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Specification and regulation of mammalian extraembryonic and pluripotent embryonic lineages *in vivo* and *in vitro*

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Stephanie Blij

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The dissertation of Stephanie Blij is approved:

_____
Dr. Amy Ralston, chair

_____
Dr. Susan Strome

_____
Dr. David Feldheim

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Tyrus Miller
Vice Provost and Dean of Graduate Studies

Table of contents

List of figures

Abstract

Specification and regulation of mammalian extraembryonic and pluripotent embryonic lineages *in vivo* and *in vitro*

Stephanie Blij

Pluripotency establishment and maintenance are critical for both the development of mammalian embryos and the production of pluripotent stem cells. Investigations into the molecular mechanisms underlying pluripotency have revealed multiple signaling networks that generate diverse pluripotent states. The relationship between these networks, their upstream transcription factors, and their associated pluripotent states is complex and poorly understood in both pluripotent stem cells and embryos. In this thesis I examine three aspects of pluripotency regulation: the role of maternal transcription factors in embryogenesis, novel effects of genetic background and naivety on differentiation to placental stem cells, and the contribution of known stem cell factors to regulation of pluripotency *in vivo*.

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T. Frum is a listed co-author in this publication and directed and supervised a portion of the research which forms the basis for chapter 2 of this dissertation.

-Eryn Wicklow is a co-contributor to the data presented in chapter 4 of this dissertation.

-Tristan Frum generated and compiled the data shown in figure 26.

-Yoshikazu Hirate and Hiroshi Sasaki performed experiments shown in figure 20 B-E.

-Richard Lang generated the $Sox2^{fl/fl}$ mouse line used for experiments in chapter 4.

Abbreviations

Cdx2	Caudal type homeobox 2
E	Embryonic day
EPI	Epiblast
EpiSCs	Epiblast stem cells
ESCs	Embryonic stem cells
Fl	Floxed
ICM	Inner cell mass
IPSCs	Induced pluripotent stem cells
M null	Maternal null
MZ null	Maternal & zygotic null
PE	Primitive endoderm
TE	Trophectoderm
TSCs	Trophoblast stem cells
XEN cells	Extraembryonic endoderm stem cells
Z null	Zygotic null

CHAPTER 1 - Introduction

Generating multiple cell types during development

Most organisms begin life as a single cell, and while many organisms such as bacteria and yeast remain as cell singles, others generate many cells which differentiate into unique types. Having many different cell types in an organism allows more diverse and complex biological processes to occur. A diverse set of mechanisms has evolved to allow a single cell to divide asymmetrically and generate different daughter cells. While the basic steps leading to the initiation of asymmetry in many model organisms such as *Drosophila melanogaster* (*D. melanogaster*) and *Caenorhabditis elegans* (*C. elegans*) have been known for some time, the initiation of asymmetry in mammalian embryos has remained elusive until recently. This is in part because the basic mechanisms involved in the generation of the first lineages in mammals are quite different than mechanisms utilized by non-mammalian model organisms. In non-vertebrate animals, asymmetry is typically generated within the oocyte prior to fertilization or in the zygote before the first cell division (Wodarz, 2002). For instance, in *C. elegans* the sperm point of entry leads to asymmetric localization of maternal factors. These maternal factors are differentially inherited during the first cell division, and the resulting cells have different fates, germ line versus soma. In *D. melanogaster* the body axes are predetermined in the oocyte due to differentially localized maternal factors. In the vertebrate *Xenopus laevis*, maternal factors are also differentially localized in the oocyte and ultimately lead to the specification of different germ layers (Mowry and Cote, 1999). In mammals there do not appear to be any similar types of asymmetries in the oocyte or zygote.

In the mouse, the first distinct lineages do not appear until several days after fertilization and are the inner cell mass (ICM) and trophectoderm (TE). The TE will ultimately contribute to the placenta and the ICM will further subdivide into the epiblast (EPI) and primitive endoderm (PE). The EPI will form the fetus and the PE will contribute to the yolk sac (Fig. 1). The extraembryonic tissues, generated from the TE and PE, serve to provide nutrients to the embryo throughout development and are also critical for inducing many major developmental events in the embryo (Arnold and Robertson, 2009). The TE mediates implantation of the embryo into the uterine wall, and after implantation extensive signaling among the TE-derived extraembryonic ectoderm, EPI-derived egg cylinder, and PE-derived visceral endoderm is required for formation of the anterior-posterior axis of the embryo and induction of the primitive streak (Arnold and Robertson, 2009). The PE (also known as the hypoblast) is present during the development of other vertebrates such as birds and fish, where it also plays inductive patterning roles and contributes to yolk sac (Stern and Downs, 2012). Placental development is a feature unique to mammals and the TE from which it develops is also unique to mammals. Since TE is the first lineage generated during mammalian development, the mechanisms driving early mammalian development are fundamentally different than those initiating the development of non-mammals which specify germ line vs soma or body axes first.

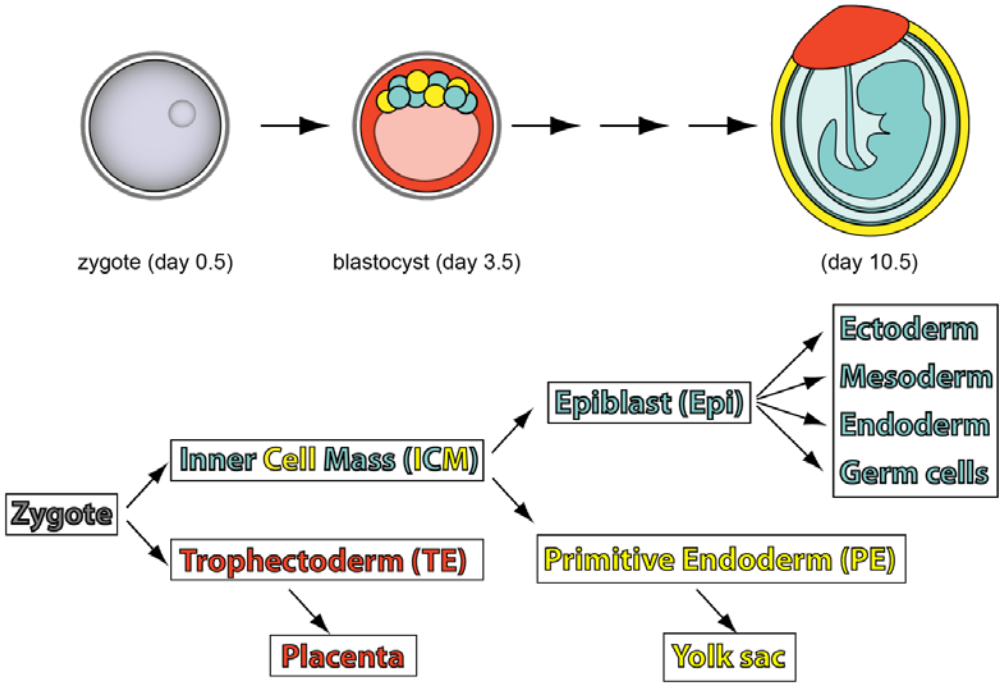


Figure 1. Development of embryonic versus extraembryonic lineages

By E3.5 the zygote develops into the blastocyst which is comprised of two lineages, the trophectoderm (red) and the inner cell mass (blue and yellow). The trophectoderm ultimately gives rise to the placenta, shown at E10.5. The inner cell mass further subdivides into the epiblast (blue) and primitive endoderm (yellow). The primitive endoderm goes on to become the yolk sac, while the epiblast generates all fetal lineages, shown at E10.5.

-adapted from (Yamanaka and Ralston, 2010)

As maternally provided factors play major roles in lineage specification in many species, their contribution to mammalian development has been extensively studied. Maternally inherited factors perform critical roles in processing the male genome, the first cell divisions, initiation of zygotic transcription, and ultimately the degradation of many maternal RNAs and proteins (Li et al., 2010; Zheng and Liu, 2012). Although several studies have reported roles for maternal factors in lineage specification using RNA knockdown techniques, genetic knockouts of maternal

stores of these factors have thus far refuted those results (Blij et al., 2012; Foygel et al., 2008; Frum et al., 2013; Jedrusik et al., 2010; Wu et al., 2010; Wu et al., 2013). Although a role for maternal factors cannot be formally ruled out, current evidence suggests that maternal factors are not involved in mammalian lineage specification.

An additional line of evidence supporting the notion that maternal factors do not drive lineage specification in mammals is the highly regulative nature of mammalian embryo development. During non-mammalian embryo development maternal factors contribute to lineage formation by their asymmetric localization within the embryo. This asymmetric localization leads to differential inheritance of maternal factors as cells divide, generating cells with unique fates. However, in 2-cell mouse embryos the cells can be separated and put back into a host mother and develop into normal adult mice, demonstrating that both cells are totipotent (Tarkowski, 1959). Up to the 8-cell stage individual cells can generate new blastocysts, but due to insufficient cell numbers they cannot fully develop (Rossant, 1976; Tarkowsk.Ak and Wroblews.J, 1967). However, individual cells from 4- and 8-cell embryos can contribute to all lineages when put into a new embryo, demonstrating that the individual cells do not have restricted fates (Fujimori et al., 2003; Kelly, 1977; Tarkowski et al., 2005). The regulative nature of mouse development is also evidenced by the regeneration of TE from ICM after removal of TE by immunosurgery (Hogan and Tilly, 1978; Spindle, 1978).

Molecular regulation of the first lineage decision: ICM versus TE

The first lineage decision in the mammalian embryo is a result of the first asymmetry observed during development. The source of this asymmetry was a mystery for many years, and only recently have molecular mechanisms regulating

this process come to light. Approximately 24hrs after fertilization the mouse embryo undergoes a series of symmetrical divisions. These divisions are known as cleavages because the size of the embryo remains constant while the number of cells increases. Therefore, cells are essentially being cleaved into smaller and smaller cells. Up to the 8-cell stage, all cells, or blastomeres, of the embryo are thought to be equivalent both spatially and molecularly (Guo et al., 2010). After another round of division, cells can no longer all occupy identical positions; some cells are on the outside of the embryo and others are on the inside, completely surrounded by other cells (Fig. 2). At this time the inside and outside cells become molecularly distinct. The inside cells will go on to generate the ICM, while the outside cells will go on to generate the TE.

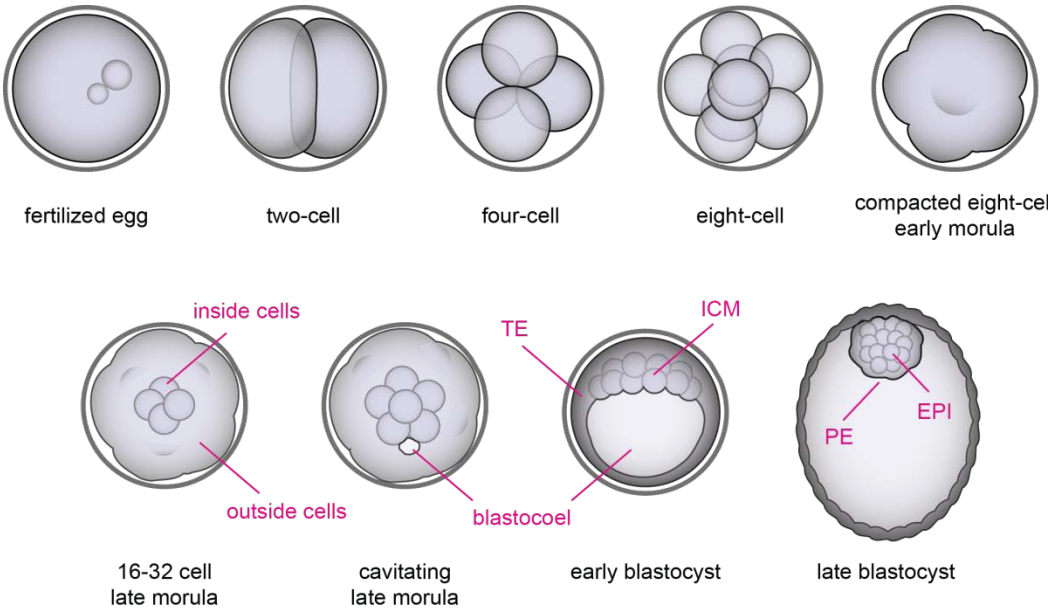


Figure 2. Stages of mouse preimplantation development. Development proceeds with a series of cleavages in which cells get smaller but embryo size in unchanged. Compaction of blastomeres occurs at 8-cells, and the 16-cell morula develops unique inside and outside cells, corresponding to the future ICM and TE lineages, respectively. The formation of the blastocoel, a large fluid-filled cavity, is initiated in the late morula and is a hallmark of the

blastocyst. Upon transition from the early blastocyst to the late blastocyst the ICM is further subdivided into EPI and PE lineages.
-adapted from (Yamanaka et al., 2006)

The transition from 8 to 16 cells is a pivotal step in establishment of asymmetry in the mouse embryo because this initiates events leading to the establishment of the first two lineages. Although there do not appear to be differences among cells at the 8-cell stage, the cells are all polarized. Each cell has an apical domain which contains many tight junction proteins, and a basolateral domain which contains many adherens junctions (Fig. 3). The basolateral domains form at places of cell-cell contact, while the apical domains are present on the most apical region of each cell, which lacks cell-cell contacts (Pauken and Capco, 2000; Plusa et al., 2005; Thomas et al., 2004; Vestweber et al., 1987; Vinot et al., 2005).

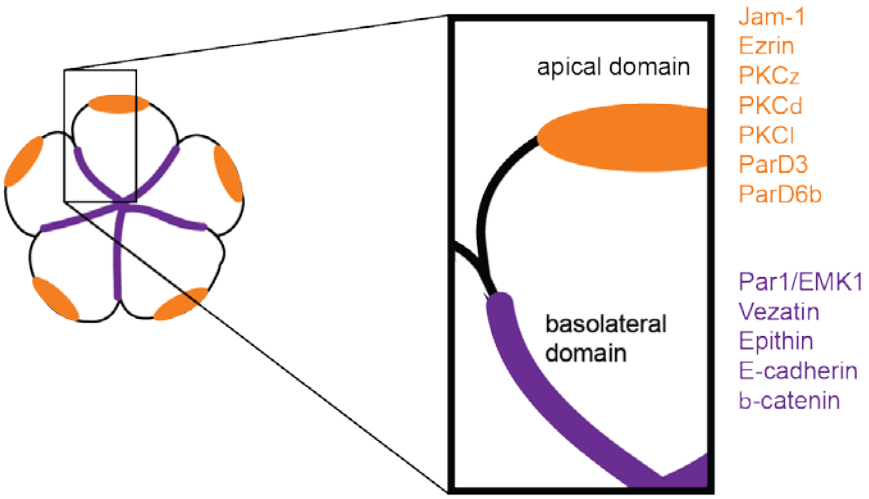


Figure 3. Polarity in the 8-cell embyo. In the 8-cell embryo each blastomere is polarized and contains an apical and basolateral domain. The apical domain consists of tight junction proteins (listed in orange) and is localizes to the outside of the embryo. The basolateral domain consists of adherens junction proteins (listed in purple) and is found at regions of cell-cell contact.
-adapted from (Yamanaka et al., 2006)

One longstanding issue in mammalian development is whether all blastomeres are in fact equivalent through the 8-cell stage or if there are differences which have yet to be detected. To understand how mammalian development proceeds we need to know when meaningful differences occur between cells and what those differences are. Assessing the ability of individual blastomeres to generate all lineages is a robust metric for determining whether meaningful differences exist among cells. Many investigations have been performed to determine the developmental potential of blastomeres during development. Up to the 8-cell stage blastomeres can be dissociated and individual blastomeres can contribute to all lineages when aggregated with a new host embryo, suggesting that blastomeres are still totipotent (Kelly, 1977). In fact, individual blastomeres from 8-cell embryos can form an entirely new blastocyst, although embryos lack sufficient cell numbers to fully develop (Tarkowski, 1959). However, when 16-cell embryos are disaggregated and individual cells are aggregated with a host embryo, outside cells are biased to become TE cells and inside cells are biased to become ICM cells (Rossant and Vijh, 1980; Suwinska et al., 2008; Ziomek et al., 1982). Although cells are biased towards one fate or another at this time, cell fate is not fixed and some cells at the 16-cell stage are in a more intermediate position and may end up either inside or outside (McDole et al., 2011). Many experimental manipulations have been done to alter cell fates, further supporting the notion that although cell fate is biased beginning at the 16-cell stage, these early stages of development are highly regulative and cells remain plastic.

The mechanism translating differences in position and polarity at the 16-cell stage to the establishment of unique cell types is not resolved. Two models have

been proposed for the generation of molecular differences between in and outside cells. The inside-outside model proposes that cell position is the driving force leading to cell identity (Tarkowsk.Ak and Wroblews.J, 1967), while the cell polarity model proposes that differential inheritance of cell polarity components as cells divide is the primary determinant of cell fate (Johnson et al., 1981). There is evidence in support of both models and it is likely that both cell polarity and position contribute to the establishment of cell fates. To what extent position influences polarity and vice versa remains unclear. In recent years, however, many studies have provided new mechanistic insights into the link between cell polarity and the initiation of unique inside and outside cell identities. The observations that the most active enhancers during preimplantation contain TEAD binding sites and that multiple *Tead* family members are transcribed during preimplantation, led to the discovery that *Tead4* is required for trophectoderm specification and blastocyst formation (Kaneko et al., 1997; Kaneko and DePamphilis, 1998; Yagi et al., 2007). However, *Tead4* is expressed in all cells during preimplantation development, implying that additional factors interact with Tead4 to restrict its activity to TE cells.

Previous work demonstrated that Hippo signaling represses the transcriptional activity of Tead proteins both in *Drosophila* and in mammals (Pan, 2007; Saucedo and Edgar, 2007). Hippo signaling is highly conserved across many species and plays a central role in regulating organ growth during development (Zhao et al., 2010). Hippo signaling depends on cell-cell contact and is typically mediated through adherens junction proteins (Fig. 4). Active Hippo (mammalian Stk3) signaling leads to activation of LATS1/2 kinases via phosphorylation, and LATS1/2 cause the transcriptional co-activators YAP and TAZ to remain in the

cytoplasm. In the absence of active Hippo signaling, LATS1/2 is inactive allowing YAP/TAZ to enter the nucleus. Here YAP/TAZ can pair with TEAD proteins and activate transcription of target genes. Therefore when Hippo signaling is active, TEAD-dependent transcription is inactive, and vice versa. Investigation into the role of Hippo signaling during preimplantation development provided the first insight into the connection between polarity and downstream transcriptional events (Nishioka et al., 2009). The presence of YAP and TAZ in the nuclei of outside cells but not inside cells indicates that Hippo signaling is active in inside cells but inactive in outside cells (Nishioka et al., 2009). Several lines of evidence demonstrate that YAP/TAZ are both necessary and sufficient to induce the expression of the outside/TE specific gene *Cdx2*, and that this activity is dependent on TEAD4 (Nishioka et al., 2009). It was also shown that LATS1/2 are necessary and sufficient to repress *Cdx2* expression and formation of the TE (Nishioka et al., 2009). It was later shown that Hippo signaling in inside cells relies on Amot and Amotl2, junction associated proteins which are part of the Hippo pathway (Hirate et al., 2013; Leung and Zernicka-Goetz, 2013). Evidence suggests that the APC in outside cells binds AMOT and prevents its function in outside cells, while its localization to adherens junctions in inside cells is critical for activation of Hippo signaling (Hirate et al., 2013) (Fig. 4).

