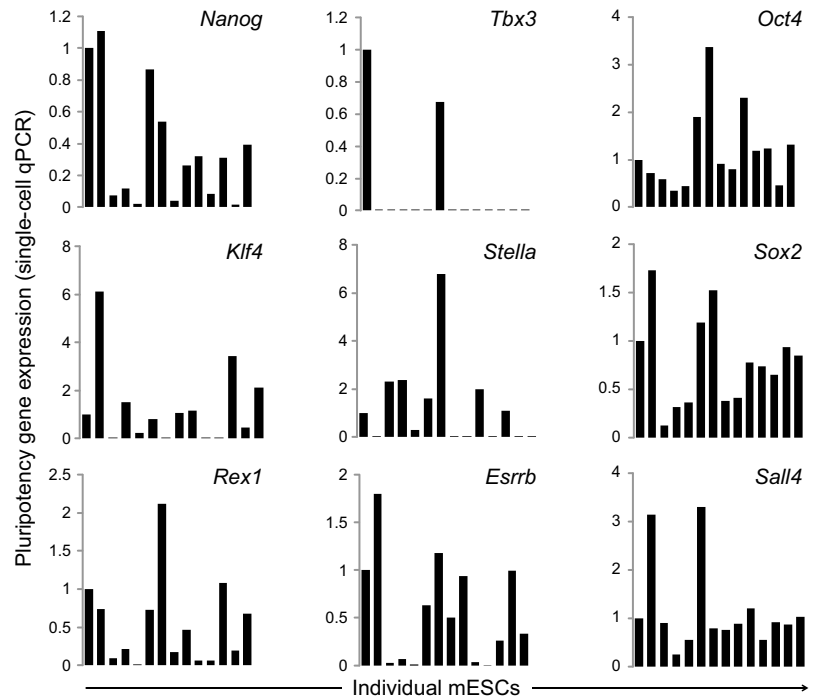
Heterogeneous expression of pluripotency and differentiation TFs throughout mESC cultures functionally engenders discrete subpopulations of cells that dramatically vary in developmental status and lineage potential. In light of these findings, referring to these cultures en bloc as “pluripotent stem cells” is a misnomer if some of these cells in fact are lineage-restricted and therefore not genuinely pluripotent.

Within mESC cultures, there is a continual intrinsic progression of naive mESCs towards a “primed” EpiSC-like identity (FIGURES 3A AND 4B). In some conditions, a subpopulation of mESCs (~2–30%) does not express pre/peri-implantation epiblast marker *Rex1* (289, 349). Instead, these *Rex1*-negative mESCs express postimplantation epiblast/EpiSC marker *Fgf5* and are incapable of embryonic complementation when reintroduced into the preimplantation blastocyst (349) (FIGURE 3B). Continual generation of EpiSC-

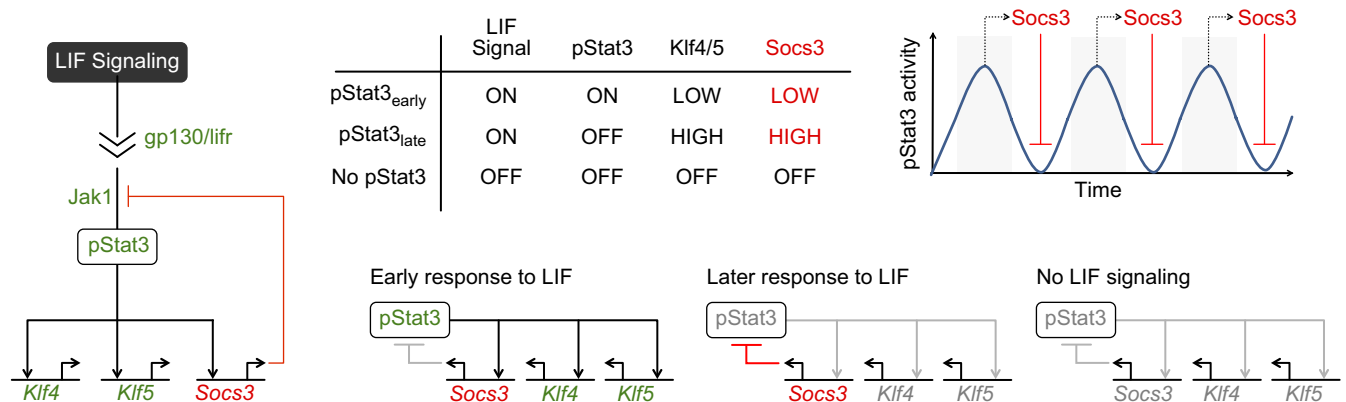
**A The fallibility of extrinsic cytokine coercion**



**B Heterogeneous pluripotency factor expression**



**C Heterogeneous LIF signal transduction by intrinsic negative feedback?**



**FIGURE 9.** The fallibility of extrinsic signaling may partially underlie heterogeneous pluripotency factor expression in ESCs. *A*: extrinsic cytokine coercion stabilizes pluripotency by maintaining the expression of pluripotency factors. However, in other cellular systems, not all cells respond uniformly towards the same extrinsic signal, as diagrammed in this cartoon (although this remains to be formally tested in the ESC system). Due to stochastic signal transduction, some transduce the signal highly, lowly, or not at all at a clonal level. *B*: this may partially explain heterogeneous expression of pluripotency factors between individual mESCs. At a single-cell level, *Nanog*, *Tbx3*, *Oct4*, *Klf4*, *Stella*, *Sox2*, *Rex1*, *Esrrb*, and *Sall4* are expressed at highly variable levels between individual mESCs grown in conventional culture conditions. Graphs (203) represent our reanalysis of single-cell qPCR data published elsewhere (337). *C*: intrinsic negative feedback may generate oscillatory signaling transduction, leading to heterogeneous signaling responses within mESC cultures. LIF signaling through gp130/Jak1/pStat3 induces pluripotency factors *Klf4* and *Klf5* but also *Socs3*, the latter of which binds to and inhibits Jak1 signaling. Consequently, *Socs3* subsides, allowing pStat3 to be reactivated and restarting the cyclic loop. These cycles manifest in periodic *Socs3* expression and hence oscillating pStat3 activity. In summary, *Socs3*-imposed negative feedback might lead to heterogeneous pStat3 activity (and thus heterogeneous pluripotency factor expression) among individual mESCs. However, this is conjectural and has not been experimentally assessed.

like cells from mESCs is driven by both extrinsic (e.g., Fgf4) and intrinsic (e.g., Mbd3) factors that perpetually propel naive cells towards a postimplantation epiblast state to ensure forward developmental progress (see sect. VD).

Unexpectedly, the reverse transition is also possible: EpiSCs may spontaneously regress to a naive epiblast-like state at some frequency (FIGURES 3A AND 4B). For instance, ~0.5% of EpiSCs spontaneously express the pre/peri-implantation

epiblast markers *Rex1* and *Stella* and regain the capacity for blastocyst complementation, and these naïvelike and primed-like subpopulations of EpiSCs interconvert (129). Likewise, *Rex1*-negative mESCs may reupregulate *Rex1* (349) (FIGURES 3E AND 4D). This therefore demonstrates that in standard culture conditions, pluripotent cells may reversibly journey between naïve and primed states, and there is spontaneous flux between these two states (FIGURE 3A).

Therefore, contrary to prior expectations, it may be an oversimplification to regard mESC or EpiSC cultures as static populations of either peri-implantation epiblast or postimplantation epiblast, respectively. Instead, in some signaling conditions, mESC and EpiSC cultures may both comprise peri-implantation and postimplantation epiblast cell populations in continual equilibrium with one another (FIGURE 3A). However, the peri-implantation epiblast compartment predominates in mESC cultures, while the postimplantation epiblast population constitutes the supermajority of EpiSC cultures (FIGURE 3C). Nevertheless, the flexibility with which some primed cells can spontaneously revert to naïvelike status is unexpected. We speculate a subset of them may resemble ~E5.0 recently implanted epiblast which has freshly downregulated *Rex1* yet can still contribute to preimplantation embryos with limited efficiency (110, 271).

