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The Battlefield of Pluripotency

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How embryonic stem cells maintain the potential to differentiate into multiple cell lineages is still unclear. In this issue of *Cell*, Niwa et al. (2005) show that a duel between the transcription factors Oct3/4 and Cdx2 can restrict embryonic stem cells to either embryonic or placental fate. The vulnerability of lineage potential to transcriptional perturbation may reflect an essential feature of pluripotency.

Pluripotency is the capacity of a cell to generate all lineages of the developing mammalian embryo, including the germline. This is the unique and essential property of the epiblast, a population of founder cells located within the developing blastocyst. These epiblast cells form in conjunction with the segregation of extraembryonic and embryonic lineages during the preimplantation phase of development (Figure 1). Epiblast cells multiply transiently until gastrulation, during which they then develop into either primordial germ cells or somatic progenitor cells. In vitro, the progression to gastrulation can be interrupted and pluripotency sustained indefinitely by deriving self-renewing embryonic stem (ES) cells. How pluripotency is acquired by a subset of cells in the embryo and how it is maintained in ES cells are questions of intellectual fascination and increasingly of biomedical significance. A series of incisive experiments by Niwa and colleagues (2005) reported in this issue of *Cell* provide a conceptual framework for considering these phenomena.

Niwa et al. (2005) focus attention on Oct3/4, a master transcriptional organizer of the POU transcription factor family. Oct3/4 expression is found exclusively in early embryos, the germline, and ES cells. In the embryo, Oct3/4 is essential to establish pluripotency (Nichols et al., 1998). Without Oct3/4, inner cells of the blastocyst fail to acquire the potential to differentiate into multiple lineages and are restricted to the generation of a single lineage, the extraembryonic trophectoderm, which eventually gives rise to the placenta. Furthermore, continuous expression of Oct3/4 is required to sustain pluripotency. The elimination of Oct3/4 from ES cells precipitates unidirectional differentiation into trophectoderm (Niwa et al., 2000).

Oct3/4 is thus positioned foremost in the hierarchy of transcriptional determinants of the pluripotent state (Chambers and Smith, 2004). One key effect of Oct3/4 is to suppress differentiation of embryonic cells into trophectoderm. Niwa et al. (2005) demonstrate that Oct3/4 achieves this by blocking both the expression and activity of the homeodomain transcription factor Cdx2. First, they observed that Cdx2 is rapidly upregulated upon elimination of Oct3/4 from mouse ES cells. Then they found that forced expression of Cdx2 is sufficient to induce trophoblast differentiation (Figure 1, lower panel), reproducing the effect of removal of Oct3/4. The gene expression changes that accompany misexpression of Cdx2 or elimination