Elucidating the role of Hippo signaling in the establishment of inside/outside cell fate was an important step towards understanding the first mammalian lineage decision, but determining the subsequent differences in gene expression between inside/outside cells is equally important. *Cdx2* is an important target of TEAD4, and is critical for maintenance of the TE and the repression of *Nanog* and *Oct4* in TE

(Jedrusik et al., 2008; Nishioka et al., 2008; Ralston et al., 2005; Strumpf et al., 2005). In the absence of *Cdx2* the TE is still specified. However, the expression of other important TE genes such as *Eomes* and *Krt8* is reduced and eventually blastocysts collapse (Ralston and Rossant, 2008; Strumpf et al., 2005). Although *Cdx2* is clearly important for TE development, the *Tead4* null phenotype is more severe than the *Cdx2* null phenotype, indicating that additional regulators of TE fate are downstream of *Tead4*. *Gata3* is also downstream of *Tead4* (Ralston et al., 2010). However, loss of *Gata3* does not impair early embryogenesis (Ma et al., 1997), suggesting that there are still additional unknown TE regulators downstream of *Tead4*. Not only will uncovering new *Tead4*-dependent genes reveal additional mechanisms required for specification of the TE, it may also provide additional insights into the regulation of pluripotency *in vitro*. ESCs must continuously repress differentiation towards extraembryonic cell fates and the same mechanisms which drive TE specification may need to be actively repressed in ESCs in order to preserve pluripotency.

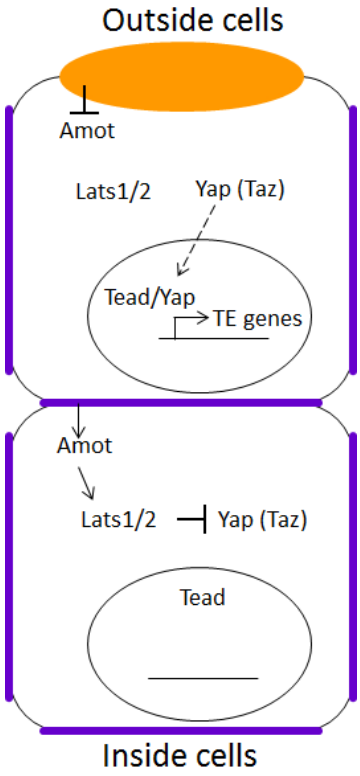


Figure 4. Hippo pathway members repress outside/TE cell fate. In inside cells of the preimplantation embryo AMOT is active and aids in the activation of LATS 1 and 2 kinases, which phosphorylate transcriptional co-activators YAP and TAZ. Phosphorylation of YAP/TAZ leads to their retention in the cytoplasm, and prevents TEAD-mediated transcription. In outside cells the apical polarity complex inhibits AMOT activity and LATS1/2 fail to be activated. YAP/TAZ translocate into the nucleus and associate with TEAD proteins to promote transcription of TE specific genes.

Molecular regulation of the second lineage decision: EPI versus PE

After specification of ICM vs TE, the next major step in mammalian development is the differentiation of the ICM into EPI and PE. In the last decade many genes involved in this process have been discovered, however, the mechanisms involved are still unclear. At E3.5 the ICM is a seemingly homogenous population of cells molecularly defined by nuclear YAP/TAZ and lack of CDX2.

Ubiquitous expression of low levels of the transcription factors *Nanog* and *Gata6* is evident until E3.5. By E3.75 *Nanog* is upregulated in half of ICM cells while *Gata6* is upregulated in the other half and the *Gata6* and *Nanog* expressing cells have a random pattern in the ICM referred to as a salt-and-pepper distribution (Chazaud et al., 2006). Interestingly, the transcription factor Sox17 and the cell surface protein Pdgfrα are both expressed in many presumptive PE cells prior to the salt-and-peppering of Gata6 and Nanog (Artus et al., 2010). Although loss of either *Pdgfra* or *Sox17* does not affect salt-and-peppering and subsequent PE specification, the expression of these genes specifically in future PE cells may reflect an underlying process required for PE specification. After establishment of the *Gata6* expressing PE progenitor population, a sequential activation of additional PE genes occurs (Artus et al., 2011). The newly formed EPI and PE cells then sort into distinct populations with the PE cells forming an epithelium between the blastocoel and the EPI (Fig. 2).

FGF4 and MAPK have a central role in the salt-and-pepppering of *Nanog* and *Gata6* in the ICM. The earliest known event in this process is the upregulation of either *Fgf4* or *Fgfr2* expression in presumptive EPI and PE cells, respectively, but the mechanism initiating this process is not understood (Guo et al., 2010; Kurimoto et al., 2006). EPI cells secrete FGF4 and PE cells may be more responsive to it due to increased expression of the receptor FGFR2 (Frankenberg et al., 2011). However, upon exogenous FGF4 treatment all cells of the ICM can express PE genes, demonstrating that EPI cells can still respond to FGF4 (Yamanaka et al., 2010). FGF4 signaling in the ICM is at least partially transduced through the Mek (Mapk) pathway (Nichols et al., 2009). In *Nanog* mutant embryos, *Fgf4* expression is greatly

reduced indicating that *Nanog* promotes *Fgf4* expression (Frankenberg et al., 2011). GATA6 is detected in all ICM cells in *Nanog* null embryos, suggesting that NANOG is required for repression of GATA6 in PE cells. However, this *Gata6* expression can be eliminated if embryos are cultured in FGFR and MAPK inhibitors prior to and during blastocyst formation (Frankenberg et al., 2011). This suggests that the repression of *Gata6* by *Nanog* relies on MAPK/FGF signaling. In *Fgf4* mutants the initial ubiquitous expression of *Gata6* is unaffected. However, its expression is lost before salt-and-peppering occurs and NANOG remains expressed in all cells of the ICM (Kang et al., 2013; Krawchuk et al., 2013). This demonstrates that *Fgf4* is not required for the initial expression of *Gata6* but is required for downregulation of *Nanog*. Together these analyses indicate that *Nanog* is required for expression of *Fgf4* and that *Nanog* is required for repression of *Gata6* in the ICM. In addition, FGF/MAPK are required for the upregulation of *Gata6*.

After the salt-and-peppering of GATA6 and NANOG, the expression of additional PE genes including *Gata4* and *Sox7* occurs (Artus et al., 2011). The individual contributions of the PE genes *Sox17*, *Gata4*, and *Sox7* are not clear, and *Gata4* and *Sox17* are not strictly required for preimplantation PE development, while the requirement for *Sox7* has not been tested (Kuo et al., 1997; Molkentin et al., 1997; Shimoda et al., 2007). The specific roles played by these factors may be masked by a degree of redundancy among them. However, the precise timing of activation of these genes may reflect a series of molecular events which specify and reinforce PE identity. One observation which suggests that *Sox7* may have a unique role is the timing of its expression, as *Sox7* is expressed when PE cells reach the

blastocoel cavity. However, it is not clear if cells must reach the blastocoel for the upregulation of *Sox7* to occur (Artus et al., 2011).

The final step in maturation of the ICM is the process of sorting EPI and PE cells from a salt-and-pepper distribution into two spatially distinct populations. Several cell surface and transmembrane proteins potentially involved in sorting are expressed in PE cells by E3.75 including LRP2 and aPKC (Gerbe et al., 2008; Saiz et al., 2013). LRP2 is a transmembrane receptor typically involved in endocytosis that can also mediate signaling by morphogens such as Sonic Hedgehog and BMP4 (McCarthy et al., 2002; Spoelgen et al., 2005). Though LRP2 is not required for PE development (Willnow et al., 1996), it is expressed in PE cells prior to sorting and becomes apically localized when PE cells reach the blastocoel (Gerbe et al., 2008). Interestingly, DAB2 is colocalized with LRP2 at the apical surface of the mature PE and is required for its endocytic functions (Gerbe et al., 2008; Maurer and Cooper, 2005). *Dab2* null embryos fail to develop functional PE derivatives and cease developing shortly after implantation (Maurer and Cooper, 2005; Morris et al., 2002; Yang et al., 2002). DAB2 likely regulates the endocytosis of proteins other than LRP2, as general endocytosis by clathrin-coated vesicles is impaired in *Dab2* mutants (Maurer and Cooper, 2005). The DAB2 null phenotype highlights the importance of establishing proper apicobasal polarity of the PE. The role of endocytosis in PE identity and maturation is not well studied and future research into these phenotypes may shed light on the mechanisms regulating proper localization of the PE at the blastocoel.

Using stem cells to understand embryo development

A common theme emerging from studies of preimplantation development is that the majority of mutant phenotypes affect the development of either the TE or PE lineage, with no obvious affect on EPI cells. Hippo signaling is required during the first lineage decision to repress outside cell fate in inside cells. However, whether Hippo signaling also directly promotes inside cell identity is unknown. The only gene reported to be specific to inside cells prior to blastocyst formation is *Sox2*. However, there are conflicting data on the protein expression pattern and there are no obvious defects in inside cell specification in *Sox2* null embryos (Avilion et al., 2003; Guo et al., 2010; Keramari et al., 2010). Even the phenotypes resulting from loss of *Nanog* or *Oct4*, genes expressed in EPI cells and important for pluripotentcy *in vitro*, do not seem to affect the EPI until around the time of implantation or after.

An outstanding question in the study of preimplantation development is: how is pluripotency established? One possibility is that pluripotentcy is the default state in the embryo, already established in the zygote and that genes such as *Nanog*, *Oct4*, and *Sox2* prevent differentiation towards extraembryonic lineages rather than initiating pluripotentcy. Although *Oct4* mutants do have ectopic *Cdx2* expression in the ICM, indicating that *Oct4* helps repress TE fate, this ectopic expression does not begin until late in blastocyst development, suggesting *Oct4* is not required for the initial repression of TE fate (Frum et al., 2013; Ralston et al., 2010). There may also be additional unknown factors that are important for ICM specification, or the loss of multiple genes may be required to reveal more severe phenotypes affecting inside/ICM specification. One roadblock to uncovering the ways in which known pluripotency factors interact with each other and their target genes in the embryo has

been the inability to utilize biochemical assays such as co-immunoprecipitation of interacting proteins and chromatin immunoprecipitation. These assays require large amounts of starting material and due to the very limited amount of material in preimplantation embryos, these assays are essentially impossible to perform on embryos.

The derivation of stem cell lines from all three lineages present in the mammalian embryo has greatly aided efforts to uncover molecular mechanisms driving the specification and regulation of these lineages. Embryonic stem cells (ESCs) were the first stem cell type to be generated from the preimplantation embryo and are derived from the EPI (Evans and Kaufman, 1981; Martin, 1981), while trophoblast stem cells (TSCs) are derived from the TE, and extraembryonic endoderm stem cells (XEN) are derived from the PE (Kunath et al., 2005; Tanaka et al., 1998) (Fig. 5). All these stem cells can all be cultured indefinitely, can differentiate into multiple downstream cell types upon growth factor withdrawal, and can contribute to their appropriate lineages in chimeric embryos. These stem cell lines have been critical for sequencing and biochemical experiments which are not possible with embryos due to the limited amount of material. For instance, the development of both the EPI and PE is impaired in *Sall4* null embryos, making it difficult to study the requirement for *Sall4* in each lineage (Elling et al., 2006). The role of the transcription factor *Sall4* was therefore investigated in ESCs and XEN cells through ChIP-Chip experiments to determine unique target genes in these lineages (Lim et al., 2008). Proteomic analyses are also quite difficult in preimplantation embryos due to lack of starting material. Therefore, a recent study performed cell-surface proteomic profiling in ESCs, TSCs, and XEN cells to find

novel lineage specific cell-surface proteins and then confirmed candidate genes by immunostaining in embryos (Rugg-Gunn et al., 2012). The use of these stem cell lines has furthered our understanding of many molecular mechanisms governing the specification and maintenance of the first lineages during preimplantation development.

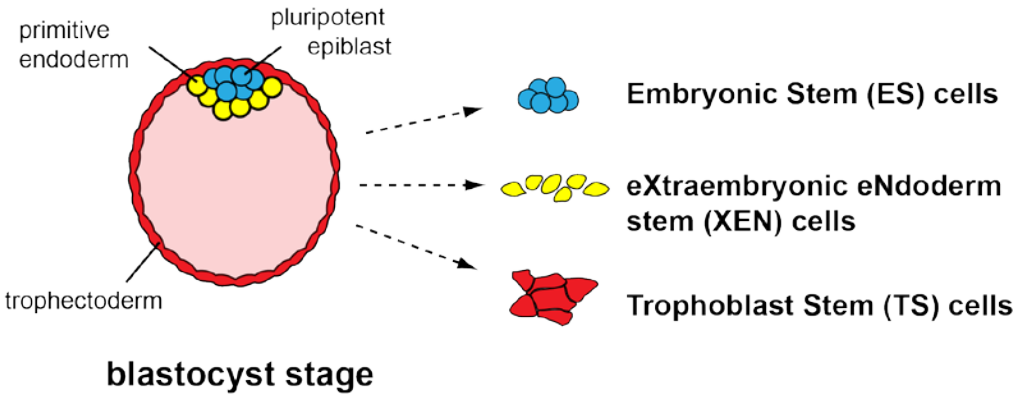


Figure 5. Stem cell lines derived from the blastocyst. Upon culture blastocysts can adhere to the culture plate and cells from a particular lineage will grow out when in the correct media. Embryonic stem cells grow out from the pluripotent epiblast when provided 2i and Bmp4. Trophoblast stem cells grow out from the trophectoderm in the presence of Fgf4, and extraembryonic endoderm cells grow out from the primitive endoderm under several conditions.
-adapted from (Yamanaka and Ralston, 2010)

Using the embryo to understand pluripotent stem cell regulation

Stem cells from the blastocyst are clearly a useful tool for studying preimplantation development. However, the embryo can also be a useful tool for studying pluripotent stem cells. Understanding the molecular regulation of ESCs is critical for their use in biomedical research and therapeutics. Maintaining the health and full potency of ESCs in culture and the ability to evaluate their health and potency will be essential for the safe and economically viable use of ESCs in

medicine. In order to fully evaluate the ESC state we must understand what molecular aspects of ESCs are the most relevant and indicative of their health and potency. Although much progress has been made in identifying key regulatory networks in ESCs, there are also many aspects of pluripotency regulation we do not understand. Mouse ESCs have significant molecular and cellular differences from human ESCs. Mouse ESCs grow in dome-shaped colonies and require LIF and BMP to maintain pluripotency, while human ESCs grow in epithelial colonies and require FGF2 and Activin to maintain pluripotency (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). Mouse epiblast stem cells (EpiSCs) are another type of pluripotent stem cell that can be derived from the postimplantation epiblast, and they are highly similar to human ESCs (Brons et al., 2007; Tesar et al., 2007). The existence of these varied pluripotent stem cell types demonstrates that pluripotency can be regulated in different ways.

Investigating the role of individual genes in the regulation of pluripotency in ESCs is limited by the interdependence of these genes. Extensive investigation into transcriptional regulation in ESCs via OCT4, SOX2, and NANOG has revealed that these factors regulate many of the same target genes and also regulate each other in both mouse and human ESCs (Boyer et al., 2005; Chew et al., 2005; Loh et al., 2006; Masui et al., 2007; Tomioka et al., 2002). This self-reinforcing gene expression network is critical for maintaining pluripotency in culture, as knockdown of any one gene leads to loss of expression of the other genes. Although OCT4, SOX2, and NANOG regulate many of the same genes, they also regulate unique sets of genes and their interdependence makes studying the unique contributions of each gene more challenging. In addition, knockdown of pluripotency genes in ESCs

leads to heterogeneous differentiation of cells, adding to the difficulty of determining the precise role of a gene. By contrast, loss of *Oct4* or *Nanog* in the embryo does not prevent expression of other pluripotency genes (Frum et al., 2013; Messerschmidt and Kemler, 2010), making the embryo an attractive model system for studying the contribution of individual genes to pluripotency.

Studying the acquisition of pluripotency in the embryo may also provide insights into the establishment of induced pluripotent stem cells (iPSCs). Induced pluripotent stem cells (iPSCs) are quite similar to ESCs. However, they originate from more differentiated cells which are reprogrammed by overexpression of transcription factors known as reprogramming factors (Takahashi and Yamanaka, 2006). The original reprogramming factors were genes already known to be important for pluripotency: *Oct4*, *Klf4*, *Sox2*, and *cMyc* (OKSM). Since the advent of this technology many additional factors that can be used in reprogramming have been identified, and unique combinations can be used for generating human IPSCs (Feng et al., 2009; Heng et al., 2010; Redmer et al., 2011; Wernig et al., 2008; Yu et al., 2007). One major area of research interest is determining whether ESCs and iPSCs are equivalent. If iPSCs are truly the same as ESCs, they hold the potential for generating autologous cell types of interest for cell based therapies. However, if they obtain genetic mutations or other potentially harmful epigenetic differences in the process of reprogramming, they may not be a useful source of cells for medical purposes. In addition to their potential medical applications, patient-derived iPSCs can also be a powerful tool for studying molecular mechanisms of disease progression *in vitro*. Patient-derived iPSCs have already been generated from patients with a variety of disease conditions including Hutchinson-Gilford progeria

syndrome and Parkinson's disease, and these iPSCs have demonstrated disease-specific phenotypes upon differentiation to target cell types (Ebert et al., 2009; Kim et al., 2013; Liu et al., 2011; Sanchez-Danes et al., 2012; Yagi et al., 2011; Zhang et al., 2011). Understanding the process of reprogramming and the potential for creating genetic or epigenetic defects that will impact the differentiation of iPSCs is critical for their research and therapeutic uses. Although many important steps in the establishment of induced pluripotency have been elucidated, the heterogeneities observed during reprogramming make identification of the fundamental steps difficult (Hanna et al., 2010). Studying pluripotency acquisition in the embryo allows issues of heterogeneity to be overcome. In the embryo, the EPI develops through a regulated series of events that happens the same way in every embryo, allowing the requirement for different genes and pathways to be studied in a more controlled context. As mechanisms underlying the specification of pluripotency in the embryo are uncovered, these mechanisms can be investigated during reprogramming.

CHAPTER 2 – Maternal *Cdx2* is dispensable for mouse development

Abstract

In many invertebrate and vertebrate species, cell fates are assigned through the cellular inheritance of differentially localized maternal determinants. Whether mammalian embryogenesis is also regulated by deterministic mechanisms is highly controversial. The caudal domain transcription factor CDX2 has been reported to act as a maternal determinant regulating cell fate decisions in mouse development. However, this finding is contentious because of reports that maternal *Cdx2* is not essential for development. Notably, all of the previously published studies of maternal *Cdx2* relied on injected RNA interference constructs, which could introduce experimental variation. Only deletion of the maternal gene can unambiguously resolve its requirement in mouse development. Here, we genetically ablated maternal *Cdx2* using a Cre/lox strategy, and we definitively establish that maternal *Cdx2* is not essential for mouse development.