Stochastic dedifferentiation of EpiSCs into a naïvelike mESC state can be accelerated by destabilizing the postimplantation epiblast state and/or providing naïve self-renewal cues. Initial work suggested LIF, serum, and feeder coculture could inefficiently convert EpiSCs into naïve cells (17). Inhibiting critical EpiSC self-renewal signals FGF and TGF- $\beta$  and concomitantly providing naïve stimuli Wnt and LIF augments EpiSC dedifferentiation into mESCs (119). EpiSC regression is maximized by eliminating *Mbd3* (a suppressor of the naïve state) (281) or by overexpressing naïve epiblast-specific TFs *Klf2*, *Klf4*, *Nanog*, *Nr5a2*, or *Tfcp2l1* (124, 126, 130, 213) that directly impose mESC identity, indicating that a naïvelike fate can be resuscitated in primed cells (FIGURE 3A).

### C. Embryonic Stem Cells Moving to and From Differentiated Lineage Options

Beyond progressing to a postimplantation epiblast state, mESCs can also spontaneously journey further to access specific lineage pathways (FIGURES 3A AND 4, B AND C). For example, subsets of mESCs express ~E6.5 primitive streak marker *Brachyury* (324, 376) or ~E4.5 hypoblast marker *Hhex* (53, 232, 347), both of which are absent from epiblast *in vivo*. Expression of lineage determinants in ESCs reciprocally correlates with decreased expression of pluripotency factors. mESCs spontaneously expressing *Brachyury* or *Hes1* are biased to differentiate into mesoderm and are largely incapable of differentiating into other lineages (174, 324). Sim-

ilarly, mESCs expressing *Hhex* are predisposed for hypoblast differentiation and poorly differentiate into any fetal lineage (53). Despite their abundance in mESC cultures, these cells are on the verge of lineage commitment and therefore do not fully qualify as “pluripotent” because they are partially lineage-restricted.

Strikingly, these digressions from pluripotency are reversible *in vitro*. mESCs expressing lineage markers are not fully committed. *Hhex*-positive mESCs coexpress epiblast marker CD31/PECAM1 (232) (which is not expressed in hypoblast, Ref. 288) and therefore lie somewhere between epiblast and hypoblast states. Indeed, *Hhex*-positive hypoblast precursors (53) and *Brachyury*-positive primitive streaklike progenitors (324) may flexibly downregulate their respective lineage specifiers and discard their former biases, seemingly returning to naïve epiblast status (53, 324). Upon *Rex1* reexpression, mESCs downregulate *Brachyury* and become refractory to mesodermal differentiation once more (349) (FIGURE 3A).

In summary, in steady-state culture conditions, there is continual transition between naïve pluripotent, primed pluripotent, and lineage-committed compartments (FIGURE 4B). Lineage predispositions are temporary conditions (53, 324), but are significant while they last. For example, when captured at any given time, *Brachyury*-expressing ESCs exclusively differentiate into mesoderm (324), and *Hhex*-expressing ESCs preferentially differentiate into hypoblast (53). Therefore, the reverse journey from lineage-committed to pluripotent compartments is likely too slow to be realized on the short time scales on which most primary differentiation protocols operate. Nevertheless, such excursions towards committed fates can be reduced (379), but not fully eliminated (232), in stringent culture conditions that preferentially capture naïve cells (e.g., “2i”).

### D. Pluripotency Factors Intrinsically Drive Lineage Progression and Induce ESC Heterogeneity

We suggest the incessant excursions of ESCs towards differentiated fates are intrinsically driven by pluripotency factors themselves, some of which continuously specify differentiation to ensure forward developmental progression *in vivo*. This can therefore generate considerable lineage heterogeneity *in vitro*. Crucially, *Oct4* itself suppresses naïve pluripotency TFs *Klf4* and *Esrrb* and thereby serves as a constitutive stimulus to induce mESCs to differentiate into a postimplantation EpiSC-like state (171). Thus heterozygous deletion of *Oct4* almost paradoxically anchors mESCs in the naïve state, eliminating spontaneous differentiation towards EpiSCs (171). Additionally, *Nanog* autorepresses itself (101), destabilizing naïve pluripotency. Therefore, autorepression or cross-repression of pluripotency TFs by one another perennially drives naïve mESCs to differentiate.

Furthermore, as aforementioned pluripotency factors also upregulate a repertoire of auxiliary factors in mESCs that constitutively induce differentiation and destabilize self-renewal, including the transcriptional repressors Mbd3 (167), Tcf3 (72, 276, 335), and Zfp281 (101) as well as the signaling ligand Fgf4 (179, 375). Each of these suppresses naive pluripotency genes, explaining why naive mESCs continually “drift” towards a postimplantation “primed” EpiSC-like phenotype in culture (FIGURE 6B). In particular, the role of DNA methyltransferases in constitutively propelling the differentiation of naive mESCs has been recently emphasized. It seems that when conventionally grown mESCs divide, they often generate daughter cells with diverging transcriptional profiles, perhaps foreshadowing a tendency towards lineage commitment (156). Yet, *Dnmt1*<sup>-/-</sup>*Dnmt3a*<sup>-/-</sup>*Dnmt3b*<sup>-/-</sup> mESCs bereft of DNA methyltransferases seem to divide almost symmetrically (156), indicating that the presence of DNA methyltransferases continually spurs onwards differentiation and induces ESC heterogeneity (151).

### E. ESC Heterogeneity: Fact or Artifact?

However, extensive heterogeneity within ESC cultures may merely be a cell culture artifact (309), as there is no *in vivo* correlate for such heterogeneity. For instance, although naive and primed cells may coexist in mESC cultures, at no time are these two lineages commingled *in vivo*, where preimplantation epiblast synchronously differentiates into postimplantation epiblast after implantation (271).

*In vitro* heterogeneity is partially attributable to insufficient provision of self-renewal signals. For example, in typical culture environs, only a subset of mESCs transduce Wnt signaling (342), which is crucial for stabilizing the naive state (see sect. IV). Limiting amounts of paracrine Wnt therefore generates a continuum of cells, some of which are naive mESCs whereas others outside the influence of Wnt signaling are likely more EpiSC-like. Adding further complexity, mESCs are often cultivated in serum, which may coax differentiation and artificially generate apparent heterogeneity.

Limiting access to self-renewal signals may owe in part to upregulation of negative-feedback signaling inhibitors. For instance, Wnt and LIF/STAT3 signaling respectively induce expression of negative-feedback inhibitors Axin2 (208) and Socs3 (195), which dampen subsequent signaling through their respective pathways and might lead to heterogeneous transduction of extrinsic signals depending on historical antecedents (FIGURE 9C). Such autonomous negative feedback may induce oscillatory STAT3 activity (e.g., Ref. 397) within LIF-treated mESCs (FIGURE 9C), potentially generating considerable heterogeneity in STAT3 activity among individual mESCs, with some cells exhibiting low STAT3 activity and others, high STAT3 activity despite continuous LIF provision. It would be informative to assess whether

STAT3 activity is truly heterogeneous among individual mESCs (171) and whether this could contribute to heterogeneous pluripotency gene expression, for example, in regards to LIF target genes such as Klf4 (256). Thus heterogeneous reception of extrinsic signals by ESCs could generate correspondingly heterogeneous pluripotency factor expression and corresponding lineage biases.

If stochastic signal transduction is one cause of heterogeneity, it should be possible to minimize heterogeneity by constitutively activating a given pathway in all ESCs across a population. Indeed, uniform activation of Wnt/ $\beta$  signaling (through GSK3 inhibition) in “2i” culture conditions generates a significantly more homogeneous population of cells, with 90–100% of mESCs expressing naive pluripotency markers including Rex1, Nanog, and CD31/PECAM1 (379, 380). The apparent homogeneity of “2i”-grown mESCs has led to widespread speculation that a type of naive epiblast “ground state” has been uniformly instilled in these signaling conditions (212, 380) (see sect. IV). However, is this truly the case?

