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New and renewed perspectives on embryonic stem cell pluripotency

Jackson A. Hoffman, Bradley J. Merrill

Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, IL 60607

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1. ABSTRACT

Although "stem cell biology" is frequently described as a young field, the examination of pluripotency and its effects on embryonic cells has had an interesting and somewhat unusual history. After decades of research into the pluripotency of mammalian embryonic cells, the use of pluripotent cells came into prominence as mouse embryonic stem cells (ESC) provided the foundation of knockout mouse technology; however, the basic biology of pluripotency in embryonic cells was not extensively examined for roughly another twenty years until the creation of human embryonic stem cell lines. With the burgeoning potential of cell based therapies and roles of cancer stem cells in disease, understanding basic biological mechanisms regulating stem cell characteristics now presents great new opportunities. Therefore, it is not surprising that the underlying genetic and epigenetic forces allowing ESC to maintain pluripotency have been the focus of intense scientific scrutiny in recent years. In order to fully appreciate the importance of new discoveries regarding pluripotency in ESC, it is necessary to understand the role of pluripotency in normal embryonic development. The main purpose of this review is to highlight recent discoveries in the context of what was known about pluripotency and lineage commitment in the embryo prior to the bioinformatics and genomics age. In doing so we attempt to elucidate the importance and limitations of recent discoveries and identify important avenues for future research.

2. INTRODUCTION / FUNCTION OF LINEAGE IN DEVELOPMENT

Each of the cells in an adult human's body was derived from a single totipotent cell, the fertilized egg (zygote). Obviously, the trillions of cells that arose from the zygote to produce the hundreds of distinct cell types were formed in a reliably programmed manner such that physiologically functioning organ systems are correctly positioned within a defined body plan. Given the similarity of many microenvironments and the finite effects of an individual molecular stimulus, the creation of such a diversity of cellular dynamics requires the function of lineage. For the purposes of this review, cell lineage effects can be defined as the remnants of previous progenitors that affect cell characteristics and responses. Many factors can transmit lineage information; chromatin modifications, stable proteins, and RNA have all be shown to affect cellular progeny after their synthesis. Distinct cellular lineages allow cells exposed to the same microenvironment and stimuli to produce disparate responses and thus add to the potential diversity of cell types. One illustrative example of the effects of cell lineage in mammals can be seen in the fur of the calico or tortoiseshell cat. Female felines heterozygous for the X-linked *Orange* gene exhibit distinct spots of orange and black hair due to the epigenetic inactivation of the X-chromosome during early stages of embryogenesis (1, 2). Cells in which the X-chromosome bearing the dominant allele (*O*) is inactivated will contribute to black-colored hair, whereas cells in which the

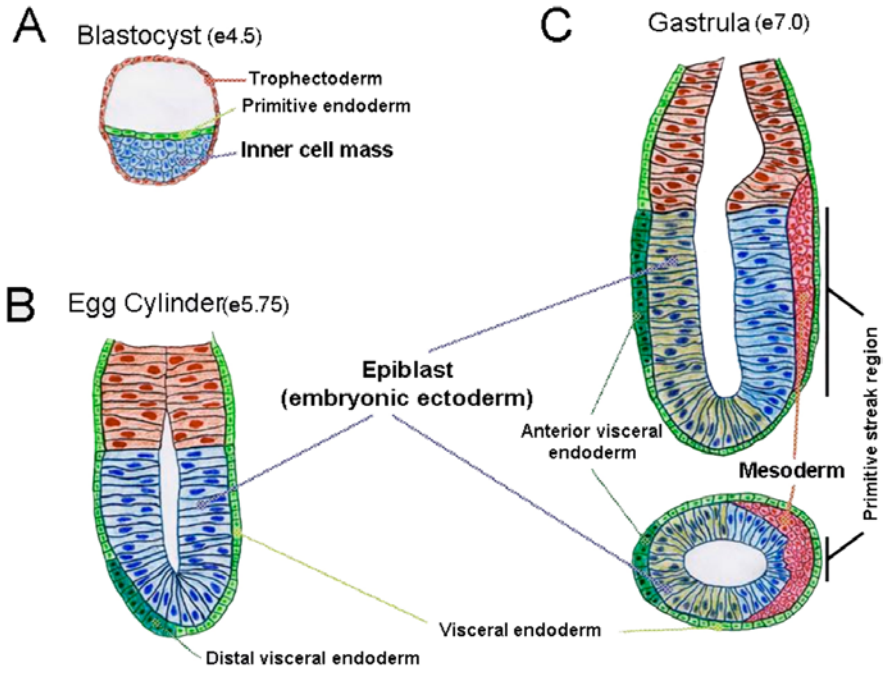

Figure 1. Early mouse embryonic development. A-C) Diagram of a sagittal view of mouse embryos of the given days of embryonic development (e4.5-e7.0) C-bottom) Diagram of a transverse section through an e7.0 embryo. A) Prior to implantation in the uterus, the blastocyst consists of three cell lineages. Trophectoderm (orange) and primitive endoderm (green) lineages are extraembryonic. Inner cell mass cells (blue) are pluripotent and will contribute all adult cell types. B) Upon implantation, the number of pluripotent cells expands (from 20-25 ICM cells to ~500 cells in the epiblast (embryonic ectoderm) (blue). The embryonic ectoderm is thought to be pluripotent and no asymmetries have been described for the embryonic ectoderm at the egg cylinder stage. The extraembryonic endoderm (green) establishes a three dimensional asymmetry through the migration of the distal visceral endoderm (dark green) towards what will become the anterior. C) During gastrulation, the mesoderm (red) germ layer forms in the primitive streak region, defining the posterior of the embryo. Based on lineage tracing and cell transplantation experiments, anterior embryonic ectoderm (brown) and posterior embryonic ectoderm (blue) begin to display different capacities for lineage commitment during gastrulation. Posterior embryonic ectoderm cells (blue) display pluripotent capabilities whereas anterior embryonic ectoderm transitions towards ectodermal (epidermal and neural) lineages.