Introduction

In animals RNA and proteins generated during oogenesis, referred to as maternal factors, initiate and drive embryogenesis, however, the degree to which maternal factors drive development varies. For instance, in flies maternal factors are responsible for the establishment of all body axes, while nematodes and frogs rely on the sperm point of entry in addition to maternal factors to initiate cell fates and body axes. Maternal factors are also important for mouse development (Li et al., 2010), however it is unclear whether these maternal factors contribute to the establishment of cell fates. Many genes with important roles in establishment of the

first three embryonic lineages are transcribed from the zygotic genome but are also found in the oocyte. Using knockdown strategies to eliminate maternal transcripts also eliminates zygotic transcripts, making this an ineffective way to study the potential unique roles of maternal transcripts. Using genetic knockout models, the loss of maternal transcripts, zygotic transcripts, or both can be studied. This type of analysis has revealed unique roles for maternal and zygotic *Cadherin 1* (*Cdh1*). *Cdh1* zygotic (Z) null embryos compact normally, but once maternally provided *Cdh1* is degraded, blastomere adhesion is lost, leading to embryo death (Larue et al., 1994). The *Cdh1* maternal and zygotic (MZ) null phenotype is more severe than the Z null phenotype, and compaction never occurs (de Vries et al., 2004). However *Cdh1* M null embryos, which only lack maternal *Cdh1*, are viable. Although initially M null blastomeres do not adhere and compaction does not occur on schedule, once zygotic transcription of *Cdh1* is initiated embryos recover (de Vries et al., 2004). Although loss of both maternal and zygotic stores of *Cdh1* is more severe than loss of only zygotic *Cdh1*, maternal *Cdh1* is ultimately not required for embryo development.

The role of maternal *Cdx2* in development has been examined by two groups using RNA knockdown techniques which eliminate both maternal and zygotic mRNA. One group found that total loss of Cdx2 led to cell cycle defects, increased apoptosis, embryo arrest by the morula stage, and failure to express many TE and ICM genes (Jedrusik et al., 2010). The other group found that knockdown of both maternal and zygotic *Cdx2* led to the same phenotype as the *Cdx2* genetic knockout, which involves loss of only zygotic *Cdx2* (Wu et al., 2010). Given the dramatically different nature of these results, further investigation into the role of maternal *Cdx2* is

necessary. If maternal *Cdx2* is required for proper establishment of ICM and TE lineages at the morula stage, this would be the first time a maternally provided factor has been shown to directly regulate lineage establishment in mammalian embryos. To investigate the role of maternal *Cdx2* in mouse development we utilized a genetic knockout strategy to remove maternal, zygotic, or total *Cdx2* from embryos and assessed their development.

Materials & Methods

Mouse strains

The following alleles or transgenes were maintained in an outbred (CD1) background: *Cdx2*^{tm1Fbe} (a null allele) (jedrisukChawengsaksophak et al., 1997), *Tg(Zp3-cre)93Knw* (de Vries et al., 2000), and *Cdx2*^{fl} (a new conditional allele). All animal work conformed to the guidelines and regulatory standards of the University of California Santa Cruz Institutional Animal Care and Use Committee.

Generation of the *Cdx2* conditional allele

To generate the *Cdx2* conditional allele, the targeting construct was created by PCR amplification of 129X1/SvJ genomic DNA using Pfu Turbo Hotstart DNA Polymerase (Stratagene) to amplify regions of the Cdx2 locus. Amplified regions were assembled in ploxPF1pneo plamid. PvuI-linearized plasmid was then electroporated into R1 ES cells. NheI-digested genomic DNA from a total of 480 neo-resistant clones was then screened by Southern blot, using a probe complementary to the 3' region. Ten positive clones were then ScaI-digested and screened by Southern blot using the 5' probe, and by PCR using P1 and P2 primers (GAATACGTCGTGTAATTAGCA AND CAAAGCCAACAACTGGAC). A single

correctly targeted clone was selected for injection into C57BL/6J blastocysts to produce chimeric mice. Germline transmission was observed in 4/11 male chimeras, which were then bred to establish founder *Cdx2*$^{fl/+}$ mice. Subsequently, the neo cassette was removed by crossing founder lines created from clone F-42 to B6;SJL-Tg(ACTFLPe)9205Dym/J mouse (Rodríguez et al., 2000).

Embryo genotype determination

Embryo genotyping was performed blind, without prior knowledge of phenotypes. To genotype embryos, genomic DNA was extracted from individual embryos using the Red Extract-N-Amp kit (Sigma) in a final volume of 10 µl extraction/neutralization buffers. Subsequently, 0.5-1 µl lysate was used for PCR detection of the various alleles using the following primers: wild type and *Cdx2*^{tm1Fbe} (AGGGACTATTCAAACTACAGGAG, TAAAAGTCAACTGTGTTCGGATCC, and ATATTGCTGAAGAGCTTGGCGGC), Zp3Cre (GCGGTCTGGCAGTAAAAACTATC and GTGAAACAGCATTGCTGTCACTT), and wild type, *Cdx2*^{fl} and *Cdx2*^{del} alleles using P1 and P2 primers (see above).

Immunofluorescence, embryo culture, and microscopy

Embryos were collected from timed natural matings by flushing dissected oviducts or uteri with M2 medium (Millipore). Embryos were either fixed and stained as previously described (Ralston and Rossant, 2008), or cultured in KSOM (Millipore) at 37ºC and 6% CO_2 to monitor morphological development. Primary antibodies included mouse anti-Cdx2 (Biogenex CDX-88), rabbit anti-Nanog (Cosmobio), rat anti-Ecadherin (Sigma), rat anti-Krt8 (Troma-1, R. Kemler, Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242). Secondary

antibodies, nuclear stain (Draq5), and confocal microscopy methods are described elsewhere (Ralston and Rossant, 2008).

Real-time PCR

To obtain oocytes, female mice were superovulated by subcutaneous injections of 5 IU each PMS and HCG (Sigma), 46 hours apart. MII oocytes were collected 23 hours after HCG. The ampulla were dissected and oocytes were then denuded of cumulus cells by incubation and gentle pipetting in 300 µg/mL Bovine Type IV-S hyalurionidase (Sigma) in M2 medium. RNA was extracted from around 20 pooled oocytes or individual blastocysts using the PicoPure RNA Isolation Kit (Arcturus), according to manufacturer's instructions. cDNA was prepared and genes amplified by qPCR (Ralston and Rossant, 2008) using *Actb* (CTGAACCCTAAGGCCAACC and CCAGAGGCATACAGGGACAG) and *Cdx2* primers (AAACCTGTGCGAGTGGATG and TCTGTGTACACCACCCGGTA).

Results

Oocytes express lower levels of *Cdx2* than do embryonic stem cells

The presence of maternal *Cdx2* mRNA has been reported in multiple studies and has led to the idea that maternally provided *Cdx2* may have an earlier role in development than zygotic *Cdx2* (Jedrusik et al., 2010; Wu et al., 2010) . However, both immunohistochemistry and mass spectrometry have failed to detect any maternal CDX2 protein (Beck et al., 1995; Wang et al., 2010). Maternal *Cdx2* transcripts may not have any functional relevance, as pluripotent and totipotent cells are known to have open chromatin and low levels of transcription of many lineage-associated genes. *Cdx2* mRNA can be found in ESCs, but *Cdx2* null ESCs can be

generated and stably propagated, demonstrating that Cdx2 is not required in ESCs (Chawengsaksophak et al., 2004). We sought to determine the level of maternal *Cdx2* transcripts relative to both blastocysts, where *Cdx2* is essential, and to ESCs, where *Cdx2* is dispensable. Previous studies only compared the level of *Cdx2* in oocytes to blastocyst (Jedrusik et al., 2010; Wu et al., 2010). We harvested mRNA from oocytes, single blastocysts, and R1 ESCs and measured *Cdx2* levels by RT-qPCR. *Cdx2* levels were over 100x higher in blastocysts than oocytes, and more than 10x higher in ESCs than oocytes (Fig. 6). This analysis suggests that maternal *Cdx2* may be unnecessary for normal embryo development.

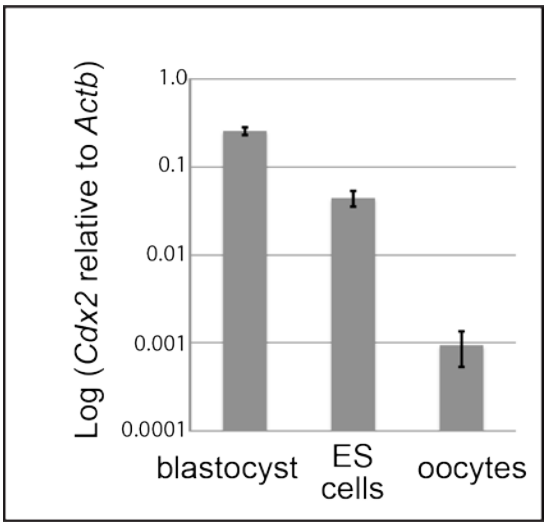


Figure 6. Cdx2 levels are lower in oocytes than in ESCs. Average *Cdx2* levels, normalized to *β-actin* (*Actb*), in E3.5 blastocysts, oocytes and ES cells. Averages were calculated from three biological replicate measurements: three wild-type (wt) blastocysts, three ES cell lines (R1, E14 and G4), and oocytes from three mice.

Generation of viable mice lacking maternal *Cdx2*

The level of *Cdx2* present in oocytes suggests that maternal *Cdx2* may not have a role in embryo development. Previous studies relied on injection of siRNAs, morpholinos, or double-stranded RNA into oocytes or one-cell embryos to knockdown *Cdx2* mRNA (Jedrusik et al., 2010; Wu et al., 2010). Despite testing identical constructs, Jedrusik *et al*. found that maternal *Cdx2* was required for progression past the morula stage and establishment of TE and ICM fates, while Wu *et al*. found no requirement for maternal *Cdx2*. To test the requirement for maternal Cdx2 we used a genetic strategy to remove *Cdx2* from oocytes prior to the initiation of maternal transcription. First we generated a conditional *Cdx2* allele (Fig. 7), in which excision by Cre recombinase leads to removal of the transcription start site and introduces a nonsense frameshift. This allele was combined with the female germline-specific *Zp3:Cre* (de Vries et al., 2000), which leads to gene excision in oocytes early during oogenesis (Lan et al., 2004) (Fig. 7). To verify loss of maternal *Cdx2* mRNA, oocytes were collected from wild type and maternal null, or M null, females and *Cdx2* levels were measured by RT-qPCR. Cdx2 was undetectable in M null oocytes (Fig. 8A). *Cdx2* M null mice were then crossed to wild type males and the resulting litter size was compared to wild type crosses. There was no difference in the number of pups per litter from *Cdx2* M null females versus wild type females (n=3 litters each) (Fig. 8B). This demonstrates that embryos lacking maternal *Cdx2* are viable and maternal *Cdx2* is not required for embryo development.

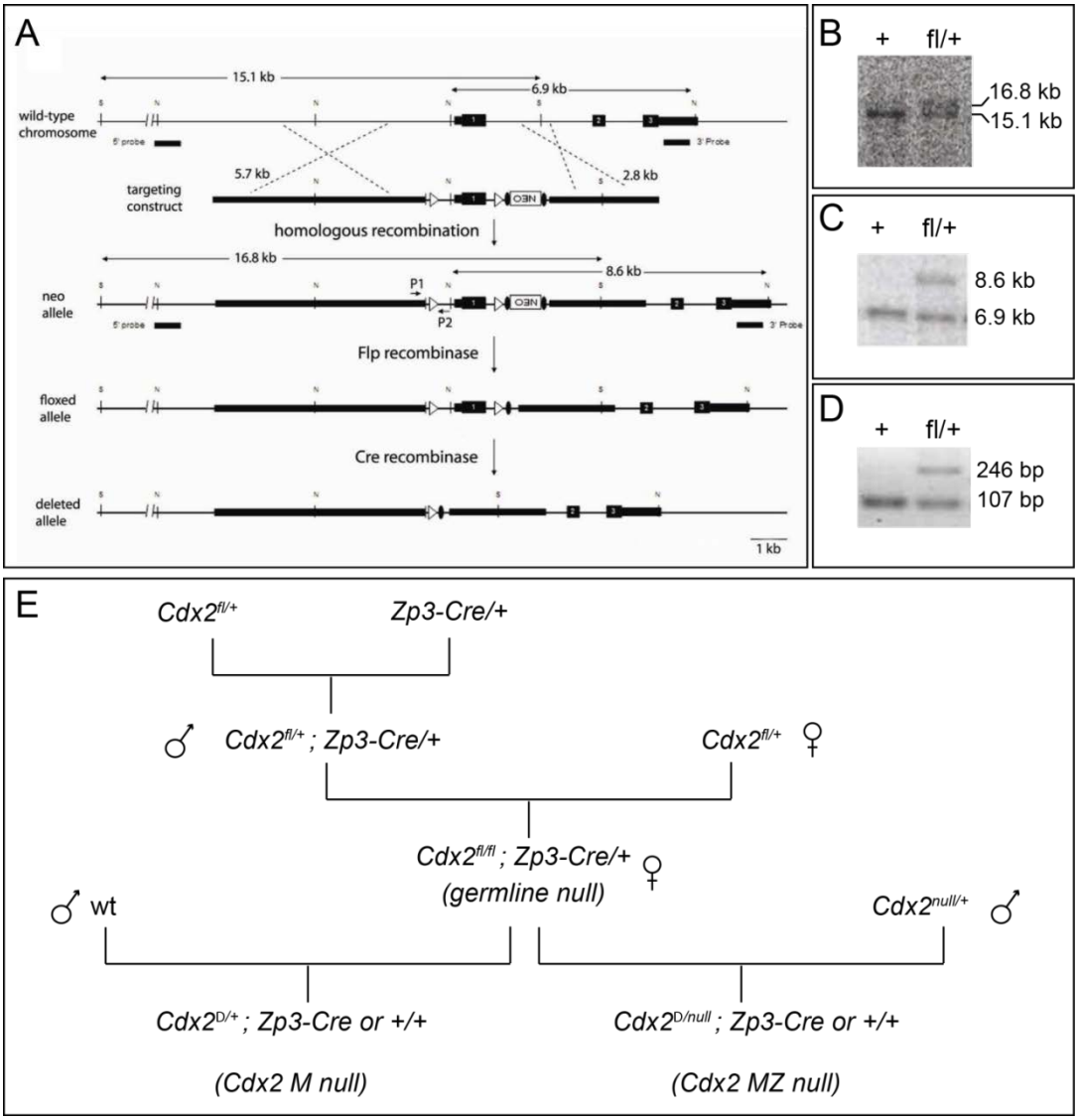


Fig. 7. Germline ablation of *Cdx2*. (**A**) Overview of the targeting strategy used to generate the *Cdx2*^{fl} allele. The targeting construct contained 5.7 and 2.8 kb homology arms, loxP sites (triangles) flanking the Cdx2 5'UTR and exon 1, and a neo selection cassette flanked by FRT sites (ovals). Homologous recombination replaces the 15.1 kb ScaI fragment with a 16.8 kb fragment, detectable by Southern blot analysis with 59 probe, and replaces the 6.9 kb NheI fragment with an 8.6 kb fragment, detectable by Southern blot analysis with 39 probe. Homologous recombination also introduces a 247 bp P1/P2 PCR product (primer positions indicated), whereas the wild-type P1/P2 product is 107 bp. (**B**) Southern blot analysis of wild-type ES cell and a correctly targeted ES cell clone using the 59 probe. (**C**) Southern blot analysis of wild-type and targeted ES cell clone using the 39 probe. (**D**) PCR genotyping of

wild-type and targeted ES cell clone using P1 and P2 primers. (**E**) Overview of crossing schemes used to create *Cdx2* germline null females and *Cdx2* M null and *Cdx2* MZ null animals or embryos.

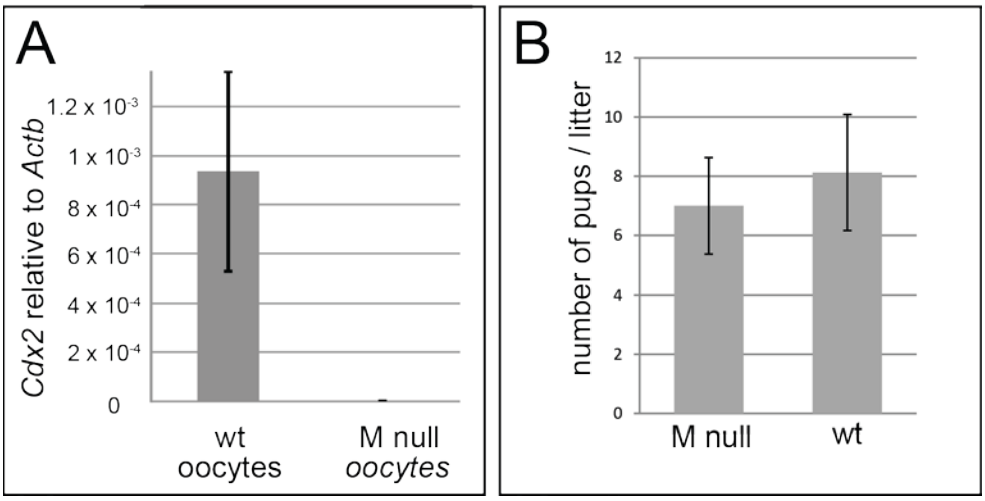


Figure 8. Maternal *Cdx2* is not required for embryo development (**A**) Levels of *Cdx2*, relative to *Actb* mRNA, in wild-type and *Cdx2* M null oocytes (average of three biological replicates for each genotype). (**B**) Average number of pups per litter from wild type and M null females crossed with wild type males, N=3 litters each. Error bars indicate s.d. of biological replicates.

Zygotic *Cdx2* does not compensate for maternal *Cdx2*

It has been proposed that zygotic *Cdx2* may compensate for loss of maternal *Cdx2*, thereby masking a maternal null phenotype (Jedrusik et al., 2010). To determine if zygotic *Cdx2* is compensating for the loss of maternal *Cdx2* we crossed M null females to *Cdx2*$^{null/+}$ males to generate *Cdx2* MZ null embryos (Fig. 7E). Control (wild type and *Cdx2*$^{null/+}$), Z, and MZ null embryos were collected and examined between E3.5-E4.25. At E3.5 MZ null embryos were morphologically indistinguishable from wild type and Z null embryos and expressed both *Krt8*, a TE marker, and *Nanog*, an ICM marker (Fig. 9A). As previously reported, there was a

reduction in *Krt8* expression and ectopic *Nanog* expression in the TE of *Cdx2* Z null embryos (Ralston and Rossant, 2008; Strumpf et al., 2005). There was no significant difference in total cell number or the proportion of TE and ICM cells among control, Z null, or MZ null embryos (Fig. 9C,D). By E4.25 both Z and MZ null embryos collapse and ectopic *Nanog* expression persists (Fig. 9B). *Cdh1* was detected at normal levels in Z and MZ null embryos, but was mislocalized in some TE cells as previously reported for Z null mutants (Strumpf et al., 2005) (Fig. 9B). In contrast to the findings of Jedrusik, et al. 2010, embryos lacking both maternal and zygotic *Cdx2* phenocopied embryos lacking only zygotic *Cdx2*. These findings support the conclusions found in Wu, et al. 2010, that maternal *Cdx2* has no role in mouse embryo development.