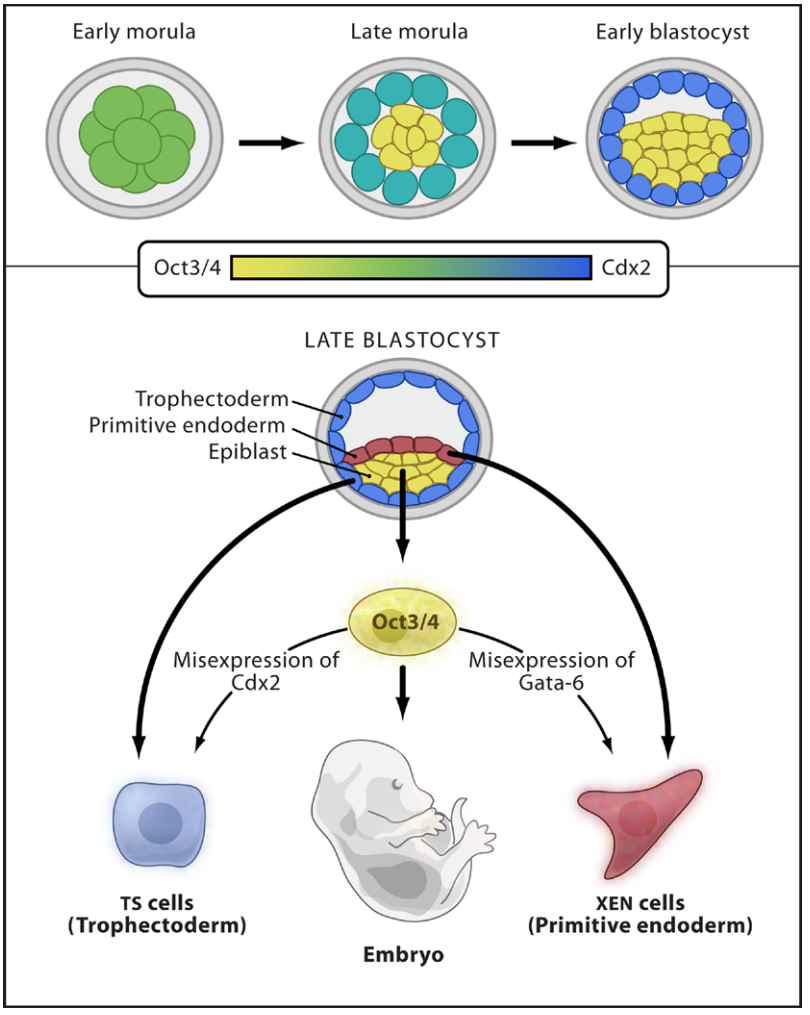

Figure 1. Mutual Exclusion of Oct3/4 and Cdx2 Specifies Lineage Potential in the Mouse Blastocyst and Embryonic Stem Cells

(Upper panel) In the early morula stage embryo, antagonistic transcription factors Cdx2 (blue) and Oct3/4 (yellow) are uniformly expressed (green). With loss of symmetry at the 8–16 cell division, this balance is perturbed. Cdx2 is rapidly extinguished in the inner cells where Oct3/4 expression is high (yellow) but progressively becomes dominant in the outer layer (aquamarine) during the late morula stage, culminating in the specification of pluripotent and trophectodermal identity, respectively, in the early blastocyst.

(Lower panel) In the late blastocyst, three lineages are determined by distinct transcription factors: trophectoderm by Cdx2 (blue), primitive endoderm by Gata-6 (purple), and epiblast by Oct3/4 (yellow). Derivative cell lines faithfully retain lineage specification (bold arrows). However, misexpression of Cdx2 or Gata-6 in embryonic stem cells extinguishes pluripotency and imposes extraembryonic fates.

of Oct3/4 are strikingly similar. Furthermore, introduction of Cdx2 leads to downregulation of Oct3/4 expression, pointing to a reciprocal repression loop. Consistent with this, Cdx2 can interfere directly with the positive transcriptional autoregulation of Oct3/4, and Oct3/4 in turn inhibits Cdx2 autostimulation. In addition to mutual repression of transcription, the two proteins appear to bind to one another in a complex which neutralizes their respective transcriptional activation effects. This tug-of-war explains the finding that overexpression of Cdx2 induces differentiation of ES cells into trophectoderm and silences pluripotent-specific genes even when Oct3/4 expression is maintained from a transgene.

But is this an artifact of intricate genetic manipulations of the mouse ES cell culture system? The question is pertinent because trophectoderm lineage commitment and segregation are implemented in the embryo prior to formation of the epiblast; therefore, this fate choice would be expected to be excluded for ES cells. Indeed, trophectoderm differentiation is not normally exhibited by mouse ES cells in vitro or in mouse chimeric embryos. It is therefore important to show that cells expressing markers of trophectoderm (such as Cdx2) are actually part of the trophectoderm lineage. To test this, Niwa et al. (2005) engineered mouse ES cells to express a form of Cdx2 that can be regulated by a hormone and then assayed their developmental potential by injecting these ES cells into blastocysts. Mouse ES cells in which Cdx2 is not induced populate the embryo, but not the placenta, as expected. In contrast, ES cells in which Cdx2 is induced do not contribute to the embryo but instead colonize the placenta. Importantly, these Cdx2 induced cells are morphologically differentiated and integrated into the placental cytoarchitecture. Thus, they meet stringent criteria for both trophectodermal lineage restriction and functional trophoblast differentiation.