X-chromosome bearing the recessive allele (*o*) is inactivated will contribute to orange-colored hair (1, 2). Thus, hair color depends upon the molecular inactivation of genes that occurred weeks earlier when the cat was an embryo of only a few thousand cells (3). Although the effects of cell lineage are frequently more subtle than differently colored hair, and X-inactivation is not typically associated with most lineage decisions, it provides an epigenetic mark that effectively distinguishes groups of cells based on their cellular ancestors. As such, the origin of a given cell can have significant effects on the way it responds to stimuli when compared to similar cells of a different origin.

While the zygote has the ability to contribute progeny to all fetal and extraembryonic lineages, the ability of embryonic cells to contribute to different cell lineages becomes progressively restricted as embryogenesis continues. Two well defined points of lineage commitment occur during early stages of mammalian embryogenesis. The first point occurs as the extraembryonic cell lineages are established as opposed to a group of cells that maintain pluripotency and are thus defined as embryonic. The establishment and maintenance of pluripotency in a small number of cells during early embryogenesis provides for the creation of enough cellular material with the potential to contribute to all cell lineages in the adult. Recent reviews nicely capture the molecular and cellular regulation of this process (4, 5); therefore, we will focus on other questions here. The second major point of lineage commitment occurs through a process called gastrulation, in which the pluripotent cells of the epiblast become restricted to contribute to defined cell lineages and consequently lose the ability to maintain pluripotency. The most basic separation of lineages comprises the three primary germ layers (ectoderm, mesoderm, endoderm). The creation of these germ layers provides crucial lineage information such that mesoderm, endoderm, and ectoderm cells are able to initiate different, cell lineage-specific responses to otherwise identical stimuli. Furthermore, this process of lineage commitment occurs in a regulated manner that generates the basic body plan of the developing embryo (Figure 1). In this review, we will examine how discoveries made through classic embryology and modern molecular genetic and bioinformatic approaches performed primarily in the mouse have combined to reveal how pluripotency is regulated and how this regulation may be important for commitment to lineages during gastrulation.

Table 1. Definitions of terms.

Totipotent	property of zygote that enables it to produce an entire organism
Pluripotent	property of embryonic stem cells that enables them to form all adult lineages
Lineage commitment	process in which the ability to give rise to cell types is restricted. Transition from totipotent zygote to pluripotent epiblast cells is an example of lineage commitment
Body plan	the basic three dimensional architecture of an organism's adult form defined by anterior–posterior, dorsal-ventral, and left-right body axes
Primitive streak	a transient structure defining the posterior of an embryo and the site of commitment to mesoderm and endoderm lineages
Orthotopic transplant	tissue or cells from one position in a donor embryo that has been placed into the **same** position in a recipient host embryo
Heterotopic transplant	tissue or cells from one position in a donor embryo that has been placed into a **different** position in a recipient host embryo

3. THE EMERGENCE OF THE CONCEPT OF PLURIPOTENCY

In recent years, ESC have been increasingly manipulated to study properties of pluripotency, self-renewal, and differentiation. In large part, this is because ESC boast a robust cell culture system that makes them amenable to many forms of molecular and genetic manipulation while maintaining pluripotency (6). These attributes have made ESC the preeminent system in which pluripotency is studied. As a result of the wonderful benefits of using ESC, the discovery of new regulators of pluripotency in this *in vitro* system has substantially outpaced the elucidation of the roles of these regulators in the authentic *in vivo* context in which pluripotency is lost. To examine how the wealth of discoveries made using ESC has affected our understanding of embryonic development, one should consider carefully how ESC relate to cells within an intact embryo and how pluripotency factors elucidated in ESC function in embryos. To examine this topic, key aspects of the origin of ESC cultures and pluripotency in mouse embryos are explored below.

3.1. Teratoma Formation

The origin of ESC cell culture can be traced to work initiated in the 1950's. Through a series of experiments that received relatively little attention from the scientific community at the time, Dr. Leroy Stevens at the Jackson Laboratories identified the 129 strain of mice as being susceptible to the formation of teratomas and teratocarcinomas (7, 8). Teratomas are tumors that are frequently benign and characterized by the complexity of cell types that they contain (9). They can possess cell types from each germ layer and can develop histologically identifiable tissues and organs, such as hair follicles, teeth, muscle, intestine, and lung all within the boundaries of a single tumor (9). Examining the origin of teratomas in 129 strain mice, Stevens found that they arose from genital ridge of embryos (10). When cells of teratomas were dissociated and placed into a mouse strain resistant to teratoma formation, individual teratoma cells could form new tumors displaying the same complexity of cell types and tissues; this ability to passage teratomas to new recipient mice led to the concept that teratoma cells possessed the property of pluripotency (7). To examine the origin of pluripotency important for maintenance of teratoma formation, intact three-day and six-day old embryos were grafted onto mouse testes for the formation of new teratomas (11). The formation of teratomas displaying tissue derived from each germ layer suggested that cells in these normal embryos also possessed the quality of pluripotency.