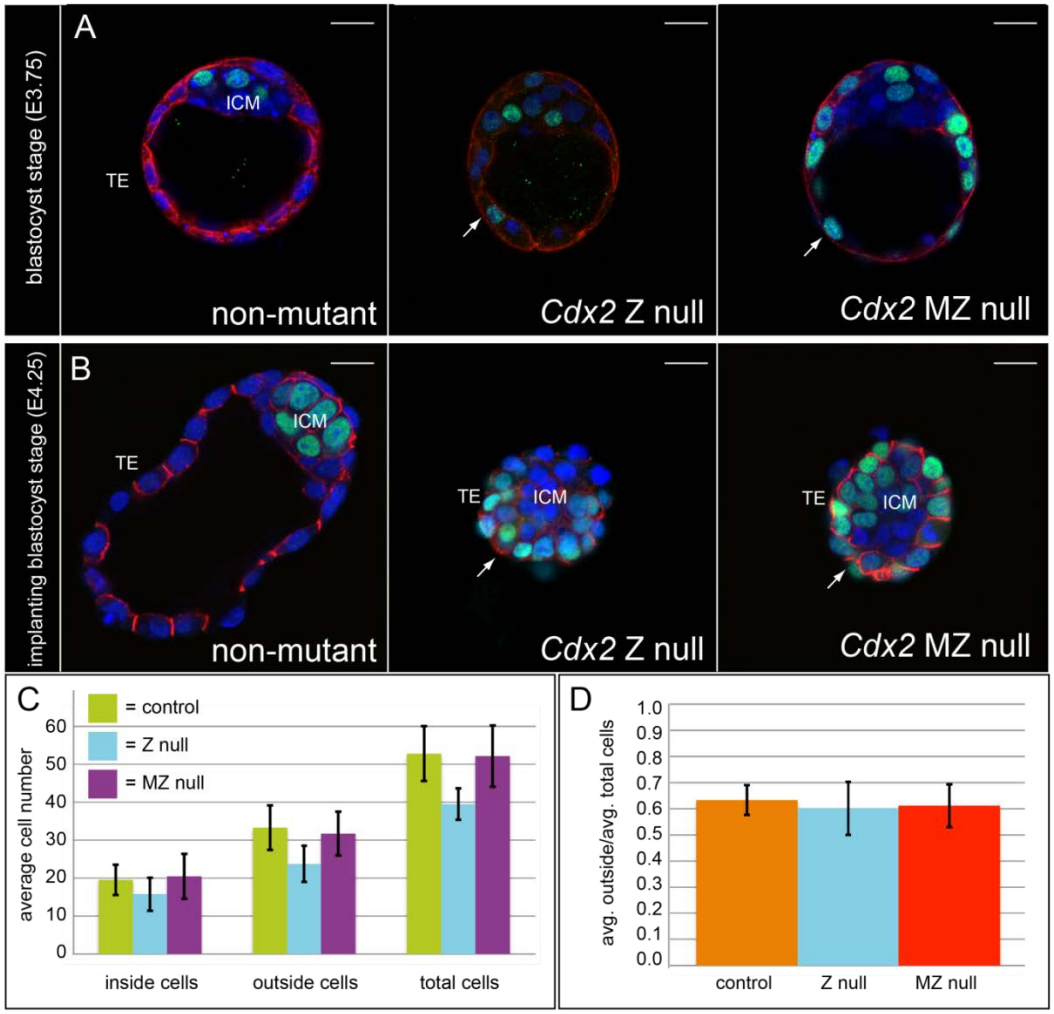


Figure 9. Loss of maternal *Cdx2* does not worsen the *Cdx2* zygotic null phenotype. (**A**) Expression of KRT8 (red) and NANOG (green) in confocal transverse sections of preimplantation mouse blastocysts at E3.75 (nuclei, blue). Images are representative of *n*=20 control (*Cdx2*$^{+/-}$ or wild type), *n*=6 Z null, *n*=5 MZ null blastocysts. In control blastocysts, KRT8 is restricted to the trophectoderm (TE) and NANOG is restricted to the inner cell mass (ICM). In *Cdx2* Z null and MZ null blastocysts, KRT8 is still expressed in the TE and NANOG is ectopically expressed in the TE (arrows). (**B**) Expression of CDH1 (red) and NANOG (green) in implantation stage blastocysts at E4.25 (nuclei, blue). Control blastocysts are expanded and *Cdx2* Z null and MZ null blastocysts are collapsed. CDH1 and NANOG are detectable in *Cdx2* Z null and MZ null blastocysts and NANOG is ectopically expressed in the TE of both mutants (arrows). Representative of *n*=7 control, *n*=7 Z null, *n*=9 MZ null blastocysts. (**C**) Average numbers of inside, outside and total cells in control (*Zp3-Cre*/+; *n*=28), *Cdx2* Z null (*n*=4) and *Cdx2* MZ null (*n*=22) blastocysts at E3.5. Inside and

outside cells were counted on the basis of morphological position in the blastocyst. (**D**) Data from C showing the average proportion of outside cells per embryo, indicating no difference in the proportion of TE cells for any genotype ($P>0.05$, *t*-tests). Error bars indicate s.d. Scale bars: 20 μm.

Discussion

We have used a genetic knockout strategy to examine the role of maternal *Cdx2* in mouse embryo development. These data have clearly demonstrated that maternal *Cdx2* is not required for development and that it does not appear to contribute to the establishment of TE or ICM. Potential reasons for the phenotype seen by Jedrusik *et al*. have been discussed (Wu and Scholer, 2011), including different injection techniques, use of live fluorescent imaging, and different genetic background, however the reasons are still unclear. Similar discrepancies exist between RNAi knockdown and genetic knockout of maternal *Oct4* (Chia Le Bin et al., 2014; Foygel et al., 2008; Frum et al., 2013; Wu et al., 2013) and maternal *Sox2* (Keramari et al., 2010). The propensity to see phenotypes by RNA knockdown but not by genetic knockout highlights the need for genetic models of murine maternal mutants. Though Wu *et al*. demonstrates that successful RNA knockdown can be done to study maternal phenotypes, if a phenotype is found by this method, the creation of genetic knockout is critical to verify the phenotype.

A contribution of maternal factors to the establishment of TE and ICM cannot be ruled out, however, several observations indicate that lineage specification in the preimplantation embryo does not rely on maternal factor. Thus far the knockdown/out of individual maternal factors has led to lethality prior to initiation of TE/ICM or no phenotype. The majority of known maternal effect genes in mice are involved in regulation of the genome in processes such as zygotic nucleus formation,

zygotic genome activation, and maintenance of imprinted genes (Zheng and Liu, 2012). Maternal factors are also responsible for the degradation of other maternal factors. By the 2 cell stage ~90% of maternal RNA is degraded (Alizadeh et al., 2005; Paynton et al., 1988), and ~50% of maternal protein is degraded (Merz et al., 1981). Molecular events underlying TE/ICM specification are initiated at the 8-16 cell transition, more than 24 hours after the massive loss of maternal RNA and proteins at the 2 cell stage, supporting the idea that maternal factors do not contribute to TE/ICM specification. In non-mammalian organisms maternal factors contribute to lineage specification through their asymmetrical localization in the embryo. The totipotency of cells and apparent lack of asymmetry among cells prior to the 8 cell stage also suggests that maternal factors do not contribute to lineage specification in the mammalian embryo. Our findings in this study highlight two important factors for ongoing research of maternal effect genes: 1) not all maternally inherited factors are important for early embryo development, even if those factors are known to be important for later developmental steps and 2) genetic knockout models are essential for confirming the phenotype of maternal effect genes.

CHAPTER 3 – Trophoblast stem cell forming potential reveals a functional distinction between naïve and primed pluripotent stem cells

Abstract

Diverse pluripotent stem cell lines have been derived from mouse, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), embryonal carcinoma cells (ECCs), and epiblast stem cells (EpiSCs). These lines can self-renew or differentiate into germ layer derivatives, but differ in origin, morphology, gene expression, and signaling. These differences are thought to define two distinct pluripotent states, termed naïve and primed. Whether the pluripotent state influences developmental potential or the competence to respond to overexpressed transcription factors is unclear, but knowing this is crucial for optimizing directed differentiation protocols. To determine whether pluripotent stem cell lines differ in developmental potential, we compared the capacity of ESCs, iPSCs, ECCs, and EpiSCs to form trophoblast stem cells (TSCs), a stem cell of the placental lineage. ESCs do not normally form TSCs, but the transcription factor Cdx2 is sufficient to drive TSC formation in ESCs. Similarly, we found that iPSCs and ECCs could give rise to TSCs following *Cdx2* overexpression, although EpiSCs did not. Since ESCs, iPSCs, and ECCs can contribute to blastocyst development, while EpiSCs cannot, TSC-forming ability could be a property of naïve, but not primed, pluripotency. Consistent with this, we noted an inverse correlation between the expression level of naïve pluripotency genes in undifferentiated pluripotent stem cell lines and the TSC-forming efficiency of those cell lines. Culturing ESCs or iPSCs in conditions that promote naïve pluripotency improved TSC-forming efficiency in multiple stem cell lines. This work demonstrates that differences in the pluripotent state can influence the developmental potential of pluripotent stem cells.

Introduction

Pluripotent stem cell lines have been derived from diverse sources, including mouse and human germ cell tumor-derived Embryonal Carcinoma Cells (ECCs) (Kahan and Ephrussi, 1970), mouse and human preimplantation epiblast-derived Embryonic Stem Cells (ESCs) (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998), mouse post-implantation epiblast-derived Epiblast Stem Cells (EpiSCs) (Brons et al., 2007; Tesar et al., 2007), and mouse and human somatic cell-derived induced Pluripotent Stem Cells (iPSCs) (Takahashi and Yamanaka, 2006). All these pluripotent stem cell lines are thought to be equivalent in terms of self-renewal and developmental potential, since they can all give rise to differentiated cells from all three embryonic germ layers. However, differences in growth factor requirements, morphology, and gene expression exist among these pluripotent stem cell lines, even within a species (Ohtsuka and Dalton, 2008). It is not yet clear whether these differences influence each cell line's ability to reproducibly differentiate into specific lineages during directed in vitro differentiation protocols. Resolving the differences in developmental potential among pluripotent stem cell lines will critically inform the decision as to whether iPSCs can replace ESCs as both a model for basic biology, and as a tool for regenerative medicine.

The mouse provides a powerful system for resolving differences in developmental potential among pluripotent stem cell lines for several reasons. First, both naïve and primed pluripotent stem cell lines exist, allowing for direct comparisons of pluripotent states within a single species. Second, mouse ESCs and iPSCs can be shifted between naïve and primed pluripotency, by addition or removal, respectively, of GSK3 and Mapk inhibitors (2i) (Marks et al., 2012; Wray et

al., 2011; Ying et al., 2008). Third, the use of inbred mouse lines allows for examination of developmental potential within a fixed genetic background. Finally, analyses of mouse pluripotent stem cell developmental potential can benefit from studies of stem cell origins during mouse development.

Here we exploited the strengths of the mouse model to examine the ability of diverse pluripotent stem cell lines to generate cells of the first lineage that is specified during embryonic development, the trophoblast, which gives rise to the entire fetal compartment of the placenta (Rossant and Cross, 2001), as well as trophoblast stem cells (TSCs) (Tanaka et al., 1998). Mouse ESCs do not contribute efficiently to trophoblast in chimeras (Beddington and Robertson, 1989), and are not thought to differentiate to trophoblast cells spontaneously *in vitro* because ESCs are derived from the blastocyst after trophoblast has already been set aside. Overexpression of the transcription factor Cdx2 in ESCs in the presence of TSC self-renewal factors FGF4, Heparin (Hep), and mouse embryonic fibroblasts (MEFs) is sufficient to convert ESCs to TSCs (Niwa et al., 2005). The ESC/TSC lineage restriction therefore provides an assay by which to systematically evaluate the developmental potential of pluripotent stem cell lines. TSCs differ from pluripotent stem cell lines in morphology, gene expression, and developmental potential. In addition, morphological and molecular markers of self-renewing and differentiated TSCs are well defined (Adachi et al., 2013; Niwa et al., 2005; Ralston et al., 2010; Tanaka et al., 1998). These features make evaluating the ability of *Cdx2* overexpression to induce TSC formation straightforward, and enable a direct comparison of TSC-forming potential between naïve and primed pluripotent stem cell lines.

Methods & Materials

Cell culture

TSCs were maintained on MEFs in TS medium (RPMI + 20% FBS + 1 µg/mL FGF4 and 1 U/mL Heparin (R&D Systems) as described (Tanaka et al., 1998), unless otherwise indicated. ESC and iPSC lines were maintained on mitotically inactivated MEFs in standard ESC medium (DMEM with 15% fetal bovine serum (FBS; Hyclone) and Leukemia Inhibitory Factor (LIF; Millipore), or in 2i medium (15% knockout serum replacement (KOSR; Gibco) replaced FBS, 1 µM PD0325901 and 3 µM CHIR99021 (Stemgent). EpiSCs were maintained on MEFs in EpiSC medium (1:1 DMEM /F12 (Gibco), 20% KOSR, 100 µM 2-mercaptoethanol, 2mM L-glutamine (Gibco), 1 mM non-essential amino acids (Gibco), 50ug/mL penicillin/streptomycin (Gibco), and 5 ng/mL FGF2 (R&D Systems). EpiSCs were split 1:4 every 2-3 days with type IV collagenase (Gibco). EC cells were maintained in DMEM with 15% FBS, L-Glutamine, and Penicillin/Streptomycin. Linearized pCAG-hCdx2ERT2-ires-puro^r construct was introduced into cells by electroporation and stable transformants were selected and screened as previously described (Ralston et al., 2010). The *Cdx2*-overexpression assay was carried out by harvesting confluent wells of pluripotent cells and seeding at a 1:100 split ratio. The next day medium was replaced with TSC medium with FGF4, Heparin, and 4-hydroxytamoxifen (4-OHT) (Sigma) to induce transgene activity. After 6 days of 4-OHT treatment cells were passaged and maintained in standard TSC conditions for an additional 2-3 passages prior to gene expression analysis.

Reprogramming

Mouse iPS cells were generated by reprogramming as described (Takahashi and Yamanaka, 2006). Briefly, retroviral reprogramming vectors were produced by transfecting 293T cells with pCL-ECO and pMXs plasmid containing *Oct4*, *Klf4*, *Sox2*, or *cMyc* (OKSM) cDNAs (Addgene). OKSM tissue culture supernatant was harvested 48 hr later and stored at -80ºC until use. Subsequently, passage 2 E13.5 MEFs were seeded at a density of 5000 cells/mL on gelatin in MEF culture medium (DMEM + 10% FBS + 200mM glutamax + 10000 U each pen/strep) in 96-well plates. 24 hr later, MEFs were cultured in OKSM supernatant for 24 hr. 24 hr later, OKSM supertantant was replaced with MEF medium, and then standard ESC medium on days 2 and 4, and finally ESC medium w/ KOSR. On day 18 after retroviral treatment, iPSC colonies were picked, expanded, and characterized after passage 10. For immunofluorescent characterization, iPSCs were plated on gelatinized cover slips and grown overnight. Cells were then fixed with 4% formaldehyde, washed with PBS, and incubated in 0.5% Triton x-100 in PBS for 30 min. Cells were then blocked in PBS with 10% FBS and 0.2% Triton x-100 for 1 hour at room temperature, then incubated in mouse anti-SSEA-1 (MC-480, Developmental Studies Hybridoma Bank) at 1:1000 in blocking buffer, overnight at 4°C. Cells were then washed with PBS and incubated with secondary antibody (Cy3-conjugated donkey anti-mouse IgM; Jackson Labs) at 1:1000 and 1:1000 DAPI (Sigma) in blocking buffer for 1 hour. For chimera characterization, iPS cells were injected into CD1 blastocysts, which were then transferred to pseudopregnant recipient females, whereupon they were allowed to come to term. All animal work conformed to the guidelines and regulatory standards of the University of California Santa Cruz Institutional Animal Care and Use Committee.

Gene expression analysis

RNA was harvested from cells using Trizol (Invitrogen). cDNA was generated from 1 µg RNA using the Quantitect Reverse Transcription Kit (Qiagen). qPCR was performed using SYBR Green and LightCycler 480 (Roche) in 12 µl reactions. All reactions were performed in triplicate, with 100-200 ng cDNA and 300 nM primers per reaction. For each primer pair (Supplementary Table 1), a standard curve was generated to determine PCR efficiency using either R1 ESC or TSC cDNA. Relative levels of gene expression were subsequently calculated using the empirically determined efficiency.

Results

EpiSCs do not give rise to TSCs following overexpression of *Cdx2* in TSC conditions

ESCs become TSCs when *Cdx2* is overexpressed in the presence of FGF4, Heparin, and mouse embryonic fibroblasts (MEFs) (Niwa et al., 2005). One study showed that EpiSCs express some trophoblast genes upon BMP4-induced differentiation (Brons et al., 2007). However, another study showed that trophoblast fate is not induced in EpiSCs by this treatment (Bernardo et al., 2011). Therefore, it is unclear whether EpiSCs respond to *Cdx2* overexpression by becoming TSC cells. On the other hand, if pluripotent stem cells are all equivalent in terms of developmental potential, then we predicted that EpiSCs should also produce TSCs following overexpression of *Cdx2* in TSC conditions (FGF4, Heparin, and MEFs).

To compare the developmental potential of ESCs and EpiSCs, we compared their abilities to give rise to TSCs following *Cdx2* overexpression. To overexpress

Cdx2, we introduced the *Cdx2ER* plasmid (Niwa et al., 2005) into EpiSC (Tesar et al., 2007) and R1 ESC lines, and selected multiple subclones expressing *Cdx2ER* by qRT-PCR. Using this plasmid, *Cdx2ER* is constitutively expressed, but the CDX2ER protein remains inactive until 4-hydroxytamoxifen (4-OHT) is added (Eilers et al., 1989). After selecting EpiSC and ESC clones expressing *Cdx2ER* at least 1x the level of Cdx2 in TSCs (Fig. 18), we attempted to induce formation of TSCs by treating cells with 1 µg/mL 4-OHT in TSC medium on MEFs for six days (Fig. 10A), as previously performed (Niwa et al., 2005; Ralston et al., 2010). As negative controls, parental EpiSC and ESC lines lacking the *Cdx2ER* plasmid were treated with 4-OHT in TSC medium in parallel (Fig. 10A). After the six-day differentiation all cells were passaged 2-3 times in TSC medium without tamoxifen to test self-renewal ability, and cell morphology and gene expression were then examined to determine whether TSCs were generated.

We evaluated the TSC morphology of the *Cdx2*-overexpressing cell lines by qualitative comparison to TSCs derived from E6.5 extraembryonic ectoderm (Tanaka et al., 1998). TSCs grow as flat, epithelial colonies with smooth borders (Fig. 10B), while ESCs grow as small, domed colonies of cells (Fig. 10C). TSC morphology was evident in most ESC+*Cdx2ER* subclones after the Cdx2-overexpression assay (5/6 subclones; Fig. 10D). By contrast, TSC morphology was not observed in ESCs cultured in TSC medium without *Cdx2ER* (Fig. 10E), confirming that the morphological TSC phenotype was *Cdx2*-dependent. We then evaluated TSC gene expression in all of these cell lines. Endogenous *Cdx2, Eomes, and Rhox4b* (*Ehox*) are all highly expressed in TSCs, and are rapidly downregulated during their differentiation (Jackson et al., 2003; Tanaka et al., 1998). In all of the ESC-derived

TSCs exhibiting TSC morphology, TSC genes were detected at levels comparable to TSCs after *Cdx2*-overexpression (Fig. 10J), indicating that the cells had acquired a TSC gene expression pattern, consistent with prior reports (Niwa et al., 2005; Ralston et al., 2010). TSC gene expression was not detected in control ESCs that had been cultured in TSC conditions without *Cdx2ER* (Fig. 10J).