So how do these results relate to the mechanism of segregating trophectoderm and establishing pluripotency in the embryo? Cdx2-deficient blastocysts are unable to undergo implantation into the uterus, which indicates defective trophoblast differentiation. Oct3/4 and a second key pluripotency gene Nanog show ectopic expression in these blastocysts (Strumpf et al., 2005) consistent with a function of Cdx2 to repress pluripotent gene expression in wild-type trophectoderm. How do Cdx2 and Oct3/4 normally localize to the outer trophectoderm and inner pluripotent cell layers, respectively? Niwa et al. (2005) found that Cdx2 and Oct3/4 proteins are transiently coexpressed throughout the early embryo (Figure 1, upper panel). Reciprocal inhibition of Cdx2 and Oct3/4 activity may create a delicately poised see-saw

between the two factors. A marginal shift in balance would result in a rapid and inexorable amplification of one transcription factor culminating in the elimination of the other and the specification of cell fate (Figure 1, upper panel). An initial imbalance between the transcription factors might occur stochastically, followed by sorting of cells to the appropriate tissue location. Alternatively, cell polarity or cell size could influence the relative expression, stability, and asymmetric allocation of Cdx2 and Oct3/4 between sister cells.

Cells in the mouse embryo cease to be identical after compaction (the morphological change that blastomeres undergo after the third cleavage), when changes in cell-cell contacts lead to apicobasal polarization and asymmetric divisions from 8 to 16 cells generate large polarized outer cells and small apolar internal cells (Johnson and Ziomek, 1981). This event and the subsequent differentiation of the outer cells into an epithelium are independent of any duel between Cdx2 and Oct3/4 because mouse mutants lacking Cdx2 form blastocysts. However, the outer cells of these blastocysts do not sustain epithelial integrity and subsequently degenerate. In addition to misexpression of Oct3/4 and Nanog, Cdx2-deficient outer cells fail to upregulate the protein eomesodermin, a T box transcription factor required for functional trophoblast differentiation (Strumpf et al., 2005). Therefore, the initial allocation and separation of inner and outer lineages is not dependent on Cdx2, but in its absence the outer cells are unable to differentiate properly. This is analogous to the effect of *Oct3/4* deletion on the development of pluripotency in the inner cells (Nichols et al., 1998). Overall, the genetic and cell biological evidence suggest that the outer and inner blastocyst cell lineages are established separately from specification of trophectodermal and pluripotent identity. How cellular asymmetry is coupled to differential distribution of Cdx2 and Oct3/4 and hence to appropriate cell specification remains an open question.

Although trophoblast differentiation in the mouse embryo is blocked in the absence of Cdx2, Niwa et al. (2005) unexpectedly found that Cdx2-deficient ES cells can differentiate into trophoblast-like cells upon elimination of Oct3/4. They attribute this to the protein eomesodermin. Eomesodermin is necessary for trophoblast differentiation in vivo and, like Cdx2, appears to be both transcriptionally repressed and functionally antagonized by Oct3/4 in ES cells. The authors show that high overexpression of eomesodermin can induce trophoblast-like differentiation of ES cells. Placental chimeras have not been generated in this case, so the evidence is limited to morphology and marker expression, and indeed the differentiation appears incomplete. This may be because, unlike Cdx2, eomesodermin does not inhibit Oct3/4 activity. Failure of trophoblast differentiation in the *cdx2* mutant blastocysts might therefore be due to the dominance of persistent Oct3/4 over eomesodermin in the nascent epithelial cells. This can be tested experimentally: if the unique and critical function of Cdx2 is to eliminate Oct3/4 and thus to liberate eomesodermin, then trophoblast differentiation should be restored in mutant blastocysts lacking both Cdx2 and Oct3/4.

The authors propose that resolution of the conflict between Cdx2 and Oct3/4 may be decisive for imbuing internal cells of the blastocyst with the capacity for pluripotency. But is mutual inhibition both necessary and sufficient to drive Cdx2 out of the blastocyst inner cells, or is this a secondary mechanism to consolidate segregation established by some other process? Examination of Oct3/4-deficient blastocysts should be informative. Inner cells of Oct3/4 mutant blastocysts initially may not be specified to follow a trophectodermal fate (Nichols et al., 1998). Thus, the question arises whether Cdx2 is maintained continuously, consistent with primary repression by Oct3/4, or is extinguished only to reemerge at a later stage.