Unexpectedly it seems that hypoblast marker *Hhex* is expressed in 35–65% of “2i”-cultivated mESCs (FIGURE 3C), and such *Hhex* expression is only enhanced by LIF (232). These “2i”-grown, *Hhex*-expressing mESCs robustly contribute to extraembryonic (e.g., hypoblast) lineages *in vivo* (232) (FIGURE 4C). A developmental explanation for these findings remains elusive, as extraembryonic fates are inaccessible to pluripotent epiblast *in vivo* (see sect. IIE). However, it is possible that in “2i” conditions where forward differentiation into primed EpiSCs is strongly inhibited (by FGF/MAPK inhibition and Wnt agonism) that naive mESCs are instead shunted into a more immature developmental state that can access alternative blastocyst fates (FIGURE 4C). For example, Wnt agonism (part of the 2i regimen) upregulates naive epiblast TF *Tbx3* in mESCs, which as aforementioned bestows mESCs with hypoblast potential (206).

Therefore, “2i” culture conditions do not eliminate “lineage priming” in mESCs (cf. Ref. 212) but instead prime different developmental outcomes. While conventional serum conditions prime mESCs for germ-layer differentiation by generating a subpopulation of EpiSC-like intermediates, “2i” signaling conditions may instead prefigure extraembryonic differentiation by capturing a more developmentally primitive state capable of previewing alternate blastocyst fates. For instance, in “2i”-grown naive mESCs, presumably pluripotency TFs such as Oct4 and *Tbx3* continually stimulate hypoblast differentiation (see sect. III), contending with hypoblast-suppressing regulators including Prdm14 (209). Such transcriptional indecision phenotypically manifests as lineage heterogeneity during prolonged *in vitro* culture, depending on which set of lineage-

specifying pluripotency TFs has temporarily prevailed in a given cell.

However, such heterogeneity may be minimized *in vivo* due to the transience of developmental pluripotency. During embryogenesis, extrinsic signals continuously direct pluripotent cells to progress forward, leaving little time for pluripotency TFs to oscillate in expression and compete with one another. This may restrict cells from previewing parallel fates.

In any case, we believe *in vitro* heterogeneity results from innate priming of multilineage possibilities by pluripotency TFs. Such lineage priming (94) is at the heart of pluripotency and confers the developmental competence to subsequently differentiate into subordinate lineages. The next section explores how such preconfiguration of lineage competence is implemented by pluripotency factors at the level of chromatin.

## VI. PRESAGING DIFFERENTIATION: PLURIPOTENCY FACTORS POTENTIATE DEVELOPMENTAL FATES IN CHROMATIN

How are uncommitted progenitors endowed with the competence to access unrealized developmental fates? In operational terms, developmental competence (362) must mean that upon extrinsic direction, progenitors can readily upregulate differentiation genes affiliated with immediately available lineage outcomes (94). Such genes are not yet expressed in undifferentiated ESCs but nevertheless appear “poised” for activation at the chromatin level, allowing them to be inducibly expressed upon developmental command. This therefore foreshadows fates available to pluripotent cells. Indeed, there is cumulative evidence that both proximal promoter elements (16, 28) and distal enhancer elements (76, 278) associated with developmental genes are primed for activation within ESCs. Preparatory priming of differentiation genes in ESCs contrasts with the notion that pluripotency TFs exclusively suppress differentiation (153, 305, 398). Here, we propose that pluripotency TFs are pre-deployed to the loci of developmental genes in ESCs, where they preconfigure such genes in anticipation of subsequent activation. In so doing, pluripotency TFs confer pluripotent cells with their developmental competence at the level of chromatin.

### A. Chromatin Preconfiguration and Developmental Promoter Poising in Pluripotent Cells

It was initially asserted that ESCs are typified by genome-wide “open chromatin” and such global transcriptional permissiveness enabled ESCs to access diverse lineage op-

tions (112, 252). For instance, when genome-wide averages are considered, ESC chromatin is visually less compacted, and repression-associated histone modifications are typically decreased relative to other cell-types (223, 410).

However, whether “globally open chromatin” is truly responsible for pluripotency remains unclear. Pluripotent cells can directly access several immediate lineage options: they do not have all fates encoded by the genome directly available as would be implied by global open chromatin. Furthermore, whether open chromatin is truly ubiquitous in ESCs should be considered in light of the fact that repressive chromatin features are still rather abundant within the pluripotent genome (138, 373). Additionally, the number of genes robustly expressed in ESCs is fairly limited, arguing against promiscuous transcription (212). Therefore, in our view the notion of pluripotency as a globally-open epigenetic tabula rasa may be overstated (305). We instead suggest that pluripotency is endowed by the precise poising of certain developmental regulatory genes that specifically presage germ-layer competence (as opposed to a miscellaneous variety of distal fates).

A number of developmental genes within ESCs bear so-called “bivalent” promoters distinguished by coexistence of both “active” (H3K4me3) and repression-associated (H3K27me3) histone marks (16, 28). Such bivalent labeling may signify that these silent developmental genes can be imminently upregulated. Upon lineage commitment, bivalent genes associated with the chosen lineage become expressed, exclusively inheriting the “active” H3K4me3 mark and losing the “repressive” H3K27me3 mark (28). In contrast, bivalent genes linked to mutually exclusive unrealized fates only acquire H3K27me3 and thereby become silenced (28). Such promoter bivalency therefore provides a conceptually accessible example of how chromatin priming in the pluripotent state could be preparatory for lineage commitment.

However, several issues regarding bivalent promoters have arisen in recent years. First, a number of bivalent promoters appear to be technical artifacts (7). Second, some “bivalent” developmental regulators are associated with much later stages of embryogenesis (e.g., *neurogenin* genes; Ref. 225). Teleologically, it is unclear why such genes should be bivalently marked in primitive ESCs. Finally, whether bivalent promoter marking has any functional consequence remains equivocal. For example, if the H3K4me3 methyltransferase *Mll2* is ablated, H3K4me3 is lost at bivalent promoters yet mESCs can still readily upregulate bivalently marked genes during differentiation (145). Indeed, despite the significance ascribed to them, there is limited evidence that histone modifications themselves (e.g., histone tail methylation) directly influence transcription; they may be more akin to markers (320). In summary, it remains to be

determined whether promoter bivalency functionally underwrites lineage potential.

Another form of promoter poising that might portend more direct functional relevance is the prepositioning of RNA polymerase II (the essential executor of transcription) at the promoters of numerous silent developmental genes in ESCs (122, 212). Such genes are not expressed because RNA polymerase is “stalled” at these promoters and does not initiate transcriptional elongation (122, 212, 404). Nevertheless, RNA polymerase predeployment may allow transcription of these developmental regulators to rapidly ensue upon morphogen signaling and polymerase “release” from the promoter (190). Conceivably, preparatory RNA polymerase prepositioning permits germ-layer genes to be swiftly upregulated upon extrinsic signaling ensuring pluripotent cells remain acutely responsive to developmental cues by permitting rapid differentiation (181). Indeed, this mechanism is critical for *Drosophila* mesoderm development where it ensures cells swiftly and synchronously differentiate in a narrow timeframe (181).

Interestingly, RNA polymerase predeployment is especially prevalent on bivalent promoters in ESCs (226). The conflicting active and inactive chromatin environments juxtaposed at such promoters might respectively recruit RNA polymerase yet prohibit transcriptional elongation (43). However, whether promoter bivalency and RNA polymerase prepositioning are causally linked remains to be fully resolved.