We further verified that ESCs had acquired TSC properties by evaluating self-renewal and differentiation of the ESC-derived TSCs. First, we verified that the ESC-derived TSCs were capable of long-term self-renewal by serial passaging of TSCs for more than 50 days (10 passages), during which time they preserved their stereotypical TSC morphology (not shown). Second, we verified that ESC-derived TSCs could differentiate upon withdrawal of FGF4/Hep and MEFs. TSC differentiation is characterized by the downregulation of TSC genes, the concomitant upregulation of differentiation genes such as *Prl3d1* (*Placental lactogen 1*), *Prl3b1* (*Placental lactogen 2*), and *Tpbpa*, and formation of syncytial and giant cells (Niwa et al., 2005; Ralston et al., 2010; Simmons et al., 2007; Tanaka et al., 1998). We therefore differentiated ESC-derived TSCs alongside TSCs by withdrawing FGF4/Hep and MEFs. After 7 days of differentiation in the absence of FGF4/Hep and MEFs, cell morphology and gene expression were evaluated. Both the differentiated TSCs and the differentiated ESC-derived TSC lines appeared large and flat, with giant or multinucleated nuclei (3/3 ESC-derived TSC lines; Fig. 10F,G), consistent with prior analysis of *in vitro* differentiation of ESC-derived TSCs (Niwa et al., 2005; Ralston et al., 2010). In addition, endogenous *Cdx2*, *Eomes*, and *Rhox4b* were all downregulated (Fig. 10K), and *Prl3d1*, *Prl3b1,* and *Tpbpa* were upregulated (Fig. 10L) in all of the ESC-derived TSC lines examined (3/3 ESC-derived TSC

lines). These data demonstrate that TSCs were successfully generated from ESCs after *Cdx2* overexpression.

Having established methods for generating and evaluating ESC-derived TSCs, we next evaluated the ability of overexpressed *Cdx2* to induce TSC morphology and gene expression in EpiSCs. Untreated EpiSCs are epithial and grow in large, compact colonies (Fig. 10H). Following *Cdx2* overexpression in TSC medium for six days, EpiSCs did not exhibit TSC morphology (5/5 subclones; Fig. 10J). Rather, these cells lost their epithelial appearance, and appeared similar to control EpiSCs without overexpressed *Cdx2* (Fig. 10I), suggesting that the TSC culture medium, rather than the overexpressed *Cdx2* induced the morphological phenotype. Next, we evaluated expression of TSC genes in EpiSCs after the six-day treatment. TSC genes were not upregulated in EpiSCs following *Cdx2* overexpression in TSC medium (Fig. 10M). We conclude from these observations that even though the levels of overexpressed *Cdx2* were comparable between EpiSCs and ESCs, the developmental potential of EpiSCs is fundamentally different from ESCs, evidenced by a differing ability to form TSCs in response to overexpressed *Cdx2*.

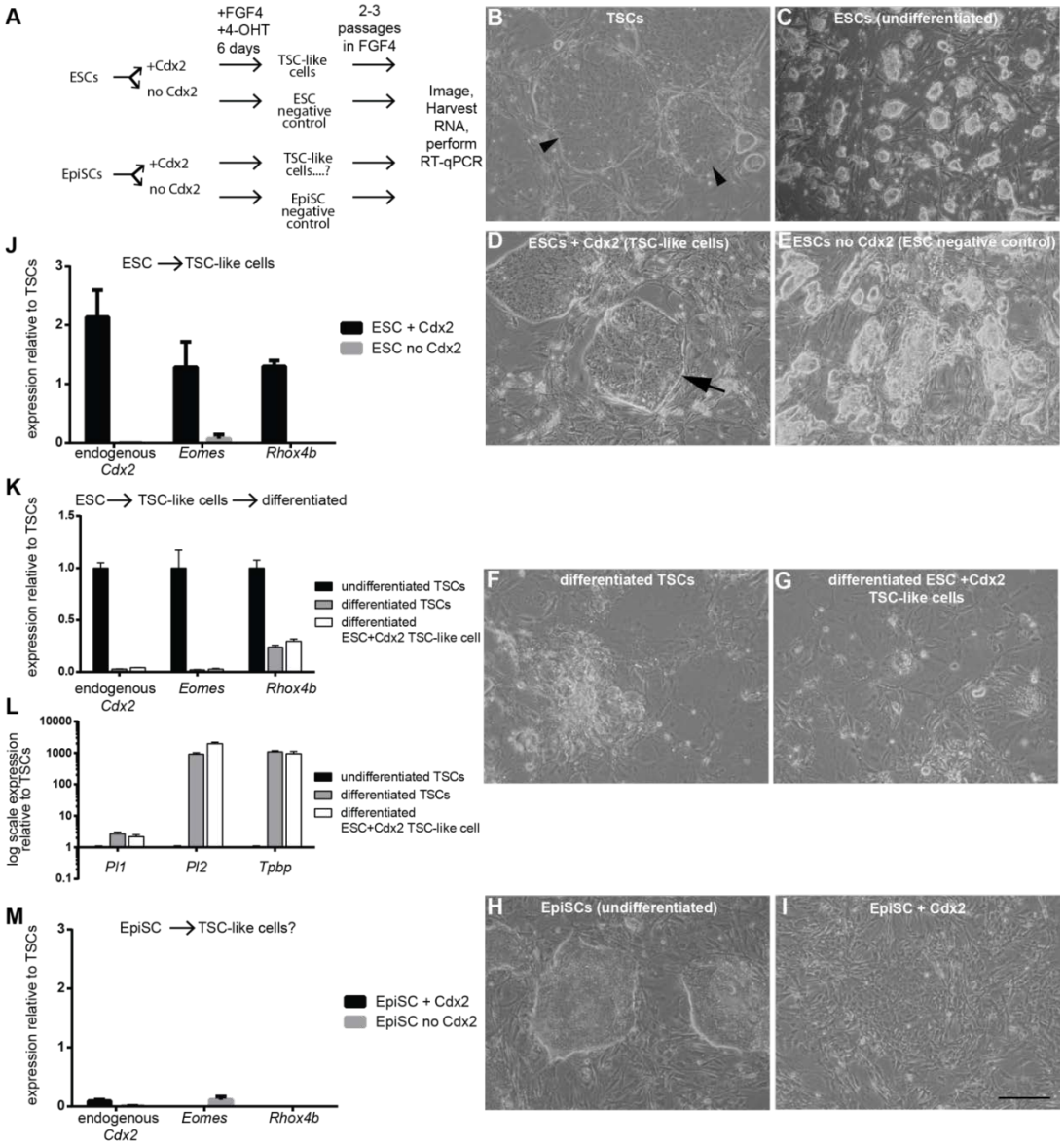


Figure 10. EpiSCs do not form TSCs following *Cdx2* overexpression. (**A**) Experimental outline of the *Cdx2* overexpression assay. (**B**) Typical morphology of TSCs in TSC medium (with FGF4/Hep) (arrowheads, colonies). (**C**) Typical ESC morphology, (**D**) TSCs derived from ESCs after *Cdx2* overexpression for 6 days in TSC medium (arrow, TSC colony). (**E**) ESCs lacking *Cdx2* cultured in TSC medium for 6 days do not form TSCs. (**F**) TSCs differentiated in the absence of FGF4/Hep for 7 days form giant cells. (**G**) ESC-derived TSCs differentiated in the absence of FGF4/Hep for 7 days form giant cells. (**H**) Typical morphology of EpiSC colonies. (**I**) EpiSCs overexpressing *Cdx2* for 6 days in TSC medium (with FGF4/Hep). (**J**) qRT-PCR determination of expression levels of TSC markers *Cdx2*

(endogenous), *Eomes*, and *Rhox4b* in indicated cell lines following 6 days of *Cdx2* overexpression in TSC medium. (**K**) Expression levels of TSC markers *Cdx2* (endogenous), *Eomes*, and *Rhox4b* in TSCs, 7 day differentiated TSCs, and 7 day differentiated ESC-derived TSCs (**L**) Expression levels of markers of differentiated trophoblast *Prl3d1*, *Prl3b1*, and *Tpbpa* in indicated cell lines after 7 days of differentiation, (**M**) Expression levels of TSC markers *Cdx2* (endogenous), *Eomes*, and *Rhox4b* in EpiSC and a representative EpiSC+*Cdx2ER* subclone after overexpression of *Cdx2* for 6 days in TSC medium. All images are displayed at same magnification, scale bar = 500 μm. qRT-PCR values are all normalized to *Hprt1* and displayed relative to expression levels in TSCs, error bars = standard error among three qPCR replicates.

ECCs generate cells with TSC properties following *Cdx2*-overexpression

ECCs are derived from germ cell tumors but can contribute to development in chimeras (Andrews, 2002; Blelloch et al., 2004; Mintz and Illmensee, 1975; Papaioannou et al., 1975), suggesting that ECCs and ESCs are comparable in terms of developmental potential. To determine whether ESCs and ECCs are similar in developmental potential, we evaluated the ability of ECCs to give rise to TSCs following *Cdx2* overexpression in TSC medium. As described above, we introduced the *Cdx2ER* overexpression construct, selected for ECC subclones stably expressing *Cdx2ER* (Fig. 18), and then attempted to derive TSCs using the *Cdx2*-overexpression assay.

The morphology of unmanipulated ECCs differs from TSCs (Fig. 11A versus 10B). After the *Cdx2*-overexpression assay ECC subclones were morphologically similar to TSCs (Fig. 11B) (6/6 subclones), while ECCs cultured in TSC conditions without overexpressed *Cdx2* failed to adopt TSC morphology (Fig. 11C). In addition, TSC genes were upregulated in ECCs following *Cdx2* overexpression but not in control cells (6/6 subclones; Fig. 11G), indicating that ECCs have a similar

developmental potential as ESCs. To confirm their TSC properties, ECC-derived TSCs were differentiated as described above. ECC-derived TSCs underwent differentiation following withdrawal of Fgf4/Hep and MEFs, although the rate of ECC-derived TSC differentiation was slower than the rate of TSC differentiation (Fig. 11E,F,H,I). While TSCs have completely lost their epithelial characteristics and adopted giant cell morphology by 7 days of differentiation, giant cells were not visible in ECC-derived TSC cultures, and cells maintained a basically epithelial appearance, although cell-cell junctions appeared somewhat loosened (Fig. 11E). After 7 days of differentiation, TSC genes were downregulated (Fig. 11H), but markers of differentiated trophoblast *Prl3d1*, *Prl3b1*, and *Tpbpa* were not yet fully upregulated in ECC-derived TSCs (Fig. 11I). By 14 days of differentiation, ECC-derived TSCs acquired differentiated TSC morphology (2/2 clones Fig. 11F), and upregulated *Prl3d1* and *Tpbpa*, but not *Prl3b1* (2/2 clones Fig. 11I). These data indicate that ECCs and ESCs respond to *Cdx2*-overexpression and TSC culture conditions by generating TSCs, and suggest that ESCs and ECCs are equivalent in terms of developmental potential. Moreover, these observations indicate that the ability to generate TSCs in response to ectopic *Cdx2* is not unique to ESCs, but is also a property of pluripotent cells of non-blastocyst origin.

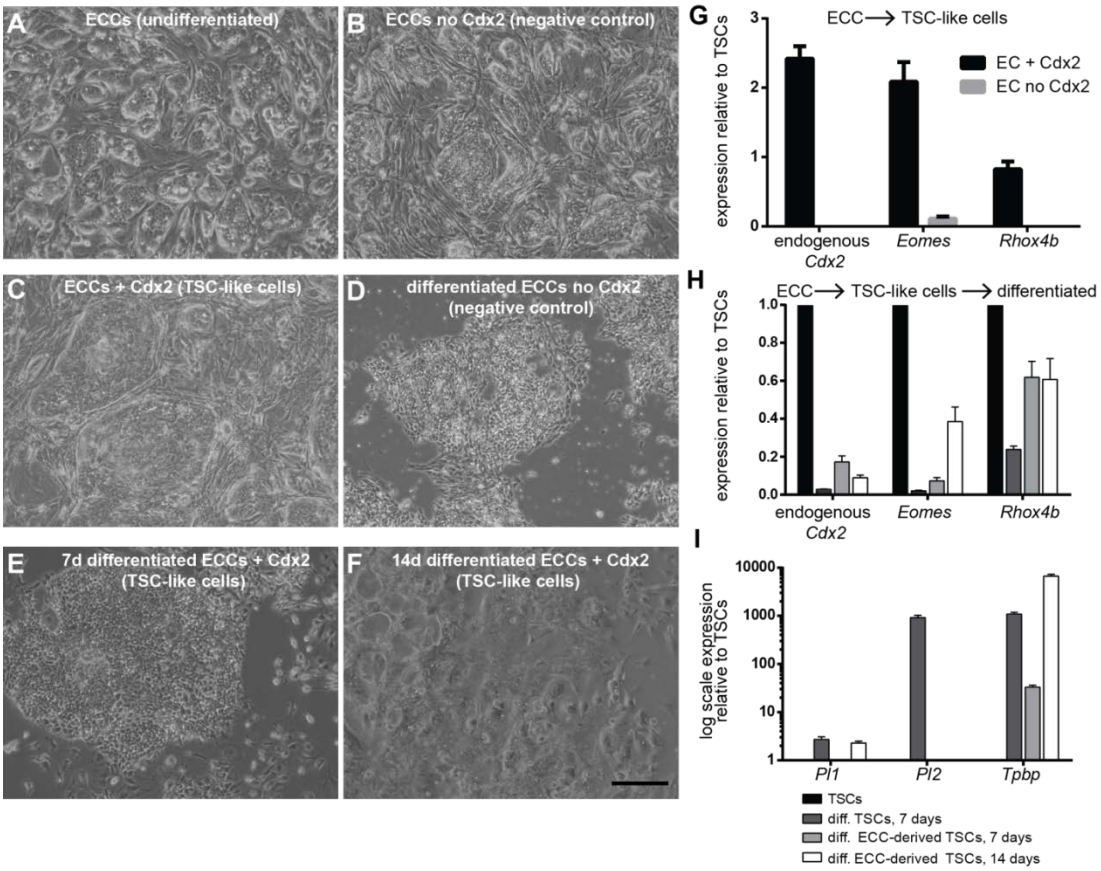


Figure 11. ECCs form TSCs efficiently upon *Cdx2* overexpression (**A**) Typical ECC morphology, (**B**) control ECCs lacking *Cdx2*, after *Cdx2* overexpression assay (**C**) TSCs derived from ECCs after *Cdx2* overexpression assay, (**D**) control ECCs lacking *Cdx2* (panel B), subsequently cultured for *7* days in the absence of FGF4/Hep, (**E**) ECC-derived TSCs (panel C) after *7* days differentiation in the absence of FGF4/Hep, (**F**) 14 day differentiated ECC-derived TSCs (**G**) q-RTPCR measurement of expression levels of TSC markers *Cdx2* (endogenous), *Eomes*, and *Rhox4b* in indicated cell line after *Cdx2* overexpression assay (**H**) Expression levels of TSC markers *Cdx2* (endogenous), *Eomes*, and *Rhox4b* in indicated cell Which lines after 7 and 14 days of differentiation. (**I**) Expression levels of differentiated trophoblast genes *Prl3d1*, *Prl3b1,* and *Tpbpa* in indicated cell lines after 7 and 14 days of differentiation. All images are displayed at same magnification, scale bar = 500 µm. qRT-PCR values are all normalized to *Hprt1* and displayed relative to expression levels in TSCs, error bars = standard error among three qPCR replicates.

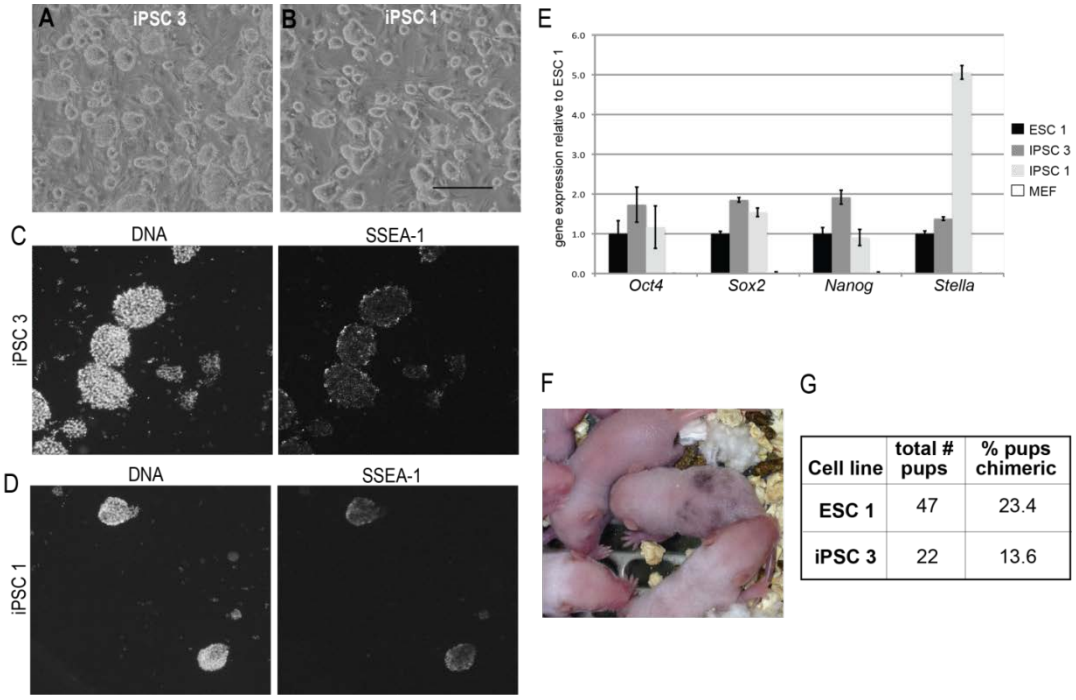
The ability to form TSCs varies among IPSCs

The inability of EpiSCs to generate TSCs following *Cdx2* over-expression in TSC conditions indicates that pluripotent stem cells can differ dramatically in their developmental potential. Given that EpiSCs and ESCs are derived from the embryo at different developmental stages, our results suggested that pluripotent stem cell origins could limit a cell line's competence to respond to TSC factors. On the other hand, the fact that TSCs could be derived from ECCs, which are of germ cell origin, suggests that the origin of the pluripotent cells may not present a barrier to forming TSCs. iPSCs are thought to be very similar, if not identical to ESCs, based on gene expression and developmental potential, despite originating from more differentiated cell types (Yamanaka, 2012). We therefore hypothesized that, like ESCs, iPSCs should give rise to TSCs very robustly. To test this hypothesis, we attempted to derive TSCs from five different iPSC lines (Table 1). Three of these iPSC lines have been shown to contribute to embryonic development in chimeras (Blelloch et al., 2007; Judson et al., 2009; Woltjen et al., 2009). In addition, we generated two new iPSC lines and validated these by examining expression levels of pluripotency genes (Fig. 12A-E). We also confirmed that one of these lines could contribute to fetal development in chimeras (Fig. 12F-G). All five iPSC lines were first cultured beyond passage 11, to ensure that the lines had acquired ESC gene expression profiles (Polo et al., 2010). We introduced the *Cdx2ER* expression plasmid into each iPSC line (Fig. 18), and then attempted to derive TSC lines from each of these 27 iPSC subclones as described above.