The passage of teratoma forming ability depended upon cells with an "undifferentiated" appearance. The location of these pluripotent cells within tumors was predictable when teratomas were passaged as "embryoid bodies" in mouse ascites (12). Using this technique, cells from a teratoma derived from a six-day embryo were passaged more than 200 times over a period of eight years (11, 13). Remarkably, when the undifferentiated embryonic carcinoma cells (EC) were removed from this embryo-derived teratoma after eight years of passaging and then injected into a normal mouse blastocyst, they contributed to adult lineages including the germ line in chimeric mice (13). Thus, mating of chimeric mice allowed for apparently normal adult mice to be produced essentially from teratoma cells. It should be noted that most EC lines have not displayed the same potential for successful germline transmission (13, 14, 15). Nonetheless, these experiments, initiated with the basic interrogation of the origin of teratoma tumors, were monumentally important for demonstrating that the pluripotency of embryonic cells could be maintained for an essentially unlimited length of time and number of cell divisions.

3.2. Pluripotency in developing embryos

The effort to understand how pluripotency is controlled during mammalian embryogenesis and the effects of this regulation on mammalian development has been addressed by several directions of investigation. Experiments in the 1970's-80's focused on questions such as which embryonic cells possessed pluripotency and how was pluripotency regulated to promote primary germ layer formation within the structure of a nascent body plan. Orthotopic and heterotopic transplantation studies using differentially marked donor and recipient cells were used to investigate the first questions of developmental plasticity of embryonic cells in mammals (Table 1). Early experiments showed that when individual cells from the blastocyst inner cell mass (ICM) were removed from donor embryos (e3.5 or e4.5) and injected into recipient blastocysts, the injected cells could contribute to all fetal cell lineages (16, 17). In contrast, transplantation of mesoderm or endoderm cells failed to generate ectodermal cell types. These classic embryology experiments suggested that a transition occurred from ICM cells, which possessed pluripotency, to lineage committed mesoderm and endoderm cells during gastrulation.

To address the how the transition from pluripotent to lineage committed cells occurred, transplantation experiments were used to test whether cell lineages were determined in the embryonic ectoderm prior to the formation of mesoderm and endoderm lineages. Comparison of regions of the embryonic ectoderm during

the formation of the primitive streak mesoderm (the hallmark of the onset of gastrulation) provided interesting results. Transplantation of embryonic ectoderm from early-streak embryos into a new embryo did not substantially affect the cell types generated from the transplanted region, i.e. heterotopically transplanted cells behaved similarly to orthotopically transplanted cells (18, 19). In contrast, embryonic ectoderm removed from later stages of embryos with a fully formed primitive streak behaved differently depending on their regional origin; transplanted embryonic ectoderm from the anterior was more likely to contribute epidermal and neural cell types whereas embryonic ectoderm from the posterior contributed mesodermal cell types (20, 21). The ectodermal, mesodermal, and endodermal cell types that were generated adopted characteristics appropriate for their position in the embryo rather than the region from which the donor embryonic ectoderm was removed (20, 21). Although the lack of misplaced posterior or anterior characteristics from heterotopic transplants indicated that the placement of epiblast progeny within the body structure was not determined prior to lineage commitment into distinct germ layers, the differences in contribution to cell lineages suggested that there was a regionalized control over lineage potential and perhaps pluripotency in the embryonic ectoderm during gastrulation. In addition, these results also suggested that the ability to contribute to multiple lineages became progressively restricted in embryonic ectoderm as gastrulation progressed.

As an important complement to transplantation experiments, cell labeling experiments have been useful in determining the lineage relationships of individual cells in embryos. Although labeling does not effectively test lineage potential per se, it is valuable in determining the normal lineage decisions that occur in embryos. Labeling of individual cells in intact embryos by injection of horseradish peroxidase marker showed that regions of the gastrulating embryo contributed differently to specific cell types (22). Injection into nascent mesodermal cells marked cell types solely of mesodermal character, confirming the one-way direction of normal lineage commitment from pluripotent epiblast cells to lineage committed ectoderm, mesoderm and endoderm cell types (18, 23, 24). The injection into a single embryonic ectoderm cell at early stages of gastrulation frequently resulted in the marking of cells in different lineages (ectoderm, embryonic mesoderm, endoderm, extraembryonic mesoderm); this sign of multipotency was dependent on the location of the embryonic ectoderm cell within the embryo (22). At the onset of primitive streak formation, injection into an anterior embryonic ectoderm cell marked predominantly ectodermal cells a day after injection; anterior injections were the least likely to produce marks in multiple lineages. The injection of marker into a lateral embryonic ectoderm cell also produced predominantly ectodermally-marked cells, but there was a slight increase in the percentage of embryos displaying two lineages of marked cells (22). In contrast, injection of marker into a posterior embryonic ectoderm cell revealed that they displayed the greatest degree of multipotency by producing the greatest percentage of marked cells in three or four different lineages (22). These results were consistent with the ability of a subset of embryonic ectoderm cells to display pluripotency in teratoma and transplantation assays. Taken together with results from transplantation experiments, these data showed that pluripotent cells existed in gastrulating embryos and that the location of these cells in the embryo was dynamic. Given these experiments were performed decades before the elucidation of genetic or epigenetic factors controlling pluripotency, a more precise examination of pluripotency in embryos was not feasible at the time. Recent discoveries of molecular genetic mediators of pluripotency in ESC offer new candidates for regulators of pluripotency during gastrulation.