In mouse ES cells, circumventing Oct3/4 transcriptional repression by forced expression of either Cdx2 (Figure 1, lower panel) or eomesodermin is sufficient to overcome restriction to a trophectodermal fate. Niwa, Fujikura, and colleagues previously showed that mouse ES cells could be driven into the second extraembryonic lineage (primitive endoderm) by misexpression of the transcription factors Gata-6 or Gata-4 (Fujikura et al., 2002; Figure 1, lower panel). The mechanism by which these transcription factors are normally blocked in ES cells is unknown but may involve Nanog and the LIF/Stat3 signaling pathway (Chambers and Smith, 2004) rather than Oct3/4. However, both findings suggest that the pluripotent ES cell state may be actively sustained by dominant transcriptional and post-transcriptional regulators rather than the chromatin-based epigenetic determination that is considered critical for somatic lineage restriction. Extraembryonic lineages too may be subject to epigenetic shutdown because pluripotency is not readily reinstated in trophectoderm or primitive endoderm in response to Oct3/4 or Nanog expression (I. Chambers, T. Kunath, and A.S., unpublished data).

A molecular correlate of pluripotency, therefore, may be an inert epigenetic machinery, presenting a tabula rasa for gene expression. However, control of gene expression mediated solely by transcription factors is a metastable condition, the outcome of which could fluctuate in the artificial environment of cell culture. Variability in mouse ES cell derivation, differentiation of human ES cells into trophectoderm without genetic manipulation, and expression of Cdx2 rather than Oct3/4 in stem cells derived from rat blastocysts (Buehr and Smith, 2003) all could be explained by subtle strain and species differences in the cross-regulatory capacity of Cdx2 and Oct3/4. Conversely, resetting of transcriptional control on a background of minimal epigenetic restriction may account for reversion of primordial germ cells and germline stem cells to a pluripotent state (Kanatsu-Shinohara et al., 2004). A requirement simply to erase programming may underlie the relative success in obtaining ES cells compared with viable fetuses after nuclear transfer from somatic cells

(Wakayama et al., 2001).

Finally, high efficiency in generating mice by nuclear transfer from ES cells is consistent with the notion that the pluripotent epigenome is unprogrammed. Indeed, removal of DNA methylation does not cripple ES cells, although downstream differentiation is impaired. If epiblast and ES cells are truly unprogrammed, self-renewal and pluripotency will withstand loss of other epigenetic modifications and be sustained only by transcriptional regulators. However, reintroduction of deleted components may be necessary to rescue disabled machinery necessary for cellular differentiation.

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Solving Mysteries of DNA Replication and Frog Cloning

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Compared to sperm nuclei, nuclei from adult somatic cells replicate inefficiently in frog egg extract. In this issue of *Cell*, Lemaitre et al. (2005) show that pre-exposure of erythrocyte nuclei to a mitotic extract removes this difference, reorganizes the chromatin into shorter loops, and allows replication at much shorter intervals along the DNA. Remarkably, these observations also explain an old mystery of why serial nuclear transplantation was so successful for cloning frogs.

As science progresses, a trail of unsolved puzzles and paradoxes is left behind along with misfit results that can never be fully explained but must have some significance. This issue of *Cell* contains a remarkable paper from Marcel Méchali's lab (Lemaitre et al., 2005) that solves several such mysteries at a single stroke. The Mechali study addresses the esoteric question of why the DNA in nuclei from adult somatic cells of the frog replicates more slowly than the DNA of sperm nuclei does when both are exposed to extracts of frog eggs. The difference in DNA replication efficiency has been an unsolved mystery for 15 years. Lemaitre et al. (2005) now demonstrate that exposure of somatic-cell nuclei and sperm nuclei to an extract of mitotic cells abolishes this difference. However, the significance of the paper by Méchali and colleagues extends far beyond answering this question. Their study explains the long-standing puzzle of why the serial transplantation of nuclei from differentiated frog cells into frog eggs from which the nucleus had been removed enabled frogs to be cloned more than 20 years before the cloning of mammals became possible.

The key advance in allowing the development of tadpoles generated by nuclear transplantation was the performance of two consecutive cycles of nuclear transfer. Embryos arising from the first cycle of nuclear transfer were dissociated, and their nuclei were transplanted into a further set of enucleated eggs. This process is called serial nuclear transfer, and it produces clones of genetically identical frogs (Gurdon 1962). The frog embryos derived from the serial trans-