Our analysis showed that iPSC lines differed in their ability to efficiently give rise to TSCs. One iPSC line (iPSC2) gave rise to TSCs efficiently (6/6 subclones; Fig. 13A,C,E). However, most iPSC lines did not, since they did not consistently produce cells with TSC morphology (Fig. 13B) or gene expression (Fig. 13D,E). These results were surprising, and in conjunction with the inability of EpiSCs to generate TSCs, prompted us to consider whether TSC-forming ability could be correlated with different pluripotent states. That is, iPSCs that do not generate TSCs efficiently are in a more primed state, similar to EpiSCs, while iPSCs that do generate TSCs are in a more naïve state, similar to ESCs. To investigate this we first sought to examine the variability of TSC-like cell formation among ESC lines to determine if this variability is unique to iPSCs or if ESCs have a similar variation in TSC-forming ability.

Table 1: All cell lines used in this study

Cell line	cell type/reprogramming method	Origin	Reference	Genetic background
ESC 1	E14	Blastocyst	Nagy, et al. 1993	129/OlaHsd
ESC 2	R1	Blastocyst	Papadaki, et al. 2007	129
ESC 3	G4	Blastocyst	Hooper, et al. 1987	129/B6
EC	F9	Choriocarcinoma	Kahan, et al. 1970	129
EpiSC	EpiSC	E5.5 Epiblast	Tesar, et al. 2007	129
IPSC 1	retroviral vectors with O,K,S	MEF	see methods & figure 12	129/B6
IPSC 2	PiggyBac-transposon with O,K,S,C	MEF	Woltjen, et al. 2009	129
IPSC 3	lentiviral vectors with O,K,S,N	MEF	Blelloch, et al. 2007	B6/mixed
IPSC 4	O,K,S, miR-294	MEF	Judson, et al. 2009	B6/mixed
IPSC 5	retroviral vectors with O,K,S,C	MEF	see methods & figure 12	129/B6



Cell line	total # pups	% pups chimeric
ESC 1	47	23.4
iPSC 3	22	13.6

Figure 12. Validation of newly generated IPSCs (A-B) Phase contrast images showing morphology of A) IPSC 3 and B) IPSC 1. (C-D) Immunofluorescent images showing DNA and SSEA-1 staining of C) IPSC 3 and D) IPSC 1. (E) Expression level of pluripotency genes measured by qRT-PCR in parental MEF lines, IPSCs, and ESC 1. (F) Chimeric pups generated by injection of IPSC 3 cells into blastocysts (G) Percentage of chimeric pups born after injection of either ESC 1 or IPSC 3 cells. qRT-PCR values are all normalized to *Hprt1*, error bars = standard error among three qPCR replicates.

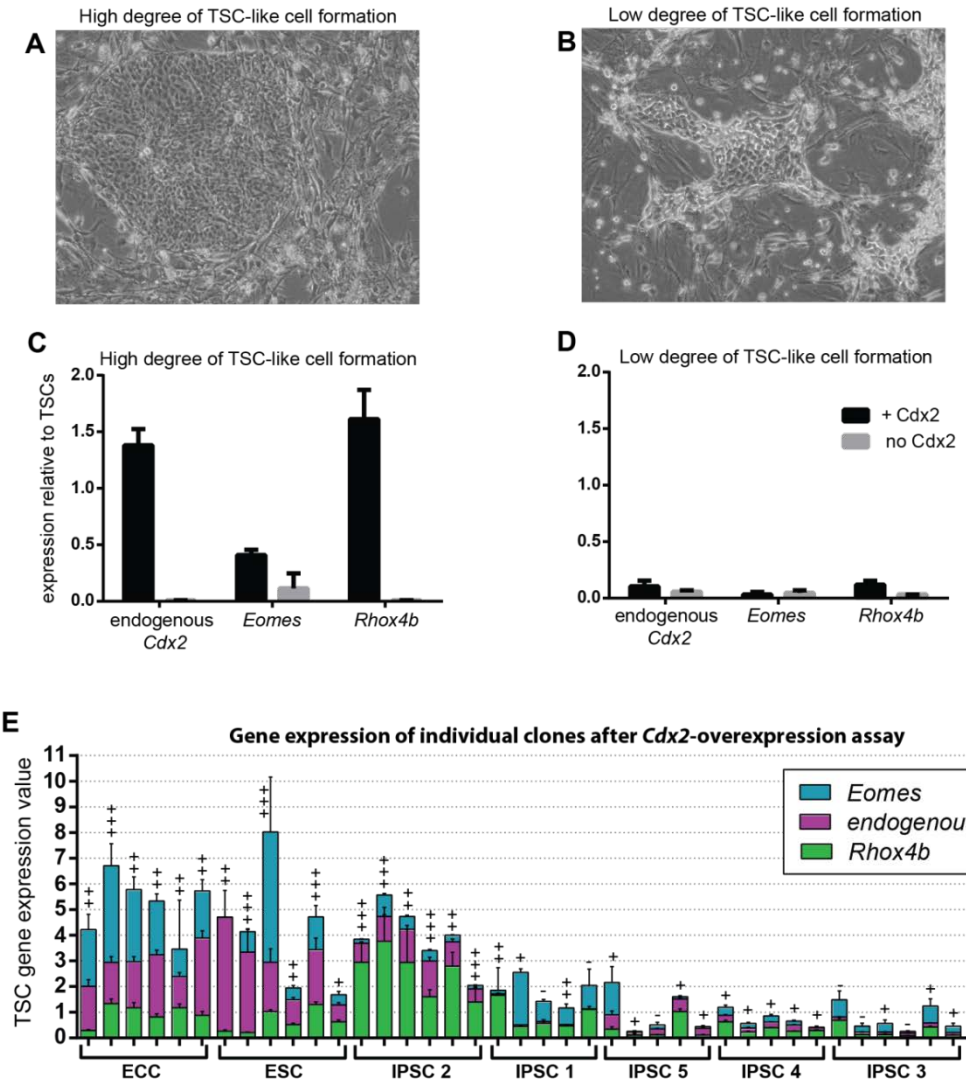


Figure 13. iPSCs give rise to TSCs with variable efficiency. (**A-B**) The morphology of one representative iPSC subclone after the *Cdx2*-overexpression assay for A) iPSC2 cell line that generated TSCs efficiently and B) iPSC5 which did not generate TSCs (**C-D**) qPCR determination of TSC gene expression levels relative to *Hprt1* of one representative iPSC subclone after the *Cdx2*-overexpression assay for C) iPSC2 and D) iPSC5 (**E**) TSC gene expression value, presented as a stacked measurement of *Eomes*, *Rhox4b*, and endogenous *Cdx2* expression levels normalized to TSCs (TSCs = 3.0), for all tested subclones for ESC, EpiSC, EC, and iPSCs. Corresponding TSC morphology for each subclone is indicated above the columns, where +++ indicates a high degree of TSC morphology and – indicates no TSC morphology. Scale bar = 500 µm , Error bars = standard error among qPCR replicates

The ability to generate TSCs varies among ESCs

We noted that the efficiency of TSC derivation from iPSCs was highly variable, but it was not yet clear whether ESCs might be similarly variable if we were to examine additional ESC lines. We therefore introduced the *Cdx2*-overexpression plasmid into two additional ESC lines (ESC 2 and ESC3; Table 1). We derived five subclones from each of these two ESCs, and then evaluated the ability of these 10 *Cdx2*-overexpressing subclones to give rise to TSCs. We were able to derive cells with TSC morphology from ESC2 and ESC3, although the efficiency of TSC derivation and the quality and consistency of TSC morphology among subclones was less than it had been with ESC1 (Fig. 14A). The average TSC gene expression levels for ESC2 and ESC3 cell lines were higher than for iPSC 1, 3, 4, and 5 (Fig. 14B). However, the average TSC gene expression was not as high among ESC2 and ESC3 as it had been for ESC1 or ECC (Fig. 14B), consistent with less efficient formation of TSCs. These data demonstrate that the ability to form TSC varies among both ESCs and iPSCs. We also observed that cell lines generating TSCs most efficiently were from a 129 background, while an intermediate TSC-forming efficiency was observed for cell lines with partial 129 genetic backgrounds, and those generating TSCs least efficiently were not 129 (Fig. 14C). This suggests that genetic background influences the ability of pluripotent stem cell lines to generate TSCs in response to *Cdx2* overexpression, with 129 being a more permissive genetic background.

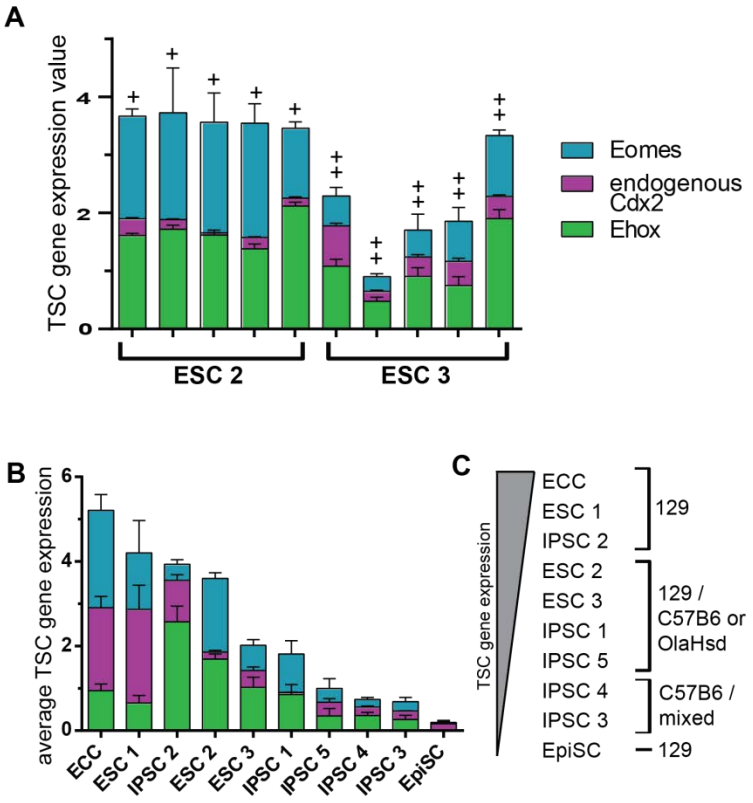


Figure 14. The ability to generate TSCs varies among ESC lines and is influenced by genetic background (**A**) qPCR determination of TSC gene expression levels, relative to *Hprt1*, for each cell line indicated after 6 days *Cdx2* overexpression in TSC medium. Corresponding TSC morphology is indicated above the columns, where +++ indicates a high degree of TSC morphology and - indicates no TSC morphology, error bars= standard error among qPCR replicates (**B**) The average TSC gene expression value of all subclones for each cell line, error bars= standard error among all subclone measurements (**C**) Genetic background of all cell lines that are listed from greatest to least expression of TSC genes

Cdx2 overexpression induces expression of non-TSC genes

One property of pluripotent stem cells that is known to vary among cell types and in different culture conditions is lineage priming (Ariasi and Brickman, 2011). Lineage priming involves the expression of both stem cell genes and lineage-specific

genes and occurs in many types of mammalian stem cells. The expression of lineage genes is thought to reflect a stem cell state which is primed for differentiation. Both human ESCs and mouse EpiSCs display increased lineage priming of endoderm and mesoderm genes relative to mouse ESCs (Tesar et al., 2007), and ESCs cultured in serum have increased lineage priming relative to those cultured in 2i (Marks et al., 2012). Although the first developmental role of Cdx2 is to drive expression of trophoblast genes (Ralston and Rossant, 2008; Strumpf et al., 2005), Cdx2 is also required for hindgut endoderm and posterior/tailbud mesoderm fates beginning at E8.5 (Beck et al., 2003; Chawengsaksophak et al., 2004; Chawengsaksophak et al., 1997; Guo et al., 2004). Since EpiSCs undergo mesoderm and endoderm lineage priming and Cdx2 can drive mesoderm and endoderm fates, we hypothesized that *Cdx2* over-expression in EpiSCs may drive expression of mesoderm and endoderm genes rather than TSC genes. To test this we first verified that the EpiSCs displayed higher levels of lineage priming than ES/IPSCs. The expression level of four lineage-associated genes (*Gata6*, *Sox17*, *Foxa2*, and *T*), which have been demonstrated to be differentially expressed between EpiSCs and ESCs (Tesar et al., 2007), was examined in three cell lines: EpiSCs, ESC1, and iPSC3, which varied greatly in their TSC-forming potential. EpiSCs expressed higher levels of these lineage-associated genes than ESCs and IPSCs, consistent with increased lineage priming. In contrast, the expression level of these genes did not differ between ESC1 and iPSC3 (Fig. 15A), indicating that there are not major differences in lineage priming among the ESC and IPSC lines tested.

To test whether mesoderm and endoderm genes are preferentially activated in EpiSCs upon *Cdx2*-overexpression, we examined the expression levels of

endoderm, mesoderm, and ectoderm germ layer markers as well as *Hoxb9* and *Isx*, which are regulated by CDX2 directly (Boyd et al., 2010; Choi et al., 2006; van den Akker et al., 2002). Gene expression was measured following Cdx2 overexpression, in the same four cell lines as above. This analysis showed that many EpiSC subclones upregulated the mesoderm markers *Meox1* and *Hoxb9* (a direct CDX2 target) relative to treated parental cells. However, none of the 3 endoderm markers tested was upregulated (Fig. 15B). Interestingly, ESC1 subclones also upregulated *Hoxb9* to a similar degree as EpiSC subclones (Fig. 15B). These data indicate that Cdx2 can direct expression of non-TSC target genes, and this occurs regardless of naïve or primed pluripotent state. These results also indicate that the expression of non-TSC genes does not interfere with the formation of TSCs since ESCs can still generate TSCs. In contrast to EpiSCs and ESC1, iPSC3 did not upregulate *Meox1* or *Hoxb9*. However, 1-2 iPSC3 subclones did upregulate *T* and *Sox1*. In addition, several iPSC3 subclones upregulated *Sox17*, *Foxa2*, and the CDX2 target *Isx* relative to the treated parental control, suggesting this cell line has a bias toward endoderm gene expression. In many iPSC3 subclones *Gata6* was upregulated. However, the treated parental controls expressed similar levels, indicating that this is not a result of *Cdx*2 overexpression. Taken together these data demonstrate that EpiSCs, which are in a primed pluripotent state, are not more likely to activate mesoderm and endoderm genes than other non-primed ESC/iPSC lines in this assay. In addition, these data indicate that although germ layer markers and non-TSC CDX2 target genes can be induced in the *Cdx2*-overexpression assay, there is no clear differentiation to an alternative lineage and expression of non-TSC genes does not interfere with the formation of TSCs.

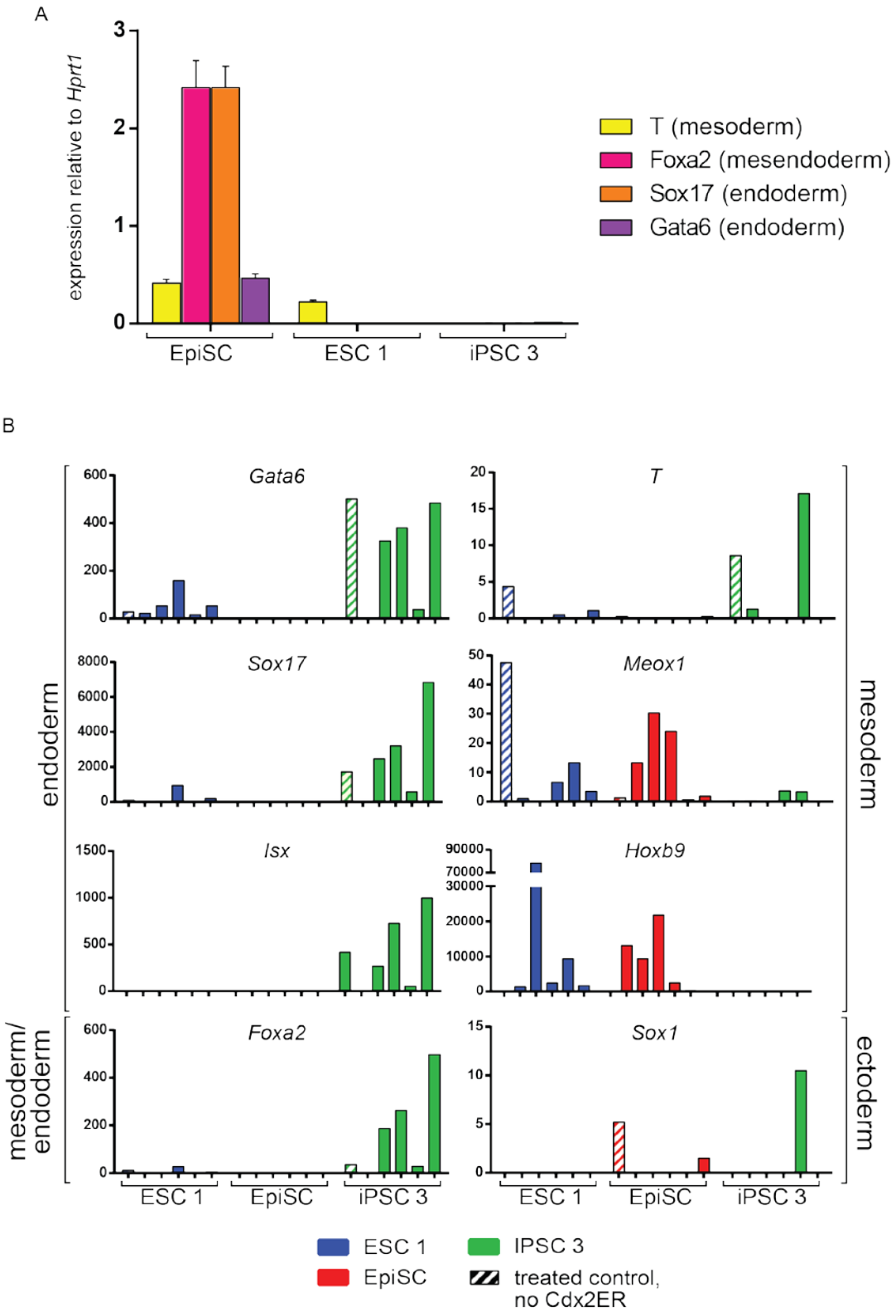

A
expression relative to Hprt1
3
2
1
0
EpiSC
ESC 1
iPSC 3
T (mesoderm)
Foxa2 (mesendoderm)
Sox17 (endoderm)
Gata6 (endoderm)
B
Gata6
T
Sox17
Meox1
Isx
Hoxb9
Foxa2
Sox1
endoderm
mesoderm
mesoderm/
endoderm
ectoderm
ESC 1
EpiSC
iPSC 3
ESC 1
EpiSC
iPSC 3
ESC 1
EpiSC
IPSC 3
treated control,
no Cdx2ER

Figure 15. Expression of non-TSC CDX2 target genes does not prevent TSC formation. (**A**) qRT-PCR determination of gene expression values of *T*, *Gata6*, *Foxa2*, and *Sox17* relative to *Hprt1* in EpiSCs, ESC 1, and IPSC 5. (**B**) qRT-PCR determination of germ layer gene expression values relative to *Hprt1* for subclones and respective treated parental controls for 3 cell lines after the *Cdx2*-overexpression assay. All values are normalized to the expression level of the respective untreated parental cell line.