Interestingly, several attempts have been made to inject cells from an implantation-stage epiblast into a blastocyst, yet reproducible incorporation of cells into recipient embryos and contribution to all fetal cell types has not been observed as one might expect from a pluripotent cell type. Several potential explanations for this result include: 1) the tremendous increase in cell proliferation rate inherent to post implantation epiblast cells prevents incorporation into the ICM, 2) altered cell-cell and cell-substratum interactions that the embryonic ectoderm cells must make to form the pseudostratified epithelium in the post-implantation embryo inhibits incorporation into the ICM, 3) the percentage of pluripotent cells in the post-implantation epiblast is sufficiently low to prevent success without an enrichment procedure, or 4) maintenance of pluripotency of embryonic ectoderm cells requires elements of the microenvironment of the post-implantation embryo, which are not present in the blastocyst. Regardless of the reason, the simplest explanation for all of the teratoma and transplantation studies is that pluripotency is maintained in embryonic ectoderm cells until gastrulation is initiated, at which point it becomes progressively restricted in a three dimensional pattern reflecting the future development of the fetus.

3.3. Embryonic stem cell culture

The experimentally demonstrated pluripotency of ICM cells and the successful cell culture of teratocarcinoma-derived EC cells provided the foundation for culturing ESC *in vitro*. In the first manuscripts describing ESC culture techniques, the proliferation of cells from the blastocyst ICM was made possible by co-culture with a so-called feeder layer or conditioned media from EC cells (25, 26). Initial experiments showed that ES and EC cells were virtually indistinguishable *in vitro*, displaying similar growth rates, morphology, and gene expression. Importantly, the property of pluripotency described for teratoma cells was also tested by injecting ESC into blastocysts; these experiments showed that ESC were much more efficient than their EC counterparts at contributing cell types to all fetal lineages including the germ line (27, 28). This property has been repeatedly demonstrated by the generation of thousands of strains of knockout mice.

Using the cell culture techniques based on these early experiments, ESC can be cultured indefinitely and still maintain pluripotency. Interestingly, this is different from the *in vivo* context of cells in the embryo, where the

pluripotency of epiblast cells is maintained for a few days through a limited number of cell divisions and is subsequently restricted by lineage commitment during gastrulation. Although ESC are frequently compared to the ICM, this difference in the sustained levels of high proliferation rates suggests that these comparisons should most likely be limited in their scope. Indeed, in their original isolation of ESC, Evans and Kaufman postulated that their cultured cells resembled the embryonic ectoderm of the post-implantation embryo (26). In some regards, it seems more likely that ESC resemble cells of the post-implantation epiblast prior to gastrulation as they possess similar robust cell proliferation and the same ability to contribute cell types to all fetal lineages. Thus as ESC continue to be used to elucidate factors controlling pluripotency, self-renewal, and differentiation, it will be important to determine how factors affecting ESC relate to normal development of intact embryos.

4. FACTORS ASSOSCIATED WITH PLURIPOTENCY OF EMBRYONIC STEM CELLS

4.1. Extrinsic stimuli

The molecular constituents necessary for ESC self renewal were identified from feeder cells (most commonly mitotically inactivated mouse embryonic fibroblasts) and from fetal calf serum. The fact that conditioned media removed from feeder cells could also support self-renewal suggested that the feeder layer provided a factor that was released into the media. The discovery that the LIF cytokine biochemically co-purified with a differentiation inhibiting activity from conditioned media suggested that LIF was the necessary factor provided by feeder layer cells (29, 30). Replacement of feeder layers with purified recombinant LIF provided formal proof that it was sufficient to support ESC self renewal in FCS-containing medium (29, 30). When LIF was withdrawn from culture media, greater than 95% of ESC colonies differentiated and lost pluripotency within a week (30).

LIF is a member of the IL6 cytokines, and by binding the extracellular surface of its receptor (LIFR), it induces intracellular dimerization of gp130 to initiate a signal transduction cascade that results in both JAK/STAT3 activation and p42/p44/MAPK activation in ESC (31, 32). Examination of the downstream effects of LIF stimulation revealed that forced expression of STAT3-ER fusion protein rescued ESC from LIF withdrawal (33), and the pluripotency factor Nanog was found to be a direct target of STAT3 transcriptional regulation in ESC (31, 34). In some aspects, the requirement for LIF for pluripotency may in fact be considered an artifact of the *in vitro* culture conditions of ESC. Neither STAT3 nor LIF signaling was necessary for pluripotent cells of intact embryos to self renew in the several mouse knockout studies inactivating the pathway *in vivo* (35, 36, 37).

A single peptide that stimulates an intracellular signaling pathway was also found to be sufficient to replace fetal calf serum. Using the rationale that bone morphogenic proteins (BMP) inhibited differentiation of neural lineages in the embryo, Austin Smith's group discovered that the combination of BMP and LIF was sufficient to support self-renewal of ESC *in vitro* in the absence of serum and feeder cells (38). A decreased rate of spontaneous differentiation was reported for ESC cultures maintained in LIF+ BMP+ media compared to LIF+ serum-containing media due to unidentified differentiation-inducing signaling molecules present in serum (38). BMP proteins function through their receptor complexes to activate nuclear Smad transcription factors. The inhibitors of differentiation (Id) family of transcriptional repressors were found to be downstream targets of BMP-activated Smads in ESC, and forced expression of the Id1 or Id2 proteins prevented the neural differentiation of ESC (38).