## B. “Preenhancer” States in Pluripotent Cells: Developmental Precedence, Poising, and Prelooping

Further foreshadowing the developmental fates available to pluripotent cells, distal enhancer elements associated with developmental genes can likewise already be primed for activation in the undifferentiated state. Such developmental enhancers acquire active mark H3K27ac upon differentiation yet lack this histone modification in the uncommitted state (76, 278). However, they reside in a “poised” enhancer state typified by open chromatin mark H3K4me1 (76, 278) among other marks. These poised enhancers are also sometimes co-decorated by H3K4me2 (95, 201) and broadly demarcate genes affiliated with the early germ layer fates available to ESCs: endoderm (*SOX17*, *EOMES*), mesoderm (*MIXL1*), and ectoderm (*GBX2*) (201, 278) (**FIGURE 10A**). Enhancer poising therefore appears to precede the lineage decisions that pluripotent cells may undertake.

H3K4me1 at poised enhancers may constitute a “window of opportunity” (51) for subsequent enhancer activation and gain of H3K27ac upon lineage commitment. It remains to be elucidated whether H3K4me1 is merely a descriptive marker of poised chromatin or if it functionally sets the

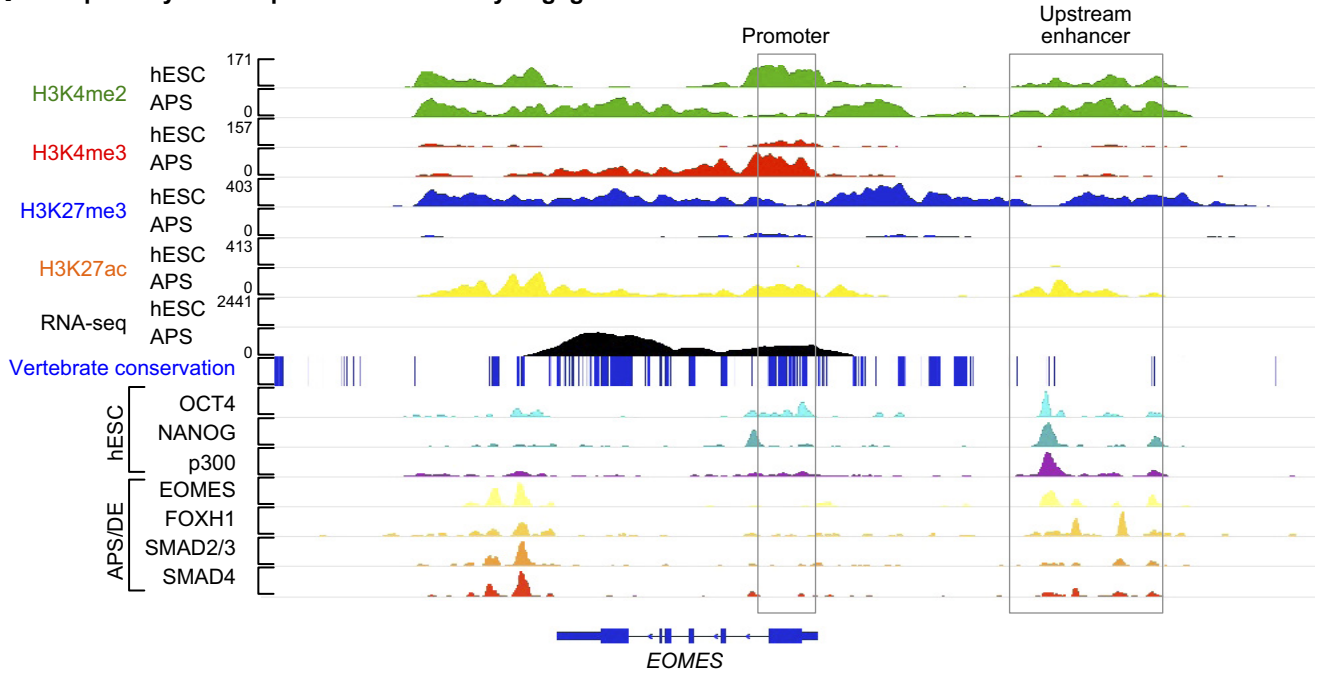
stage for future enhancer activation (51). However, H3K4me1 can directly enlist activation-associated histone acetyltransferase Tip60 (157), and enhancer-associated H3K4me1/2 methyltransferase Mll4 is functionally required for enhancer activation in some developmental scenarios (184). This hints enhancer poising may have some causal significance prior to enhancer activation.

Nevertheless, not all poised developmental enhancers are universally premarked by H3K4me1. For instance, a sizable fraction of prospective endoderm and mesoderm enhancers in hESCs are bookmarked by histone variant H2AZ (201) in the apparent absence of H3K4me1 (364). H2AZ-laden nucleosomes are unstable and readily displaced by TF binding (146, 162). Therefore, prepositioning of H2AZ at developmental enhancers within hESCs possibly attracts developmental TF binding upon differentiation, enabling these TFs to infiltrate and thus activate these enhancers (201), complaisant with a role of H2AZ in executing mESC differentiation (146, 196). In summary, H2AZ predeployment to lineage-specific enhancers in ESCs strategically presages future differentiation by functionally enabling TFs to subsequently bind and activate these enhancers.

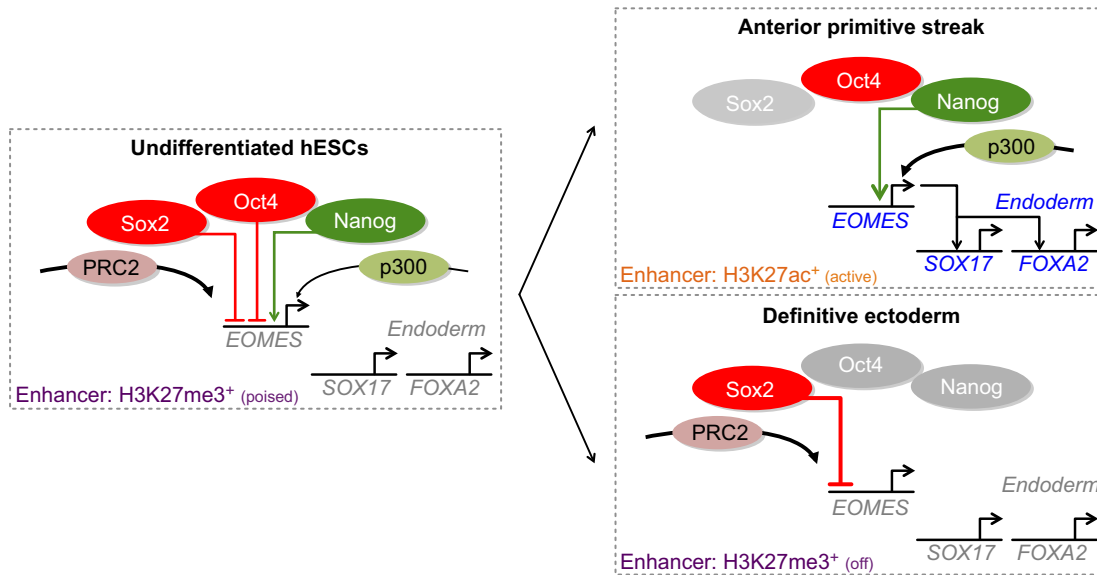
However, an integrated model of enhancer poising remains elusive. Poised enhancers are commonly thought to be characterized by both H3K4me1 and H2AZ in ESCs (51). However, in reality there is likely no single universal signature for enhancer poising. Distinct subsets of early endoderm enhancers are marked by H2AZ alone, H3K4me1 alone, both markers, or neither of them in hESCs (201).