TSC gene expression values are inversely correlated with *Myc* expression levels

In addition to differences in lineage priming, pluripotent stem cell lines also exhibit differences in their expression level of pluripotency genes depending on the cell type and culture conditions (Brons et al., 2007; Newman and Cooper, 2010; Tesar et al., 2007; Wray et al., 2011). We sought to examine whether the observed variation in the efficiency of TSC formation is correlated with the expression level of pluripotentcy genes. If the expression level of a gene correlates with TSC-forming potential, this gene could be used as a way to predict a cell line's developmental potential and could suggest a potential research avenue to study the underlying cause of the observed variation in TSC-forming potential. We measured the expression levels of twelve pluripotency genes by qPCR in the parental pluripotent cell lines, and then examined the correlation between this value and the average total TSC gene expression values resulting from the *Cdx2*-overexpression assay (Fig. 16). We observed no strong correlation between the average TSC gene expression value for each pluripotent stem cell line examined and the expression level of most pluripotency genes, with the exception of *Myc*. *Myc* levels are strongly (r=-0.70) inversely correlated with TSC gene expression values (Fig. 16). *Myc* levels are known to be lower in naïve pluripotent stem cells than more primed pluripotent stem

cells. This observation suggests that a pluripotent stem cell line's ability to form TSCs may reflect its degree of naïve pluripotency, as shown by the level of *Myc*.

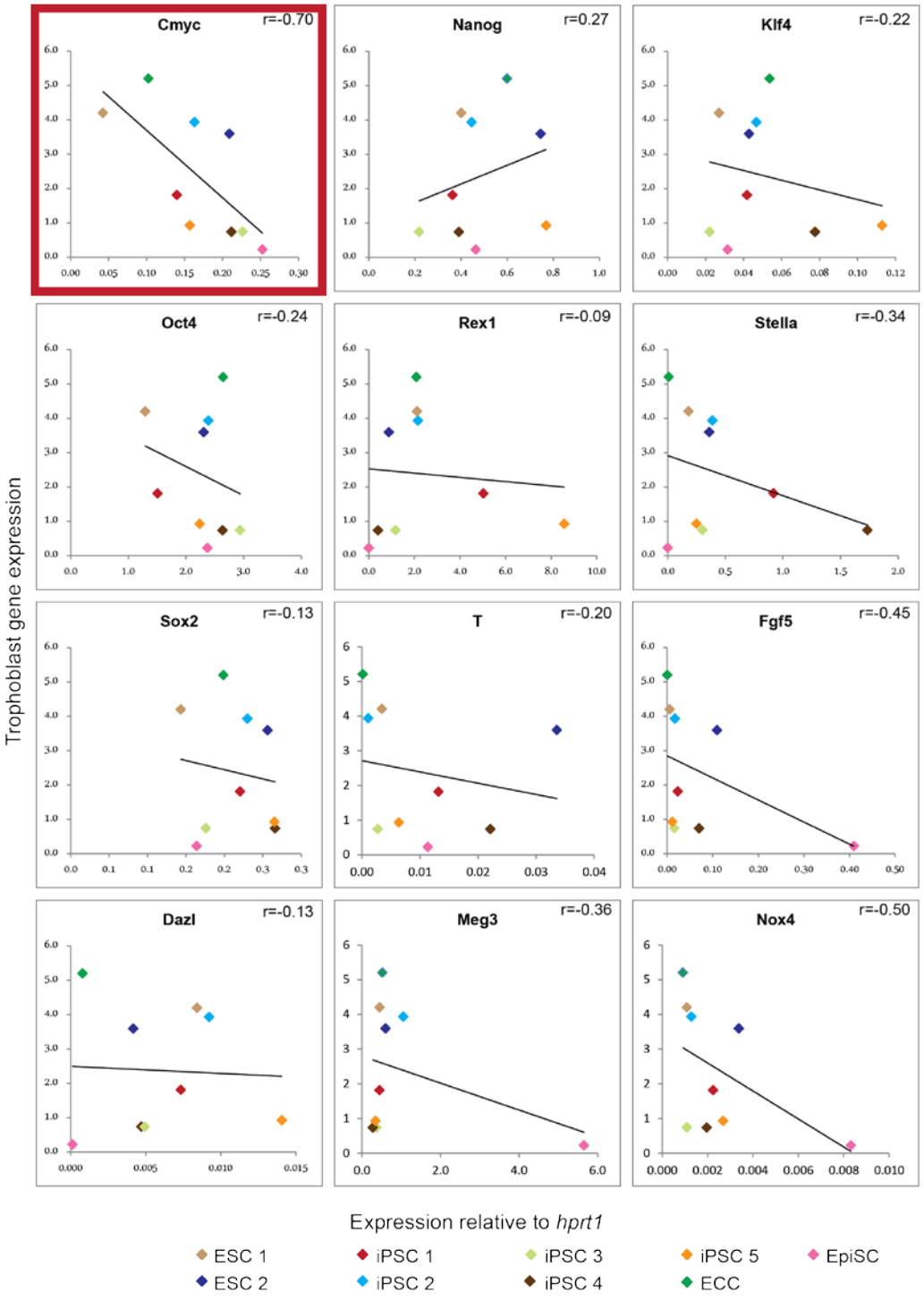

Cmyc r=-0.70
Nanog r=0.27
Klf4 r=-0.22
Oct4 r=-0.24
Rex1 r=-0.09
Stella r=-0.34
Sox2 r=-0.13
T r=-0.20
Fgf5 r=-0.45
Dazl r=-0.13
Meg3 r=-0.36
Nox4 r=-0.50
Trophoblast gene expression
Expression relative to hprt1
ESC 1
ESC 2
iPSC 1
iPSC 2
iPSC 3
iPSC 4
iPSC 5
ECC
EpiSC

Figure 16. TSC-gene expression values inversely correlates with naïve pluripotentcy gene expression levels in parental cell lines. Average TSC gene expression values relative to *Hprt1* for all pluripotent stem cell lines used in this study (Table 1), except ESC3 as this cell line was established on the basis of *Hprt1* deficiency (Hooper et al., 1987), are shown in relation to the average expression level for the indicated pluripotency genes relative to *Hprt1*. The degree correlation (r value) was calculated using Pearson's correlation.

Pushing cells to a more naïve pluripotent state improves TSC-formation efficiency

We showed that *Myc* expression levels in pluripotent stem cells are inversely correlated with their ability to form TSCs, meaning that cell lines with higher *Myc* levels were unable to efficiently generate TSCs and vice versa. Since culturing pluripotent stem cell lines in inhibitors of GSK3 and MAPK (2i) pushes cells into a more naïve pluripotent state (Marks et al., 2012; Ying et al., 2008), we hypothesized that culturing cell lines in 2i prior to the *Cdx2*-overexpression assay would increase the efficiency with which they formed TSCs. To test this hypothesis, we cultured subclones from 5 cell lines (ESC2, ESC3, iPSC3, iPSC4, iPSC5) in 2i for 7 passages, which is sufficient to transition ESCs to a more naïve state (Marks et al., 2012). We first confirmed that the 2i treatment was effective by confirming decreased *Myc* expression levels and increased *Tert* and *Dazl* expression levels in these cell lines as previously reported (Marks et al., 2012) (Fig. 17A). We then repeated the *Cdx2*-overexpression assay in these cell lines to determine whether TSC-forming ability, judged on the basis of TSC gene expression, was improved by the 2i treatment. 2/4 ESC3 subclones had increased TSC gene expression after pretreatment with 2i (Fig. 17B), while 4/4 ESC2 subclones had increased TSC gene expression (Fig. 17C). 4/6 iPSC3 subclones and 0/4 iPSC4 subclones had

increased TSC gene expression after 2i pre-treatment (Fig. 17D-E). Surprisingly, iPSC5 subclones pre-treated with 2i were unable to survive the *Cdx2*-overexpression assay and therefore could not be evaluated. These data show that the ability to form TSCs can be enhanced in some, but not all, pluripotent cell lines by first culturing cells in 2i. The variety of responses to 2i pre-treatment among cell lines also highlights the complex nature of pluripotency regulation and illustrates that not all cell lines can respond to 2i treatment in the same manner. The improvement in TSC-forming efficiency in several lines supports our hypothesis that TSC formation is influenced by pluripotent stem cell naivete. However, the efficiency of TSC formation in some cell lines was not improved after 2i treatment, indicating that naivete is not the only factor affecting to TSC formation.

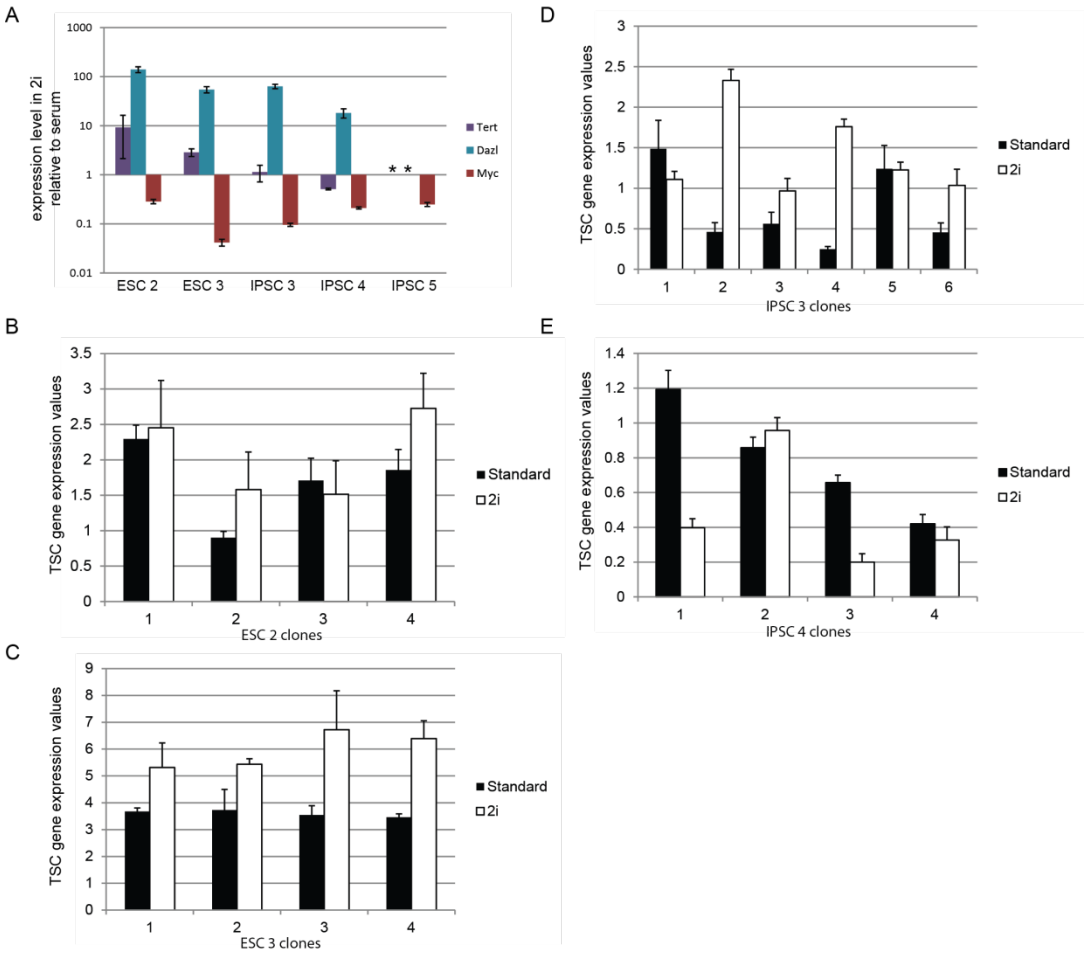


Figure 17. Treatment in 2i increases TSC gene expression levels following *Cdx2* overexpression assay. (**A**) qRT-PCR determination of *Tert*, *Dazl*, and *Myc* expression levels relative to *Hprt1* in the indicated cell lines after 7 passages in 2i and in standard ESC culture conditions, astricks indicate data not gathered. (**B-E**) TSC gene expression values (sum of *Eomes*, *Rhox4b*, endogenous *Cdx2* transcript levels) of subclones cultured in standard ESC conditions or cultured in 2i for 7 passages prior to the *Cdx2*-overexpression assay for (B) ESC 2 (C) ESC 3 (D) iPSC 3 (E) iPSC 4. Error bars = standard error among qPCR replicates.

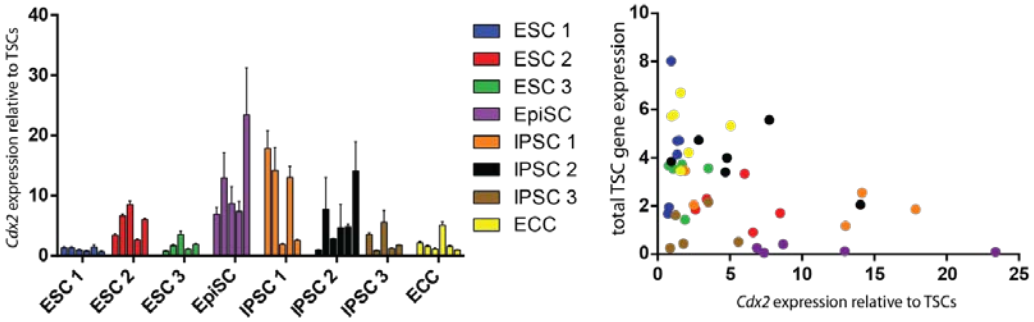


Figure 18. Cdx2 levels in undifferentiated pluripotent stem cell clones Cdx2 levels were measured by qRT-PCR in undifferentiated ESC subclones after transfection with *Cdx2ER* plasmid and normalized to *Hprt1*. *Cdx2* expression levels are displayed in comparison to total TSC gene expression after *Cdx2*-overexpression assay. Error bars= standard error among qPCR replicates.

Discussion

Characterizing the differences in developmental potential of pluripotent stem cells and deciphering the mechanisms leading to these differences is critical for efficient, predictable differentiation of pluripotent stem cells in both basic research and regenerative medicine. Many studies have shown that differences in developmental potential exist among pluripotent cell lines, yet the underlying reasons for these differences are not clear. Here we measured developmental potential using a TSC-forming assay and explored several possible causes of differences in efficiency of TSC formation, including stem cell origin, pluripotency gene expression, and primed vs. naïve pluripotent states. We examined TSC forming potential in ESCs, IPSCs, EpiSCs, and ECCs, and found that EpiSCs were the only cell type that did not generate TSCs. EpiSCs originate from the post-implantation epiblast and represent a later developmental timepoint than the other cell lines examined. EpiSCs are derived from the embryo several days after the specification of

extraembryonic lineages and therefore may no longer have the ability to generate the stem cells from these lineages. Consistent with this hypothesis previous work has shown that ESCs can give rise to XEN cells, but EpiSCs cannot (Cho et al., 2012). ESCs and IPSCs both have similarities to the preimplantation epiblast, while ECCs are derived from germ line tumors. ECCs can contribute to all lineages in chimera assays while EpiSCs do not contribute to development, suggesting that ECCs are more similar to ESC/IPSCs than EpiSCs. The ability of ECCs to give rise to TSCs further supports the idea that they are in a similar pluripotent state as ESCs.

Our data also demonstrate that ES/IPSCs derived from a 129 genetic background form TSC-like cells more efficiently than those derived from partial and non-129 backgrounds. This may reflect differences in the molecular state of pluripotent stem cells derived from a 129 background. This idea is supported by previous data demonstrating that the derivation of ESCs from embryos is much more efficient in a 129 background than in other genetic backgrounds. The importance of genetic background specific effects during development has been demonstrated by many groups. Analysis of mutant alleles on different genetic backgrounds has demonstrated that genetic background can have dramatic effects on the penetrance of many phenotypes from placental development to diabetes to neurological disorders (Kraut et al., 1998; Holmes et al., 2003; Jaquemar et al., 2003; Kulkarni et al., 2003; Lloret et al., 2006; Dackor et al., 2007). Our data indicates that genetic background can also influence the behavior of pluripotent stem cell differentiation. As humans have much greated genetic diversity than inbred than mouse lab strains, it is highly likely that genetic background will also have an influence on the differentiation potential of human embryonic stem cells.

We also observed a relationship between naïve pluripotency and TSC-forming potential, suggesting that the pluripotent state can impact the developmental potential of a cell line. Interestingly, in ESCs a unique relationship between the *Oct4* expression level and extraembryonic differentiation has been observed. ESC lines in which *Oct4* expression level can be tightly controlled through tetracycline responsive promoters were used to show that reduced *Oct4* expression in ESCs leads to trophoblast differentiation and increased *Oct4* expression leads to primitive endoderm differentiation (Niwa et al., 2000). This was one of the earliest studies in ESCs showing that pluripotency gene expression level can directly affect ESC differentiation. A mutually repressive relationship between *Oct4* and *Cdx2* in ESCs has also been demonstrated (Niwa et al., 2005). *Oct4* directly represses *Cdx2* in ESCs, and reduced *Oct4* levels allow *Cdx2* to be expressed and activate TSC genes, while *Cdx2* overexpression in ESCs leads to downregulation of *Oct4* (Niwa et al., 2005). These studies established that differentiation to extraembryonic lineages can be subject to the level of pluripotentcy gene expression. However, these studies did not address variation in pluripotency gene expression among cell lines.

Although we observed variation in *Oct4* levels among our ESC lines (values were between 1.5-2.5 relative to *Hprt*), this variation among wild type ESC/iPSCs was most likely not responsible for the observed variation in TSC-forming efficiency as *Oct4* levels were not correlated to the degree of TSC differentiation. However, we found that TSC differentiation ability was inversely correlated to *Myc* levels, which are know to be low in more naïve pluripotent stem cells (Marks et al., 2012). This finding suggests that increased expression of primed pluripotency genes reduces the efficiency of TSC formation. In support of this we found that pushing cells to a more

naïve pluripotent state could increase TSC forming efficiency in some cell lines. Interestingly, the ability of ESCs to form XEN cells was not altered when ESCs were first cultured in 2i compared to culture in standard serum conditions (Cho et al., 2012). In fact, the ability of ESCs to differentiate into most lineages *in vitro* is not affected by first culturing in 2i (Marks et al., 2012), making the enhancement of TSC differentiation in response to 2i a unique finding. The reason underlying the enhanced TSC-forming potential in more naïve pluripotent stem cells is not entirely clear. However, previous work has shown that culture in 2i causes ESCs to upregulate many genes known to be expressed in the ICM (Marks et al., 2012). Many of these same genes are downregulated when ICM cells are put into standard medium (Tang, 2010). These findings led to the idea that 2i brings ESCs into an earlier, more naïve, developmental state that is more similar to the early ICM of the embryo rather than the more developed epiblast. More naive ESCs have been pushed closer to very early developmental stages, closer to when cells are still totipotent, and totipotent cells are capable of generating all extraembryonic cell types. Our findings support the idea that more naïve pluripotent states have a greater potential to generate extraembryonic cell types. Furthermore, this study demonstrates how evaluation of a cell line's full developmental potential can reveal novel aspects about regulation of the pluripotent state.