Although the effects of Wnt signaling on ESC have remained controversial, multiple means of stimulating Wnt signaling have been shown to promote ESC self renewal in the absence of LIF. The Wnt pathway has been shown to be controlled by several positive and negative intracellular regulators that affect the stability of a dual function protein, β-catenin (39). Inhibition of negative regulators of β-catenin (Gsk3β and APC) by pharmacological inhibition (BIO-mediated inhibition of Gsk3β) or loss of function mutation ($APC^{-/-}$ ESC) increased activity of canonical Wnt signaling and prevented differentiation of ESC when LIF was withdrawn from culture media (40, 41, 42). Genome-wide microarray analysis of $APC^{-/-}$ ESC revealed a reduction of neural marker genes, suggesting that Wnt signaling inhibited neural differentiation of ESC (41). Similarly, the stimulation of Wnt signaling by treatment with purified Wnt proteins also inhibited differentiation and promoted self renewal of ESC (40, 43, 44, 45). In contrast, Wnt-treated human ESC *did not* maintain long-term self renewal *in vitro* (46), and Wnt treatments or forced expression of β-catenin in mouse ESC grown at high density promoted differentiation (47). In addition, the ability of β-catenin-deficient ESC to maintain self-renewal *in vitro* and in embryos showed that canonical Wnt signaling was not necessary for ESC self-renewal (48) Taken together, these data demonstrate that the Wnt-signaling pathway can have substantial effects on the pluripotency of stem cells; however, the mechanisms by which these effects are determined remains unclear.

4.2. Intrinsic network of transcription factors

Oct4 was the first identified intrinsic regulator of ESC self-renewal. The homeobox-domain containing Oct4 transcription factor was found to be expressed in epiblast cells during the early stages of embryonic development (prior to e8.5) and in germ cells (49). Its expression in ESC was necessary for self renewal as $Oct4^{-/-}$ ESC could not be established from mutant embryos (50). Interestingly, control of the relative level of Oct4 protein was shown to be important for the pluripotent state of ESC. By controlling expression of Oct4 with a doxycycline-regulated promoter element in an otherwise Oct4-deficient ESC line, Niwa *et al* found that decreasing the levels of Oct4 by 50% promoted the differentiation of cells into a trophoblast lineage while overexpression of Oct4 to 150% levels of endogenous Oct4 in ESC caused differentiation

into endoderm lineages (51). Thus, the relative levels of Oct4 controlled the ability of ESC to sustain self-renewal.

Another homeobox-domain containing transcription factor named Nanog was identified by two independent groups based on its specific expression in ESC (52), and by its ability to promote ESC self-renewal in the absence of LIF (53). Several independent molecular genetic approaches that either eliminated or reduced Nanog expression in ESC cultures all found that it was necessary to prevent differentiation (52, 53, 54, 55). Endodermal cell characteristics were most frequently observed when Nanog was inhibited in ESC (52, 53, 55, 56). In contrast to Oct4, forced expression of Nanog did not promote differentiation; instead, it effectively blocked differentiation induced by absence of LIF as well by the presence of retinoic acid (52, 53). Thus, Nanog has been considered both necessary and, in some contexts, sufficient for self renewal of ESC. Despite the ability of forced Nanog expression to overcome requirements for LIF and BMP, forced Nanog expression was not able to support ESC self renewal when Oct4 was mutated or inhibited (53).

The HMG-domain-containing Sox2 transcription factor has been detected in several embryonic cell types, including ESC. It has been shown to form heterodimers with Oct4 protein, and in doing so stimulated binding of heterodimers to DNA sequences containing binding sites for both proteins adjacent to each other, so called Oct-Sox sites (57, 58). Although Sox2 has been detected in a variety of embryonic and some adult cell types, its biochemical interaction with Oct4 has suggested that it may have a specific function in pluripotent cells compared to other cell types. $Sox2^{-/-}$ ESC could not be established from mutant embryos (59), and RNAi-mediated knockdown of Sox2 promoted differentiation of ESC *in vitro* (56). Thus, Sox2 was necessary for self-renewal and pluripotency of stem cells *in vitro*.

Combined bioinformatics- and molecular genetics-based approaches have revealed a fundamental relationship between Oct4, Sox2, and Nanog transcription factors and self-renewal of ESC. Mutation or knockdown of any one of the factors resulted in the loss of expression of the other two (56, 60). Classic promoter analyses showed binding of Oct4-Sox2 heterodimers to promoters was critical for the expression of Nanog, Oct4, and Sox2 (61, 62, 63, 64). Using a genome wide approach, the identity of DNA fragments precipitated by antibodies directed against Nanog, Oct4 and Sox2 was assessed in human ESC samples with microarrays of tiled genomic DNAs. This ChIP on CHIP analysis of promoter binding characteristics of Nanog, Oct4 and Sox2 indicated that they bound to a substantially overlapping set of target genes in human ESC (65). Similarly, mouse ESC DNA precipitated by anti Nanog and anti Oct4 antibodies was analyzed by a paired-end ditag cloning method and also found to represent highly overlapping genomic regions (60). Protein complexes containing Nanog together with Oct4 have been isolated from self-renewing ESC, suggesting a direct biochemical link between these three proteins (66). Many of the promoter regions bound by Nanog/Oct4/Sox2 resided near genes previously identified as regulators of embryonic development (60, 67), suggesting the possibility that a primary function of these transcription factors is to prevent expression of differentiation-inducing gene products (60, 67). Indeed, functional reduction of Nanog or Oct4 in ESC stimulated transcription of many differentiation associated target genes (60, 67). In addition, chromatin modifying factors were also found in Nanog-Oct4 containing protein complexes (66). These relationships have been used to construct a robust feedforward model that functions through the relationship between Nanog, Oct4, and Sox2 to control pluripotency (68). One important feature of this feedforward system is that although it can be modulated by external forces, it provides a stable level of Nanog, Oct4 and Sox2 to ESC in self-renewing conditions.