Clearly, poised developmental enhancers are a heterogeneous class of element. Indeed, given the functional diversity of enhancers (47), a priori it would be unexpected that there need be any singular ubiquitous enhancer “poising” mechanism. Instead, there exists a constellation of distinct developmental “preenhancer” states in ESCs, each characterized by differing combinations of chromatin marks but all of which can be readily activated upon differentiation (201). For each of these “preenhancer” states, there is likely a different regulatory sequence for enhancer priming. For example, a quarter of endodermal enhancers are exclusively marked by H2AZ in the apparent absence of other factors in hESCs (201). In these cases, H2AZ predeployment may be the “trailblazing” event enabling subsequent H3K4me1 deposition and TF acquisition upon differentiation. Yet, for other enhancers, H3K4me1 “poising” may instead precede gain of H2AZ. It will be important to decipher whether H2AZ and H3K4me1 are deposited synchronously in a coordinated manner (51) or instead whether these marks are sequentially gained, and the different order(s) in which various poising marks can be deposited. Activation mechanisms likely differ for varying preenhancer classes. Histone acetyltransferase p300 is predeployed at a subset of H3K4me1-marked poised enhancers in hESCs (278) (**FIG-**

**A** Pluripotency transcription factors already engage the *EOMES* enhancer in hESCs



**B** A transcriptional duel between pluripotency factors over the *EOMES* enhancer



**FIGURE 10.** Pluripotency factors potentiate developmental fates in chromatin by dueling over *EOMES*. **A:** chromatin state analysis of the *EOMES* gene in both undifferentiated hESCs and hESC-derived anterior primitive streak (APS) (201). In hESCs, the upstream enhancer is already bound by OCT4, SOX2 [not shown (343)], NANOG, and p300 (278). Yet, due to competition between these pluripotency factors, it is retained in a “poised” state bearing repression-associated H3K27me3 and euchromatic mark H3K4me2 (but active mark H3K27ac is absent). Within 24 h of APS differentiation, H3K27me3 is rapidly depleted and H3K27ac is acutely gained, correlating with enhancer activation and *EOMES* upregulation (RNA-seq data; black). A number of endoderm-specifying TFs including *EOMES* itself, SMAD2/3, SMAD4, and FOXH1 now engage the active *EOMES* enhancer (173, 343). ChIP-seq data are taken the authors’ own work (201) and other publicly available datasets (173, 278, 343). **B:** in hESCs, OCT4, SOX2, and NANOG duel over the *EOMES* enhancer: NANOG drives *EOMES* activation but is opposed by the countervailing forces of OCT4 and SOX2 (*left*). Both H3K27 acetyltransferase p300 and H3K27 methyltransferase PRC2 are simultaneously recruited. As a result, *EOMES* is not expressed. During anterior primitive streak differentiation (*top*), SOX2 is lost, leaving NANOG to upregulate *EOMES* together with p300 which deposits H3K27ac. *EOMES* subsequently upregulates endoderm differentiation genes *SOX17* and *FOXA2*. However, during ectoderm commitment (*bottom*), NANOG is suppressed, leaving SOX2 and PRC2 to unilaterally silence *EOMES*.

**URE 10, A AND B).** The ab initio availability of p300 might explain why these enhancers may gain H3K27ac so swiftly upon differentiation, whereas other enhancers must recruit histone acetyltransferases de novo only upon differentiation.

Interestingly, poised developmental enhancers in ESCs are frequently prelooped to their target promoters even prior to gene activation (163, 409), signifying another form of differentiation priming. Typically when enhancers become activated, the intervening chromatin between the enhancer and its cognate gene deforms such that the enhancer physically “loops” to its target promoter (165), activating gene transcription (83). Therefore, the preexisting juxtaposition of poised enhancers with their cognate developmental genes in ESCs (409) might facilitate rapid differentiation, as enhancer activation upon developmental cues (see below) can be immediately translated into gene upregulation. Significantly, RNA polymerase II is often predeployed to these “prelooped” promoters, and such promoters are also frequently bivalently marked (409). Enhancer-promoter prelooping combined with RNA polymerase predeployment at such developmental promoters likely synergize to enable rapid developmental gene expression and hence synchronous ESC differentiation. How are all these preparatory arrangements made? Prelooping of enhancers to promoters in ESCs is driven by pluripotency factors (370), which as we explain below are fundamentally responsible for “priming” differentiation genes in multiple ways.

### C. Pluripotency Factors Duel to Specify Competing Lineages Within Chromatin

How are activatory chromatin marks (e.g., H2AZ, H3K4me1, and H3K4me3) strategically deployed to developmental genes in anticipation of differentiation? This signifies some developmental “foresight” in that prospective lineage outcomes are being presciently visualized even in the uncommitted state. Chromatin modifiers often lack intrinsic sequence-specific DNA-binding activity. Therefore, for activation-associated chromatin marks to be precisely deposited at differentiation genes, their cognate depositors must somehow be guided there by sequence-specific TFs in ESCs. What TFs within pluripotent cells could poise developmental genes in this fashion? We propose that pluripotency TFs functionally prime developmental gene expression, and in this capacity, they presage differentiation.

Developmental gene loci are frequently decorated by pluripotency factors in the undifferentiated state (37). On the basis of these data, it was initially asserted that pluripotency factors suppress such developmental genes (37, 153, 305, 398). Yet this is not always the case.

How pluripotency factors potentiate differentiation is illustrated by their action on the *EOMES* gene, which is neces-

sary for anterior primitive streak/definitive endoderm development in vivo (13). Although *EOMES* is not expressed in hESCs, its upstream enhancer element is jointly occupied by OCT4, SOX2, and NANOG (343) (**FIGURE 10A**). Strikingly, endoderm-inducing pluripotency TF NANOG activates the *EOMES* enhancer in hESCs, whereas the same enhancer is reciprocally suppressed by mesoderm-inducing OCT4 and ectoderm-specifying SOX2 (343). Therefore, these pluripotency factors duel at the level of chromatin, competing to elicit their respective lineage outcomes even in the undifferentiated state (**FIGURE 10B**). As a result, *EOMES* is not appreciably expressed in hESCs. However, NANOG predeployment at the *EOMES* enhancer in hESCs serves a strategic purpose: it enables *EOMES* to be acutely expressed in the anterior primitive streak once SOX2 expression declines (343). In summary, NANOG continually accesses endodermal genes, potentiating their expression, and thereby instills hESCs with their endodermal competence.

Pluripotency factors likely capacitate developmental gene expression by instructing the deployment of chromatin regulators. For instance, the activation-associated histone acetyltransferase p300 is already predeployed to the *EOMES* enhancer in hESCs (278) (**FIGURE 10A**) and might be delivered there by NANOG (**FIGURE 10B**). The SMAD2/3 transcriptional coactivator FOXH1 is likewise prepositioned at the *EOMES* enhancer (30). In opposition, endoderm-repressing pluripotency TF TEAD4 directly recruits the NuRD histone deacetylase complex (30), and in parallel, the PRC2 H3K27me3 methyltransferase complex is presumably attracted by another opposing TF (e.g., SOX2) (**FIGURE 10B**). There is therefore a battle over the *EOMES* enhancer: NuRD histone deacetylases counteract p300 by depleting H3K27ac. In parallel, PRC2 divests H3K27me3 to install a repressed chromatin state (**FIGURE 10B**). Therefore, competing pluripotency factors might dichotomously recruit opposing chromatin modifiers to duel over lineage specification genes in ESCs.

However, the conflict is rapidly resolved upon lineage commitment. Upon endoderm induction, TEAD4 and SOX2 are displaced (30, 343) and the *EOMES* enhancer is thereby emancipated from the oppressive influence of NuRD deacetylases (**FIGURE 10B**). As a consequence, p300 becomes unopposed: H3K27ac acutely accumulates within just 24 h of anterior primitive streak differentiation (201). In parallel, H3K27me3 is totally eliminated (201) (**FIGURE 10A**), and it is likely erased by H3K27 demethylases Utx and Jmjd3 (78, 160, 170). Finally, FOXH1 cooperates with TGF- $\beta$  effectors SMAD2/3 (30) to enact full-fledged enhancer activation.

In contrast, Sox2 potentiates a neural fate by binding a number of neuroectodermal genes within ESCs (25). Potentiation of these neural genes by Sox2 in ESCs is crucial for

sustaining neural competence, because if *SOX2* is ablated, hESCs can no longer differentiate into neuroectoderm (366). Sox2 recognizes the same Sox motif as anterior ectoderm TF Sox3, and therefore, Sox2 likely “stands in” at these regulatory elements as a placeholder, maintaining local open chromatin and creating a foothold for future Sox3 binding in early neuroectoderm (25).