Interestingly, comparison of the Nanog and Oct4 target genes identified from two different species of ESC (human and mouse) and different techniques revealed only 18 transcription factor genes in common between both data sets (60, 65). Tcf3 (called TCF7L1 in humans), which is a DNA binding transcriptional regulator of the Wnt signaling pathway (69, 70, 71), was one of these 18 transcription factors bound by Oct4 and Nanog in both human and mouse experiments (60, 65). In addition, Tcf3 was one of only 38 genes whose expression was elevated in each neural, embryonic, and hematopoietic stem cells compared to differentiated progeny, and Tcf3 expression was shown to affect hair follicle stem cell characteristics in the skin (70, 72, 73, 74). A direct role for Tcf3 in this internal ESC regulatory network was revealed when it was found to directly inhibit Nanog promoter activity (75). While $TCF3^{-/-}$ ESC possessed elevated levels of Nanog, the levels of Sox2 and Oct4 remained normal, demonstrating the potential to modulate the output of the Nanog-Oct4-Sox2 network. This relationship allowed ESC lacking Tcf3 protein to produce abnormally high levels of Nanog and a delayed response to differentiation conditions *in vitro* (75). These results suggested a direct role for Tcf3 and perhaps the Wnt-signaling pathway in the control of pluripotency through the regulation of Nanog transcription.

4.3. Epigenetic controls

In contrast to the function of extrinsic factors and intrinsic networks of genes, epigenetic effects function through factors inherited from previous generations of self-renewing cells. Although the epigenetic effects described here can be altered by genetic relationships, they are presented separately because they pose potential self-stabilizing regulatory systems.

4.3.1. Cell cycle regulation

When cultured under self renewal conditions, ESC have been shown to progress through the cell division cycle with unique characteristics. Flow cytometry analysis of ESC cultured in LIF-containing media showed that they completed a cell cycle every 10 hours and progressed through G1 phase rapidly (76, 77). The levels of several cell-cycle related mRNAs (Cyclins A1, B1, E1, and Cdc2) were greatly elevated in ESC compared to other proliferative cells and differentiated progeny of ESC (76,

78). When ESC cell cycle progression was inhibited by nocodazole or aphidicolin, the levels of cell cycle regulators (Cdk2-cyclin A and Cdk2-cyclin E kinases) remained high (76, 78). Combined with the constitutively high levels of Cdk2 kinase activity throughout cell cycle stages, this observation suggested that there was a cell cycle independence of Cdk activity that could promote irregular cell cycle progression of ESC. Supporting this hypothesis were findings that Cdk4-cyclin D kinase activity was dispensable for ESC self-renewal as forced expression of $p16^{ink4a}$ Cdk-inhibitor protein caused negligible effects in ESC (79). These data have supported the conclusion that in self-renewing ESC, the pRb-phosphorylation by the constitutively high Cdk2-kinase activity uncouples pRb-regulation of E2F transcriptional activators from the cell-cycle (80, 81). Upon differentiation, the Cdk2-kinase activity collapsed, and cell-cycle dependence was imparted on Cdk activity (76). As a result of these molecular changes the cell cycle profiles of ESC undergoing differentiation changed to a G1-enriched population similar to other primary cell types (76, 78). Despite the correlation between the unique cell cycle of ESC and self-renewal, a clear cause-effect relationship has not yet been demonstrated.

One potentially interesting mechanism by which cell cycle dynamics could regulate developmental potential is through the effects of origins of replication. The onset of S-phase occurs with the firing of origins of replication and the location of origins determine what regions of the genome are replicated early in S-phase and what regions are replicate late in S-phase. Controlling origins of DNA replication could affect cell characteristics through gene expression since genes replicated early in S-phase were frequently expressed at higher levels than those replicated later (82, 83). Indeed, several stem cell genes, including Nanog, Oct4 and Sox2, were shown to be replicated early in the cell cycle of ESC (84). Compared to lineage committed cells such as MEFs, ESC express relatively high levels of regulators of DNA replication and origin initiation (ASK, Cdc6, PCNA, MCM3, MCM5, MCM7)(78). It is likely that elevated levels of proteins such as Cdc6 and ASK could affect the repertoire of origins activated at the onset of S-phase. It is possible that the unique cell cycle and expression of regulators of DNA replication could promote activation of origins which imparts important chromatin characteristics regulating nearby genes. However, it should be pointed out that several lineage-specific transcription factors (i.e. inhibitors of pluripotency) were also replicated early in ESC compared to non-pluripotent cells, yet these genes were not expressed in ESC and their promoter regions possessed histone modifications associated with transcriptional repression (84).

4.3.2. Chromatin modification

Modifications to DNA and the histone proteins bound to DNA have been shown to mediate fundamental effects on gene expression. With the model that modifications to chromatin structure affect the accessibility of DNA to transcriptional machinery, accessibility to transcription factors, and effects on elongation of transcription comes the result that chromatin modifications affect levels of gene transcription. Several modifications to histones have been characterized as allowing access to transcriptional machinery and histone acetyltransferases; these include acetylation of lysine 9 of histone 3 (H3K9) and methylation of lysine 4 of histone 3 (H3K4) (85, 86). Modifications such as trimethylation of lysine 27 of histone 3 (H3K27) have been shown to inhibit gene transcription by compacting chromatin and preventing initiation (87, 88). Examination of the roles of Polycomb protein complexes that catalyze repressive histone modifications (H3K27) revealed that they play important roles in cell lineage relationships in all metazoan organisms examined (89, 90). Since ESC display the special property of pluripotency, the examination of chromatin characteristics and chromatin modifying enzymes was an important goal that revealed some underlying characteristics of pluripotent cells.

Several approaches have been successful in revealing insights into the status and importance of chromatin modification for ESC pluripotency. Biochemical extraction of proteins from chromatin revealed that histones were more loosely associated with DNA from ESC compared to neural progenitor cells; similarly, histone-EGFP fusion proteins displayed an increased rate of fluorescence recovery after photobleaching compared to differentiated cells types suggesting that chromatin was more dynamic in ESC (91). Large-scale assessments of histone modifications across the genome by ChIP experiments identified genomic regions possessing a bivalent chromatin structure that contained both H3K27 repressive modifications together with H3K9 and H3K4 activator modifications (84, 92). Of the 68 bivalent domains that overlapped with transcription start sites of known genes, 93% of those genes encoded transcription factors which were expressed at low levels in ESC (92). Nearly all of the bivalent domains (93 of 97) discovered in ESC were resolved into monovalent (either H3K27 or H3K4) chromatin in differentiated cell types suggesting that bivalent domains were highly enriched in pluripotent cells (92).

To determine whether chromatin modifications were important for controlling pluripotency of ESC, the effects of proteins that catalyze histone modifications were examined by several independent approaches. Genome wide location analysis (ChIP-CHIP) using antibodies against core components of polycomb complexes, PRC1 (Phc1 and Rnf2 antibodies) and PRC2 (Suz12 and Eed antibodies) revealed that the repressor complexes were localized to the same regions of the genome that possessed H3K27 modifications (67). The effects of these modifications were tested by the mutation of the Eed protein necessary for PRC1 activity and H3K27 modification (67, 93). $Eed^{-/-}$ ESC were able to self-renew; however, in two independent studies $Eed^{-/-}$ ESC displayed a twofold or greater increase in Polycomb target gene expression including those gene products previously characterized as mediators of lineage commitment and differentiation (67, 84). Consequently, $Eed^{-/-}$ ESC displayed a propensity to differentiate. Consistent with a role for Polycomb-mediated chromatin modification in self-renewal, ESC could not be established from blastocysts lacking the Polycomb subunit, Ezh2 protein (94).

Interestingly, the effects on chromatin caused by loss of Eed did not significantly affect activation of origins of replication as early replicating genes were still replicated early in $Eed^{-/-}$ ESC (84). The early replicating genes that were repressed in wild-type ESC were expressed in $Eed^{-/-}$ ESC, suggesting that if replication timing affects pluripotency, it functions upstream of Polycomb-mediated chromatin modifications. Taken together, these data have been used to suggest the presence of an indexing system whereby transcription factors promoting lineage commitment are held at low levels in pluripotent cells and allowed to be activated in lineage committed cells. The identification of bivalent domains provides a potential mark or reference point by which indexing could occur. Two excellent reviews have recently covered how potential indexing systems may be organized within the genomes and how this organization could affect function (95, 96).

5. ROLE OF PLURIPOTENCY FACTORS DURING DEVELOPMENT

5.1. Nanog, Oct4, and Sox2 knockout mice

The analyses of knockout mice harboring mutations in the key determinants of pluripotency have been of significant importance in understanding pluripotency in the developing mouse embryo. In particular, mutation of Nanog, Oct4 and Sox2 genes have demonstrated the importance of these "stem cell genes" for maintaining a population of pluripotent cells in the embryo.

Oct4 is specifically expressed in the ICM of the pre-implantation mouse blastocyst and its derivative, the epiblast, after implantation (49, 97). Targeted ablation of the Oct4 gene demonstrated that the maintenance of these tissues depended upon Oct4 expression (50). $Oct4^{-/-}$ blastocysts appeared normal at e3.5, however, they failed to produce ICM derivatives following implantation, suggesting a failure to maintain the ICM. Indeed, the ICM of $Oct4^{-/-}$ blastocysts failed to be maintained through an implantation delay and also failed to expand in culture. Interestingly, upon culture of immunosurgically isolated $Oct4^{-/-}$ ICMs, typical ICM-derived cell types were absent and mutant cells instead differentiated into trophoblast giant cells (TGCs). This phenotype suggests that Oct4 is required for the maintenance and pluripotency of the ICM (50).

Sox2 expression was also detected in the ICM of mouse blastocysts and in the epiblast. Similarly to $Oct4^{-/-}$ mutants, $Sox2^{\beta geo/\beta geo}$ blastocysts appeared normal but failed to survive after implantation, suggesting a failure to maintain the ICM (59). This conclusion was supported by the failure of $Sox2^{\beta geo/\beta geo}$ blastocysts to form ICM derivatives in culture. Instead, cultured $Sox2^{\beta geo/\beta geo}$ blastocysts and isolated ICMs produce TGCs, diploid trophoblast, and parietal endoderm cells. As with Oct4 mutants, the lack of ICM derivatives and differentiation to extraembryonic cell types by Sox2 mutants indicates that Sox2 is also required for the maintenance and pluripotency of the ICM (59). The similarity of $Oct4^{-/-}$ and $Sox2^{\beta geo/\beta geo}$ mutant embryos was consistent with the biochemical data showing their function as heterodimers.

Nanog expression was also detected in the pluripotent cells of the early embryo, however the pattern of expression was slightly different than Oct4 and Sox2. Transcripts were first detected in the interior cells of morulae and persist in the ICM of blastocysts until implantation when expression of Nanog mRNA was reported to be downregulated (52, 53). Interestingly, Nanog has also been reported to be expressed near the primitive streak region in gastrulae (29). Like Oct4 and Sox2 mutants, Nanog null blastocysts appeared normal yet failed to survive past implantation or produce ICM derivatives (52). Culture of ICMs from Nanog null blastocysts resulted in differentiation to parietal endoderm-like cells. Thus, Nanog was also required for the maintenance and pluripotency of the ICM. However, the lack of differentiation to trophoblast lineages, such as seen in Oct4 and Sox2 mutants, suggested that the initial requirement for Nanog in the ICM occurs later than that of Oct4 or Sox2 (52).