Other pluripotency factors may also serve as temporary stewards of differentiation-associated enhancer elements in ESCs until the appropriate TFs can bind during differentiation. For instance, Foxd3 binds to the *albumin* enhancer in mESCs and maintains it in an unmethylated state until Foxa1 can bind the enhancer in hepatic progenitors (382, 383), and a related mechanism operates at the *immunoglobulin lambda* enhancer (197). Although pluripotency factor binding presages future binding of lineage commitment factors at these elements, these regulatory elements are not precociously activated in the undifferentiated state. This could be because fully fledged enhancer activation requires lineage-specific transcriptional coactivators that are absent in the undifferentiated state (4, 200) or because other competing pluripotency factors suppress these same elements, as is the case for the *EOMES* enhancer.

#### D. Enhancer Poising: the Pages Yet-to-Be Turned

Despite the advances summarized above, knowledge of enhancer poising still remains fragmentary. How many early developmental enhancers are truly “poised” in ESCs? A full census of poised enhancers remains out of reach because there is really no single unified enhancer poising signature given the multiple enhancer poising mechanisms detailed above (and those that remain to be discovered). Furthermore, from a functional perspective, need all major germ-layer developmental regulators even be strategically poised in hESCs?

Poising may facilitate but not be obligatory for enhancer activation. Some endoderm enhancers lack H3K4me1 and H2AZ and instead bear heterochromatic mark H3K9me3 (associated with strong repression; Ref. 411) (201). Strikingly, around one-fifth of endoderm enhancers appear “latent” (265), lacking all known chromatin marks (201). These “nonpoised” or repressed enhancers likely require distinct activation mechanisms. For instance, heterochromatic H3K9me3 accumulation on enhancers is likely inimical to TF binding (319). Therefore, these repressed enhancers may require an additional activatory step in which H3K9 demethylases are recruited prior to enhancer activation/TF binding. Latent endoderm enhancers lacking all recognizable chromatin marks may be specifically activated by so-called “pioneer factors,” which include endodermal TFs (e.g., Foxa2 and Gata4; Ref. 69) that can directly access and open compacted chromatin. Presumably pioneer fac-

tors might be capable of analogously activating latent or repressed developmental enhancers. Given such potent activatory mechanisms, how “many” developmental enhancers for a given germ layer fate need to be teleologically poised for hESCs to be able to access that lineage?

Another question concerns what degree of developmental foresight is embodied by enhancer poising. How many developmental steps ahead do pluripotent cells “see”? As is the case with bivalent promoters, a number of genes associated with much later development also appear to bear poised enhancers in ESCs (197, 278, 383), and the teleological necessity of this remains unclear. When lineage commitment occurs, do poised enhancers associated with mutually exclusive lineages become “decommissioned” (374) or do they still harbor H3K4me1 (34), perhaps reflecting latent developmental plasticity to be respecified into those cell types?

Finally, how do developmental enhancers specifically associated with the correct prospective lineage options become strategically poised in ESCs? Although developmental gene loci are likely ensconced by closed chromatin, pluripotency TFs including Oct4 and Sox2 may possess pioneer factor activity (319) and thereby access and thus poise these silenced developmental enhancers. Nevertheless, how is H2AZ (typically associated with active transcription) also targeted to as-of-yet-inactive lineage-specific enhancers in ESCs? Oct4 and Nanog both directly interact with H2AZ depositor p400/Trrap (106, 268, 355) and presumably thereby target H2AZ insertion into developmental enhancers, potentially explaining how enhancer poising is established de novo. In addition, the ability of poised enhancers to be prelooped to their target promoters (409) may also be coordinated by pluripotency TFs, which physically interact with enhancer-looping complexes (e.g., Mediator; Refs. 166, 355) perhaps bringing developmental enhancers about to promoters as a consequence (370).

#### E. Securing Lineage Choice by Chromatin Rearrangements

Although pluripotency TFs confer developmental competence unto pluripotent cells, as pluripotency segues into differentiation how is lineage choice maintained? Embryogenesis is unidirectional; therefore, differentiation regulators repress the preceding pluripotency regime to ensure forward developmental progression. Although pluripotency and differentiation programs temporarily overlap as pluripotency TFs drive the earliest steps of commitment (see sect. IV), clearly at some point these two states must be stringently separated.

Lineage commitment factors act recursively to suppress pluripotency genes. Even as NANOG initially elicits *EOMES* expression in the posterior epiblast/primitive

streak, EOMES transcriptionally represses *OCT4*, *SOX2*, and *NANOG* (343). Therefore, the transcriptional torch is passed from *NANOG* to *EOMES* as cells irreversibly depart from pluripotency and enter lineage commitment. This decision is made permanent by several mechanisms. First, caspases induce the degradation of pluripotency TFs themselves, eliminating residual protein (104). Second, differentiation-upregulated microRNAs forestall subsequent translation of pluripotency factor mRNAs (339, 340, 384). Finally, chromatin repressors attenuate pluripotency gene expression. Upon differentiation, H3K9 methyltransferase G9a deposits the heterochromatic H3K9me3 mark upon the *Oct4* promoter, indirectly recruiting DNA methyltransferases to achieve gene silencing (99). H3K4 demethylase *Lsd1* acts analogously to silence the enhancer elements of pluripotency genes (374).

As a whole, chromatin repression features prominently in lineage commitment. Interestingly, when mESCs are stricken of various chromatin repressive complexes, including polycomb repressive complex 1 [*Ring1b*<sup>-/-</sup> (357, 361)], polycomb repressive complex 2/PRC2 [*Eed*<sup>-/-</sup> (351), *Ezh2*<sup>-/-</sup> (257), *Suz12*<sup>-/-</sup> (270)], the NuRD histone deacetylase complex [*Mbd3*<sup>-/-</sup> (167)], or DNA methyltransferases [*Dnmt1*<sup>-/-</sup> (187, 191), *Dnmt3a*<sup>-/-</sup>; *Dnmt3b*<sup>-/-</sup> (152)], their self-renewal is minimally impaired. In contrast, these repressor-deficient mESCs are incapable of properly differentiating; in some circumstances they simply perish (187, 267) instead of differentiating.

Why are ESCs bereft of chromatin repressive mechanisms unable to differentiate? Perhaps such cells are unable to silence pluripotency genes during lineage commitment. For example, PRC2-deficient *Suz12*<sup>-/-</sup> mESCs express ESC markers even during differentiation and are therefore unable to generate mature cell types (270).

Another possible explanation is that ESCs lacking chromatin repressors express lineage markers from multiple competing lineage programs and are therefore unable to decisively commit to any particular lineage. Because unilateral lineage commitment requires the repression of alternative lineage options (94), spurious expression of extraneous lineage regulators may impair unilateral commitment. Indeed, repressor-deficient cells often perish upon differentiation (187, 267), and we speculate this could be driven by a hypothetical developmental safeguard that induces apoptosis of cells coexpressing multiple lineage programs. Therefore, as pluripotency segues into lineage commitment, a decisive lineage decision may only be accomplished once a dominant lineage program employs chromatin repression (e.g., DNA methylation or histone deacetylation) to subjugate its competing programs to consolidating exclusive lineage commitment.

## VII. MODELS DESCRIBING PLURIPOTENCY

### A. Differences Between the Ground State and Precarious Balance Models of Pluripotency

Historical interest in the mechanism of ESC self-renewal may have overshadowed the most quintessential characteristic of pluripotent cells, their vast developmental competence. Indeed, Zaret (402) reminds us that we should consider pluripotent cells with regard to *what they can become*, rather than with regard to *what they currently are*. In light of this, although there has been substantial emphasis on factors regulating the undifferentiated “self-renewal” of pluripotent cells, it now seems appropriate to refocus attention onto the molecular mechanisms underlying their multilineage potential, which, after all is the sine qua non of pluripotent cells.

To this end, the ground state (305) and precarious balance models (203) have both been proposed to describe pluripotency, although they differ in many ways (TABLE 5). The ground state hypothesis asserts that pluripotent cells are managed by a totalitarian regime in which pluripotency factors inhibit differentiation. In contrast, the precarious balance model avers that some pluripotency factors are instead lineage specifiers that drive lineage commitment. The ground state hypothesis predicts that pluripotency is intrinsically stable when extrinsic signals are inhibited and that this stability is attributable to the differentiation-suppressing effects of pluripotency factors. On the other hand, the precarious balance model implies that pluripotency is intrinsically unstable due to the lineage-specifying activities of pluripotency factors and therefore extrinsic signals are necessary to coerce self-renewal.

At the crux of this discussion is whether pluripotency factors truly drive differentiation and therefore equip ESCs with their developmental competence. This claim is corroborated by multiple lines of evidence. Notably, pluripotency factors are endogenously required for pluripotent cells to differentiate towards specific lineage outcomes both in vivo and in vitro (see sect. III). If an individual pluripotency factor is acutely ablated, pluripotent cells can no longer access the relevant lineage outcome. If the pluripotency factor is overexpressed, they are ectopically driven towards that lineage. These findings are altogether contrary to the notion that pluripotency factors function exclusively as antagonists of differentiation. Instead, pluripotency factors have an imperative role in potentiating lineage-specific differentiation.

Indeed, from an embryological perspective, pluripotent cells are on the developmental precipice of differentiation. They reside on one step in a long series of lineage progressions, and each step, cells must be prepared to boldly go

**Table 5.** Comparison of pluripotency models

	Ground-State Model	Precarious Balance Model
Function of pluripotency factors	Unilaterally inhibit differentiation	Some are lineage specifiers that specify differentiation to a particular lineage
Intrinsic transcriptional stability of pluripotency	Intrinsically stable (380)	Intrinsically unstable
Contribution of extrinsic signals	If all extrinsic signals are censored, self-renewal is constitutive	Self-renewal continually contingent on provision of extrinsic signals
Explanation of ESC heterogeneity	Autocrine FGF signaling downregulates <i>Nanog</i> (305)  Endogenous Tcf3 continually represses Wnt-dependent pluripotency genes, can be relieved by exogenous Wnt signaling	In addition to points raised in the ground state models, pluripotency transcription factors and auxiliary transcriptional repressors (e.g., Tcf3 and Mbd3) continually prime downstream lineage options, triggering lineage fluctuations <i>in vitro</i>
How ESC can be made homogeneous	In “ground state” culture conditions (“2i”) with concomitant Wnt agonism and MAPK inhibition	Individual ESCs will tend to display lineage biases due to the intrinsic lineage-specifying function of pluripotency factors; difficult to achieve complete homogeneity
Default “ground state” of pluripotent cells	Undifferentiated self-renewal	Lineage commitment and forward developmental progression

forth into the next. It is therefore logical that the main reason that pluripotency factors are even expressed in epiblast cells is to enable pluripotent cells to venture towards the next developmental step. Therefore, when pluripotent cells are first born, they gain pluripotency factors and a number of intrinsic transcriptional repressors (e.g., Mbd3 and Tcf3) that together continually drive forward lineage progression to ensure unidirectional developmental progress. Why pluripotency should be intrinsically transcriptionally stable (380) is questionable as developmental pluripotency is only a transitory staging point *in vivo* before lineage specification ensues. Altogether, from a teleological point of view, it seems likely that pluripotency factors are expressed in pluripotent cells solely to drive subsequent lineage progression and to enable multilineage differentiation.

We therefore suggest pluripotency and differentiation are closely intimated. In contrast to the view that pluripotency and differentiation are strictly separated states, pluripotency and differentiation regulators are transiently coexpressed during early differentiation. Specific pluripotency factors are upregulated when epiblast cells are assigned to either the prospective primitive streak or ectoderm (see sect. IV), and these pluripotency factors drive incipient lineage commitment by priming developmental genes in chromatin for activation (see sects. V and VI). Therefore, it seems apt to conclude that 1) pluripotency factors are bona fide lineage specifiers, and 2) upregulation of particular lineage-specifying pluripotency factors is the developmental mechanism through which pluripotent cells are allocated to specific fetal germ layers both *in vivo* and *in vitro*. This hypothesis (203) reconciles hitherto-irreconcilable observa-

tions over the years, specifically that pluripotency TF overexpression drives differentiation and that some pluripotency factors do not block, but are rather required for differentiation. Finally, recent evidence suggests pluripotency factors also reprise their roles as lineage specifiers during iPSC reprogramming in both mouse and human lineages (231, 303, 327) (see below).

## B. Pluripotency Factors as Lineage Specifiers During iPSC Reprogramming?

One unexpected finding in recent years was that pluripotency factors might potentially also masquerade as lineage specifiers during iPSC reprogramming (231, 303). These data have been cited as key support of the precarious balance model of pluripotency (23, 67). iPSC reprogramming is typically effectuated by overexpressing pluripotency TFs (e.g., *Oct4*, *Sox2*, *Klf4*, and *c-Myc*; “OSKM”) in committed cell types to directly instate a pluripotent identity (328).

Strikingly, *Oct4* can be functionally replaced as a reprogramming factor by various endoderm/mesoderm lineage regulators including *Gata3*, *Gata4*, and *Gata6* that have no role in pluripotency (231, 303). This coincides with the role of *Oct4* as a primitive streak specifier in ESCs that represses alternate ectoderm fates (203). If *Oct4* is removed from the reprogramming quartet, reprogramming is aborted and ectodermal TFs (e.g., *Dlx3*) become reciprocally upregulated (303). Ablating *Dlx3* allows reprogramming in the absence of *Oct4* (in “SKM” conditions), signaling that a pivotal role of *Oct4* is to suppress ectoderm genes during reprogramming (303). Why should ectoderm regulators be upregu-

lated during reprogramming in the first place? This is likely because another reprogramming factor is the ectoderm-specifying pluripotency regulator *Sox2*, which extraneously induces ectodermal genes during reprogramming (225, 303) as well as in ESCs (203).

Therefore, it seems that a critical function of *Oct4* and *Sox2* during reprogramming is to suppress one another's ancillary and counterproductive lineage-specifying activities, preventing digressions into other cell types and exclusively consolidating reprogramming to a pluripotent lineage. Therefore, a balance between *Oct4* and *Sox2* may be key. For example, *Sox2* acts reciprocally to cross-repress *Oct4*-induced endoderm/mesoderm regulators during reprogramming. Therefore, *Sox2* itself can be replaced by ectoderm lineage commitment factors *Sox1*, *Sox3*, and *Znf521* that are not associated with pluripotency but can block these endoderm/mesoderm proclivities (231, 303). Remarkably, even if mesodermal and ectodermal specifiers are respectively used to simultaneously replace *Oct4* and *Sox2*, fully fledged iPSCs can still be generated in the context of *Klf4* and *c-Myc* (231, 303), supporting the “precarious balance” viewpoint.

How could differentiation regulators that are not expressed in ESCs incipiently activate pluripotency genes in fibroblasts to execute reprogramming? Paradoxically, there is ample evidence that such lineage specifiers often repress pluripotency gene expression (see sect. VI). However, when *Oct4* and *Sox2* are concomitantly replaced using differentiation regulators, perhaps a permissive environment for iPSC reprogramming is created because these commitment factors cross-repress transdifferentiation to undesirable fates (e.g., *Oct4* replacements inhibit ectoderm and *Sox2* substitutes blockade mesoderm). In this lineage-neutralized environment, *Klf4* and *c-Myc* must then assume the role of an instructive force to upregulate pluripotency genes. Nevertheless, how *Klf4* and *c-Myc* could be sufficient to induce such ESC genes in this context remains unclear, as they may contend against the lineage specifiers themselves that were used to replace *Oct4* and *Sox2*. Therefore, the mechanisms through which lineage specifiers can induce iPSCs remain to be fully elucidated.