The early embryonic lethality of Oct4, Sox2, and Nanog mutants confirmed the requirement for pluripotency factors to establish a mass of epiblast cells sufficient to provide lineage committed cells during gastrulation. Each of these pluripotency factors was absolutely required for the production of embryonic tissue. Thus, the maintenance of pluripotency is essential for embryonic development. The similar phenotype observed in each of these mutants suggests that some form of the ESC intrinsic transcription factor network of Oct4, Sox2, and Nanog is also present during early embryonic development. These genetic results have helped confirm ESC as a relevant *in vitro* model system to exploit for the identification of regulators of pluripotency. Interestingly, Oct4, Sox2, and Nanog are also expressed in post-implantation embryonic ectoderm cells, but are not expressed in mesoderm and endoderm derivatives, suggesting that this network of transcription factors could also play an important role in regulating the loss of pluripotency during gastrulation. Unfortunately, the peri-implantation lethality of the each mutant embryo precludes the use of existing knockout mice to address the roles of the Nanog-Oct4-Sox2 network during gastrulation.

5.2. Epigenetic controls

Given the small size of the early mouse embryo, progress in understanding the roles of epigenetic factors in intact embryos has been understandably slow; however, some of the effects linked to pluripotency of ESC have been examined directly in embryos. Prior to implantation, the pluripotent cells in the epiblast display relatively slow rates of cell division. The 20-25 ICM cells that comprise the epiblast at the time of implantation expand rapidly to form a more than 500 cell epiblast within a day and a half (98). The cell cycle in these rapidly expanding epiblast cells resembles that previously described for ESC cells, i.e. approximately 10hrs long with short G1 and G2 phases (99). Similar to ESC, epiblast cells also do not require the activity of Cdk-cyclin D complexes as mutants progress through this stage of embryogenesis without defects (100). Also consistent with a requirement for the high levels of Cdk2-cyclinA activity in ESC, mutation of the gene encoding cyclin A2 prevented embryos from progressing

past the blastocyst stage; $Ccna2^{-/-}$ embryos failed to implant although they did not display defects at blastocyst stages without detectable maternal contribution (101). In contrast, progression of the cell division cycle becomes dependent upon Cdk4/6-cyclin D activity in later stages of embryogenesis (100). Effects on replication timing and the levels of Cdc6 have not been investigated in the peri-implantation stage embryo.

A recently published technique provided the potential to reveal whether the nature of chromatin modifications observed in ESC could also affect pluripotency in intact embryos. The inclusion of non-specific carrier DNA (in this case from *Drosophila* cells) in native ChIP procedures increased the sensitivity of immunoprecipitation such that material from as few as 100 cells could be used for ChIP analyses (102). This technique allowed direct comparison of H3K9 (repression), H3K4 (repression), and H4 acetylation (activation) histones on chromatin regions regulating expression of stem cell genes (Nanog, Oct4) and an extraembryonic-specific gene (Cdx2) in dissected ICM, dissected trophectoderm, and ESC samples. While the appearance of most chromatin modifications was qualitatively similar between ESC and ICM samples, the ICM samples displayed quantitatively greater signals for chromatin modifications, suggesting that silencing and activation marks were diminished in ESC compared to ICM (102). Regions of bivalent chromatin marks were not examined in intact ICM; however, using carrier ChIP techniques to examine their dynamics in intact embryos remains an interesting possibility.

6. PERSPECTIVE

In recent years, incredible progress has been made towards understanding the underlying mediators of pluripotency in ESC. In particular, the identification of an intrinsic network of transcriptional factors forming a feedforward system of regulation has been confirmed by molecular genetic experiments in embryos. Using new techniques, the importance of specific histone modifications during the establishment of pluripotency in ICM cells also has great potential to be confirmed in embryos. In addition, it is clear that the cell division cycle of pluripotent cells in embryos is regulated in special fashion that is similar in ESC. Taken together, this wealth of information has shown that a combination of genetic and epigenetic factors coordinates the establishment and maintenance of pluripotency in embryonic cells.

However, to really understand the role of pluripotency in development, it is critical that we discover mechanisms controlling the loss of pluripotency and lineage commitment that occur during gastrulation. In comparison, progress on this front has been slower, and many basic questions remain unanswered at the time of this writing: Is the lineage commitment of embryonic ectoderm into cells of the primary germ layers during gastrulation regulated by the Nanog-Oct4-Sox2 network? How are pluripotency factors identified in ESC spatially regulated to explain classic transplantation and lineage tracing results? Is spatiotemporal control of pluripotency factors necessary for coordinating lineage commitment with formation of the basic body plan? How does cell cycle regulation affect epigenetic control of pluripotency? While answering these questions may not be a simple task, the dissection of pluripotency factors in ESC has provided new approaches that have made answering these questions a realistic possibility. Based on the importance of the groundwork laid by previous generations of developmental biologists for current approaches of directing lineage commitment, one should anticipate that answers to these questions will provide new avenues for the increasingly relevant cell-based therapeutic approaches to disease.

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Send correspondence to: Dr Bradley J. Merrill, Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, 900 S. Ashland Ave, Molecular Biology Research Building Room 1268, Mail Code 669, Chicago, Illinois, Tel: 312-996-0346, Fax: 312-413-0353, E-mail merrillb@uic.edu

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