The notion that pluripotency factors can act as lineage specifiers during reprogramming was also supported by a report that *Oct4* alone can transdifferentiate fibroblasts into a mesodermal fate (blood) (327). While *Oct4* clearly instructs a primitive streak/mesodermal fate in the gastrulating epiblast (see sects. III and IV), it remains uncertain whether it reprises this exact role when ectopically expressed in fibroblasts. Besides blood, can *Oct4* transdifferentiate other mesodermal lineages directly from fibroblasts? Other *Oct* family TFs are involved in hematopoiesis, and therefore, the function of *Oct4* in this circumstance (327) might be to bind to their target motifs and broadly mimic the role of other

*Oct* homologs in hematopoiesis (as opposed to driving primitive streak differentiation per se).

In summary, pluripotency factors may act dichotomously during reprogramming to resuscitate pluripotency gene expression while concomitantly driving transdifferentiation into their own lineages of interest. The latter might constitute a molecular heirloom from ESCs, where pluripotency factors similarly instruct differentiation. Thus dampening the lineage-specifying activities of pluripotency factors may be beneficial to thwart transdifferentiation into committed fates and consolidate pluripotency within incipiently forming iPSCs.

### C. Concluding Remarks

“We shall not cease from exploration, and the end of all our exploring, will be to arrive where we started and know the place for the first time.”

T.S. Eliot, *Little Gidding* (1942)

The appreciation of mammalian epiblast pluripotency in the 1950–1970s and the subsequent isolation of ESC lines in 1981 proclaimed a new expeditionary era exploring the molecular design principles of pluripotency and more recently to recreate it. Yet the fundamental nature of pluripotency remains obscure. Is it an intrinsically stable state maintained by genes that block differentiation or a precarious situation organized by pluripotency factors functioning not as antidifferentiation factors, but rather lineage specifiers? This review has explored this question while advocating the notion that pluripotency genes function as lineage specifiers that drive lineage commitment and thereby endow pluripotent cells with their multilineage differentiation potential. Lineage-specifying pluripotency factors may promote a specific lineage whilst excluding alternative fates, therefore jointly establishing an unstable equilibrium where no fate dominates. These ideas may reconcile the paradoxical functions of pluripotency genes, seemingly maintaining ESCs in an undifferentiated state whilst conferring the potential to form all lineages. As Elliot quips, at the end of all of our explorations, we may find ourselves back at the beginning: pluripotency, from whence everything began.

### VIII. APPENDIX: X CHROMOSOME INACTIVATION AND PLURIPOTENCY

In most differentiated female cells, one of two X chromosomes is epigenetically “inactivated” (336) for the purposes of dosage compensation (leading to so-called “ $X_aX_i$ ” status; one X chromosome is active, while the other is inactive). However, in female peri-implantation epiblast (262) as well as mESCs, both X chromosomes are active ( $X_aX_a$ ), and this is a distinctive feature of naive pluripotency (247). X inactivation occurs only upon differentiation within the

postimplantation epiblast after the conceptus implants (230). Like the postimplantation epiblast, female mEpiSCs also exhibit X inactivation (17), therefore distinguishing them from  $X_aX_a$  female mESCs.

Interestingly, naive pluripotency and X chromosome activation may be mechanistically intertwined. It was originally speculated that the two active X chromosomes of female mESCs broadly reflected a “generally open chromatin landscape” within pluripotent cells. However, certain pluripotency factors explicitly intervene to prevent X inactivation in female mESCs by directly regulating various components of the X activation/inactivation machinery. For example, Oct4 and Nanog bind to and downregulate the expression of *Xist* (90, 238), a primary effector of X chromosome inactivation. Concomitantly, Rex1 upregulates the expression of *Tsix* (239), an inhibitor of X chromosome inactivation. Moreover, Oct4, Sox2, and Nanog cotarget and potentially regulate the expression of *Rnf12*, another mediator of X chromosome inactivation (164).

Because pluripotency factors directly facilitate X chromosome activation, an intuitive question is whether two active X chromosomes are beneficial for pluripotency. However, two active X chromosomes must not be absolutely required for pluripotency, as XY male ESCs are obviously still pluripotent. In contrast, two active X chromosomes might actually be detrimental to pluripotency (310), as  $X_aX_a$  female mESCs are chromosomally unstable and frequently lose part of or an entire copy of the X chromosome (287). Furthermore, female  $X_aX_a$  mESC lines are frequently afflicted by global DNA hypomethylation (412), although the etiology of this epigenetic dysfunction remains cryptic.

In fact, when considering the intimate linkage of naive pluripotency and X chromosome activation, it may make more sense to think about the issue the other way around: that X chromosome activation is not required for pluripotency, but rather, two active X chromosomes in pluripotent cells is important for the mouse’s developmental X chromosome inactivation strategy (211). Namely, preprogrammed parental X chromosome inactivation in early female blastomeres is strategically undone in naive epiblast to enable either X chromosome to be subsequently randomly inactivated during subsequent development (262).

However, the latest evidence suggests that two active X chromosomes might be beneficial for female mESC self-renewal. Intriguingly, upon differentiation, naive marker *Rex1* is downregulated more slowly in female  $X_aX_a$  mESCs relative to male mESCs (300), suggesting that an additional active X chromosome may impede differentiation to some extent. Indeed, removing an X chromosome (as in XO female mESCs) causes differentiation to proceed more rapidly (300). However, the physiological relevance of these findings remains to be established; it remains unclear whether

the naive-to-primed epiblast transition truly occurs slower in vivo in female embryos as opposed to male embryos.

Can X inactivation status inform the popular debate of the embryological ancestry of hESCs versus mESCs? It is generally assumed that all female hESC lines display X chromosome inactivation, and this constitutes proof of their postimplantation identity (247). Yet upon close examination, some, but not all, female hESC lines exhibit X chromosome inactivation (44, 306). Hence, the picture is muddy in regards to whether based on X chromosome status alone, hESCs may be likened to the  $X_aX_a$  naive mESCs or else  $X_aX_i$  primed mEpiSCs.

In fact, X chromosome status may not be a precise metric to determine whether hESCs resemble pre/peri-implantation or postimplantation epiblast. In mammals besides mice, X chromosome inactivation occurs before implantation. Prior to implantation, female rabbit ICM cells display X chromosome inactivation and human ICM cells show molecular features of X chromosome inactivation (*Xist* upregulation) without overt epigenetic inactivation of the X chromosome (263). Therefore, species considerations need to be taken into account before using measures of mouse naive pluripotency to make extrapolations about features of pluripotency in other species.

## ACKNOWLEDGMENTS

We are indebted to the members of the embryonic stem cell field, whose years of ceaseless research provided us with the experimental observations and motivation to write this review. We thank Massimo Nichane and Kian Leong Lee for discussions on pluripotency, Roger Pedersen for discussions regarding primed pluripotency, Yaqub Hanna for insight into human naive pluripotency, Irving Weissman and Roel Nusse for debate concerning self-renewal, and Austin Smith for introducing the phrase “capacitating differentiation.” We also thank S. W. S. Lim, J. Y. Zhang, V. Kumar, and S. Prabhakar for coining the neologism “preenhancer” and contributing specific data that underlie some of the ideas proposed here. Finally, we apologize to all workers in the field whose papers we were unable to refer to due to space constraints.

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## GRANTS

K. M. Loh is supported by fellowships from the Fannie and John Hertz Foundation, the Davidson Institute for Talent Development, and the United States National Science Foundation. L. T. Ang, K. M. Loh, and B. Lim are jointly sup-

ported by a grant from the Singapore Agency for Science, Technology, and Research (A\*STAR).

## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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*Physiol Rev* 95:245-295, 2015. doi:10.1152/physrev.00001.2014

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