CHAPTER 4 – Hippo regulated *Sox2* is required for normal primitive endoderm specification and maintenance of the ICM

Abstract

Embryonic stem cells are derived from the pluripotent epiblast (EPI) lineage after blastocyst formation. Therefore, understanding the molecular events that lead to segregation of EPI from differentiating trophectoderm (TE) and primitive endoderm (PE) cells during blastocyst formation is essential for understanding how to create and use pluripotent stem cells. In embryonic stem (ES) cells, the transcription factor Sox2 is essential for pluripotency, and regulates expression of pluripotency genes such as *Oct4* and *Nanog*. During development, zygotic (Z) *Sox2* is thought to maintain development of the EPI postimplantation. However, several lines of evidence have suggested that maternal (M) *Sox2* acts earlier in development, promoting blastocyst formation by specifying TE fate. We show here using null alleles that neither M nor Z *Sox2* is required for formation of the blastocyst, nor for expression of TE genes such as *Cdx2,* nor pluripotency genes such as *Oct4* and *Nanog*. We show that SOX2 protein is first detected in ICM progenitors at the 16-cell stage, and that this pattern is regulated by the HIPPO signaling pathway, and not by *Cdx2*. These results reveal that HIPPO signaling regulates the patterning of TE and ICM genes in parallel. Subsequently in the blastocyst, we show that Sox2 expression is restricted to EPI cells by FGF4/MAPK signaling, and in EPI cells, *Sox2* promotes PE development non cell-autonomously, via FGF4.

Introduction

To understand and control the growth and differentiation of pluripotent stem cells, the mechanisms to initiate and maintain the pluripotent state must be understood. The

study of individual pluripotentcy regulators in ESCs, such as Oct4, Sox2, and Nanog, has been challenging as they are required for each other's transcription and knockdown of one gene leads to downregulation of the other genes (Young, 2011). The mouse preimplantation embryo provides a unique model for studying the initiation and maintenance of pluripotentcy as the embryo is the origin of ESCs and the core pluripotency genes are expressed in the EPI. The expression of *Oct4* and *Nanog* is not thought to be as interdependent in preimplantation embryos, allowing the unique contributions of these genes to be assessed (Frum et al., 2013; Messerschmidt and Kemler, 2010). Understanding the regulation of lineage specification in the early embryo also provides insight into the earliest steps of mammalian development that set up the asymmetries responsible for the subsequent development and patterning of the embryo.

Mechanisms leading to specification of the epiblast are largely unknown. *Oct4*, *Nanog*, and *Sox2* are all required for the derivation of ESCs from the epiblast (Avilion et al., 2003; Mitsui et al., 2003; Nichols et al., 1998), however, the role of *Sox2* in the epiblast is still unclear. In recent years, careful characterizations of lineage specification in *Oct4* and *Nanog* null embryos have been performed and the phenotypes of *Oct4* and *Nanog* null embryos appear to largely affect PE development (Frankenberg et al., 2011; Frum et al., 2013; Messerschmidt and Kemler, 2010; Wu et al., 2013). *Fgf4* expression is reduced in *Nanog* mutants, leading to a non-cell autonomous loss of PE (Frankenberg et al., 2011; Messerschmidt and Kemler, 2010). *Fgf4* expression is similarly reduced in *Oct4* mutants, however there is also a cell autonomous requirement for *Oct4* in the PE (Frum et al., 2013). In ESCs, *Fgf4* expression is regulated by *Oct4*, *Nanog*, and

Sox2, suggesting that *Sox2* may also be required for normal *Fgf4* expression in the embryo.

One important step towards understanding the role of a transcription factor in development is determining where it is expressed. Although the expression of *Sox2* has been examined in several studies, there is no consensus on the *Sox2* expression pattern (Avilion et al., 2003; Guo et al., 2010; Keramari et al., 2010). Genetic knockout of *Sox2* has revealed that *Sox2* null embryos die shortly after implantation due to loss of the epiblast (Avilion et al., 2003), and RNA knockdown experiments suggest there is a role for maternal *Sox2* in TE specification (Keramari et al., 2010). However, a genetic knockout of maternal *Sox2* and detailed analysis of cell fate decisions in *Sox2* null preimplantation embryos have not been done. Previous maternal RNA knockdown experiments of *Cdx2* and *Oct4* in the preimplantation embryo have yielded phenotypes which are not consistent with maternal germline knockout phenotypes, making maternal knockouts crucial for evaluating the requirement for maternal factors in development (Blij et al., 2012; Foygel et al., 2008; Frum et al., 2013; Jedrusik et al., 2010). The mechanisms accounting for the *Sox2* phenotype have also not been explored. In this study we examined *Sox2* expression and regulation during preimplantation development and utilized a maternal knockout strategy to investigate the role of maternal *Sox2* in development. We also performed a detailed analysis of the *Sox2* null phenotype to uncover mechanisms by which *Sox2* regulates preimplantation development.

Methods & Materials

Mouse strains and genotyping

All animal research was conducted in accordance with the guidelines of the University of California Santa Cruz Institutional Animal Care and Use Committee or by RIKEN CDB and Kumamoto University. The following alleles or transgenes were used in this study and genotyped according to cited references: *Sox2*$^{tm1.1Lan}$ (Smith et al., 2009), *Tg(Zp3-cre)93Knw* (de Vries et al., 2000), *Tead4*^{tm1Hssk} (Nishioka et al., 2008), and *Cdx2*$^{tm1.1Aral}$ (Blij et al., 2012). Mice carrying the Sox2 null allele (*Sox2*$^{del+}$) were generated by crossing mice carrying *Sox2*$^{tm1.1Lan}$ with *129-Alpl*$^{tm1(cre)Nagy}$ (Lomelí et al., 2000).

Embryo collection and manipulation

Mice were maintained on a 12-hour light/dark cycle. Embryos were collected from timed natural matings by flushing dissected oviducts or uteri with M2 medium. Embryos were either fixed or cultured in KSOM (Millipore) at 37ºC and 6% CO_2. For embryos cultured in 1 µg/ml each Fgf4 recombinant human Fgf4 (R&D Systems) and Heparin (Sigma). For outgrowth assays, embryos were cultured as above until E4.5 and then transferred to individual wells of MEF-conditioned TS medium with 1 µg/mL Fgf4 and 1 U/mL Heparin. Final concentrations of Fgfr/Mapk inhibitors were 100 nM PD173074 and 500 nM PD0325901 (Stemgent).

Immunofluorescence and confocal microscopy

See chapter 2 materials & methods

Chimeras

YFP-expressing R1 ES cells (George, 2007) were cultured on MEFs in ES cell medium + 1 µM PD0325901 + 3 µM GSK3 inhibitor Chir99021 (Stemgent) (Nichols

et al., 2009). Pre-compacted 4-8 cell embryos were collected from *Sox2*$^{fl/del}$ intercrosses, zonas pellucida removed with Tyrode's Solution, and embryos aggregated with groups of 3-5 ES cells in depression wells. Aggregations were cultured in KSOM under light mineral oil at 37ºC and 6% CO_2. Chimeras were subsequently genotyped by PCR using primers that could distinguish wild type, floxed, and deleted *Sox2* alleles.

RNA isolation and cDNA preparation

See chapter 3 materials & methods

Results

SOX2 is restricted to ICM progenitors by a *Lats2/Tead4*-dependent mechanism and not by *Cdx2*

Sox2 mRNA expression begins at the 16-cell stage and is restricted to inside cells (Guo et al., 2010), making *Sox2* the earliest known factor to be restricted to inside cells, and *Sox2* mRNA remains restricted to inside cells throughout preimplantation. The expression pattern of SOX2 protein during preimplantation is still unclear, as different patterns have been reported (Avilion et al., 2003; Keramari et al., 2010), with neither pattern matching that of the mRNA. We examined SOX2 expression using immunofluorescent staining in wild type and MZ null embryos. At the 16-cell stage we found that SOX2 was detectable in the nuclei of inside cells only, while NANOG was detectable in nuclei of all cells (Fig. 19A). This pattern agrees with the mRNA expression, and this nuclear staining was absent in MZ null embryos (Fig. 19A,B). Cytoplasmic staining was seen throughout the embryo,

however, this staining was also present in MZ null embryos, demonstrating that it is non-specific, background staining. SOX2 remains restricted to the nuclei of inside cells at E3.5, and this signal was not detected in MZ null embryos (Fig. 19A,B). These data show that in contrast to OCT4 and NANOG, SOX2 is found specifically in inside cells throughout all of preimplantation development.

Next we sought to examine the regulation of *Sox2* expression. The restriction of both *Oct4* and *Nanog* expression to the ICM is dependent on CDX2 (Strumpf et al., 2005), although this restriction occurs later than the restriction of *Sox2*. *Cdx2* is expressed in all cells of the 8-cell embryo and is downregulated in inside cells at the 16-cell stage when *Sox2* expression is initiated, making it an attractive candidate for causing the inhibition of Sox2 in outside cells. To test this hypothesis we examined SOX2 expression in *Cdx2* null embryos at E3.5 and after embryo collapse due to loss of TE integrity at E4.25. Surprisingly, SOX2 was restricted to the ICM in *Cdx2* null embryos at these timepoints (Fig. 20A). The downregulation of *Cdx2* in inside cells relies on Hippo signaling through LATS1/2 kinases. Repression of LATS1/2 in outside cells allows the transcriptional co-activators YAP/TAZ to translocate to the nucleus, bind to TEAD4, and upregulate TE genes including *Cdx2* (Nishioka et al., 2009; Nishioka et al., 2008; Yagi et al., 2007). As *Cdx2* was not required for restriction of SOX2 to inside cells, we manipulated Hippo signaling components and examined the resulting SOX2 expression in outside cells. Outside cells were identified on the basis of position and lack of apical E-CADHERIN localization. In *Tead4* null embryos we found that SOX2 was expressed in ~50% of outside cells at E3.5 (Fig. 20B,C). Next we overexpressed *Lats2* via mRNA injection at the 2-cell stage, while *B-globin* mRNA injected embryos served as controls (Fig. 20D). Loss of

nuclear YAP in outside cells was observed upon *Lats2* overexpression, indicating successful activation of Hippo signaling in outside cells, and SOX2 was found in >80% of *Lats2*-injected outside cells but was absent in *B-globin* injected outside cells (Fig. 20 C,E). Together these data indicate that TEAD4 and LATS2 regulate SOX2 expression, and this regulation is independent of *Cdx2*.

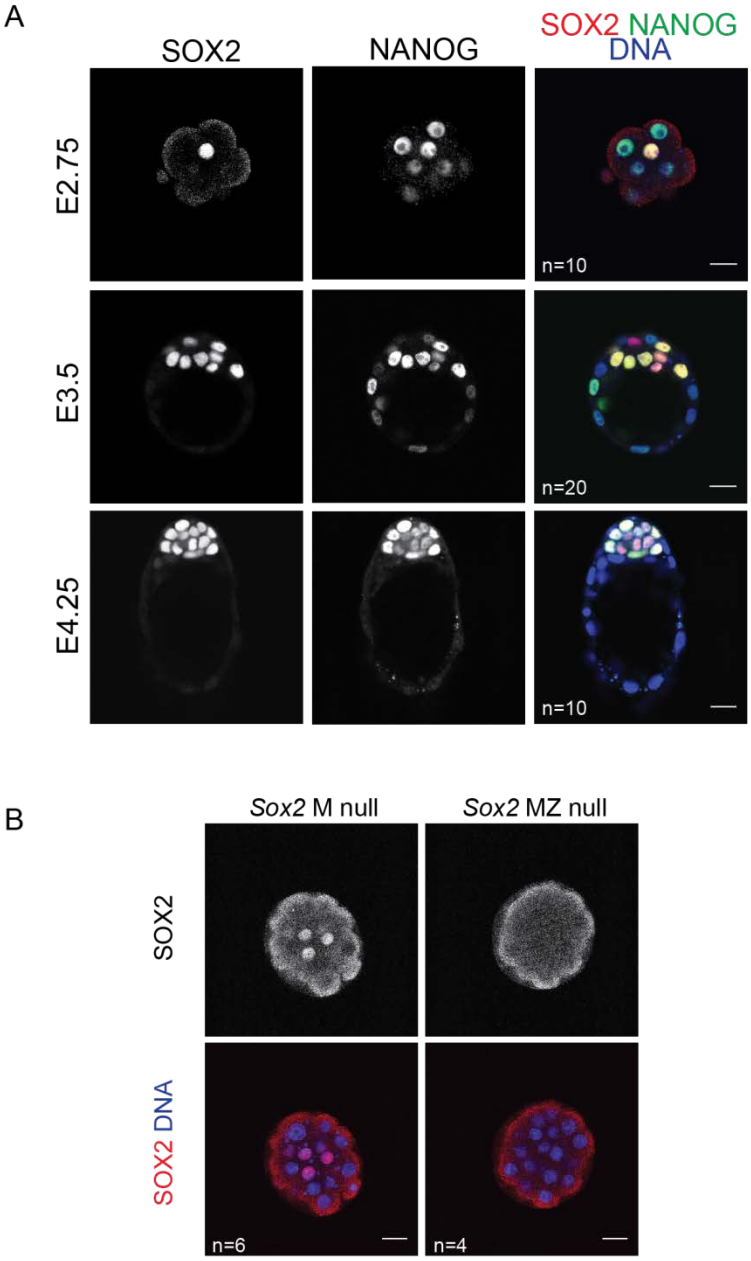


Figure 19. SOX2 is the earliest marker of the embryonic lineage (A) SOX2 is detectable specifically in inside cells beginning at the 16-cell stage (E2.75). SOX2 expression remains restricted to the ICM throughout preimplantation and at E4.25 is detectable only in EPI cells. (B) SOX2 nuclear staining is not detected in *Sox2* MZ null embryos, while the non-nuclear staining remains.

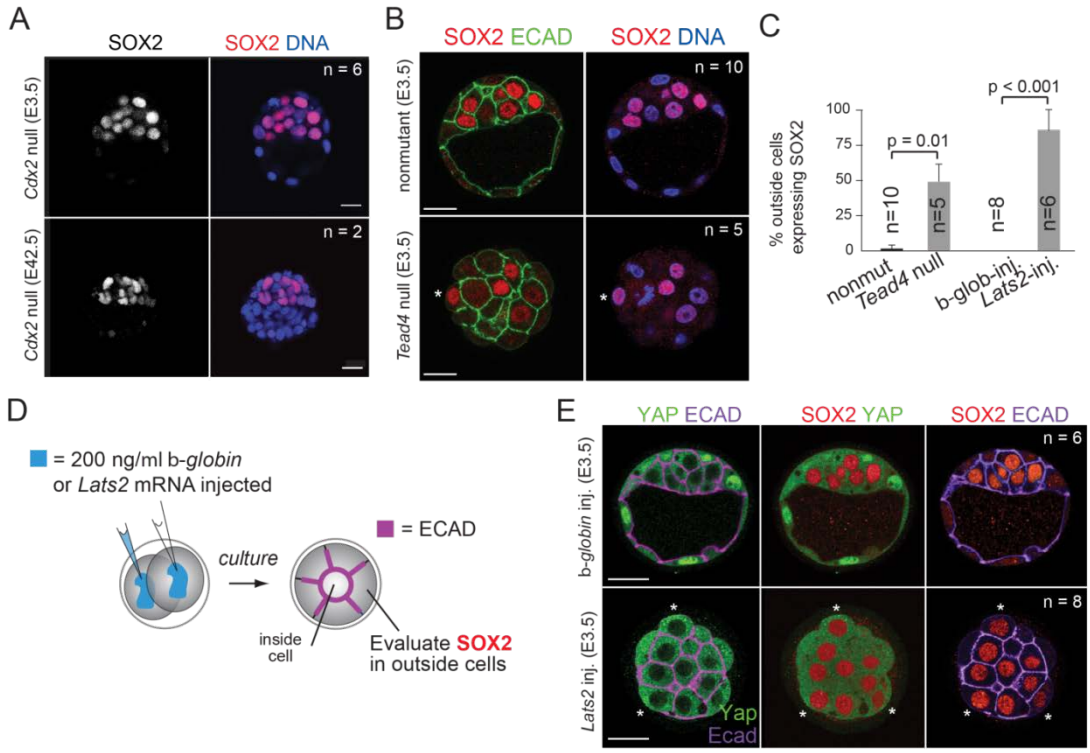


Fig. 20. SOX2 is restricted to ICM progenitors by Hippo pathway members and not by *Cdx2*.

A) SOX2 is not upregulated in the TE of *Cdx2* null embryos at early blastocyst or late blastocyst stages, indicating that CDX2 does not restrict SOX2 to the ICM. B) SOX2 is ectopically expressed in outside cells of embryos lacking the HIPPO pathway member *Tead4* (asterisk = SOX2-positive outside cell). C) The proportion of outside cells in which SOX2 was ectopically expressed was significantly increased in both *Tead4* null embryos, and in embryos overexpressing the HIPPO pathway member *Lats2*, relative to controls. D) Either *Lats2* or *b-Globin* mRNAs were injected into both cells of 2-cell embryos, and embryos were then cultured to blastocyst stage. E) Overexpression of *Lats2*, which prevents nuclear YAP localization, causes ectopic expression of SOX2 in outside cells (indicated by asterisk). In all panels, bar = 20 µm. p calculated by T-test.

Maternal *Sox2* is not required for development

Our finding that SOX2 protein is only found in the ICM conflicted with previous reports that M SOX2 is present in the TE and is required for normal TE specification (Avilion et al., 2003; Keramari et al., 2010). The role of M *Sox2* has not been tested using null alleles, and our prior work has shown that preimplantation knockdown experiments do not necessarily recapitulate the knockout phenotype (Blij et al., 2012; Frum et al., 2013). To examine the role of M *Sox2* in preimplantation development we generated M null embryos through Cre-mediated excision of a conditional *Sox2* null allele (Smith et al., 2009). Cre expression was restricted to the female germ line through use of the *Zp3* promoter (de Vries et al., 2000). To generate M null embryos, *Sox2* germline null females were crossed to wild type males. The loss of M *Sox2* was verified by harvesting litters of M null oocytes and measuring *Sox2* mRNA by qRT-PCR (Fig. 21a). The number of offspring per litter did not differ between Sox2 M null and control matings, indicating that M *Sox2* is not required for development (Fig. 21b). We next examined whether loss of M and Z Sox2 disrupts lineage specification and blastocyst formation as previously reported (Keramari et al., 2010). We crossed *Sox2* germline null females with males carrying a *Sox2* null allele, which yields 50% M null and 50% MZ null embryos. At E3.5 MZ null embryos were morphologically indistinguishable from control embryos and there were no significant differences in total cell number, or in the ratio of ICM/TE (Fig. 22a,b). *Sox2* MZ null embryos also expressed normal levels of the TE markers CDX2 and EOMES (Fig. 22c). The expression of TE markers remained normal in *Sox2* MZ null embryos at E4.25 (Fig. 22d,e). OCT4 expression also appeared normal in MZ null embryos, confirming that *Sox2* is not required for *Oct4* expression

(Fig. 22f). These findings are of particular interest as knockdown of *Sox2* in ESCs leads to loss of *Oct4* expression and upregulation of TE markers (Masui et al., 2007). These data suggest that upregulation of TE markers upon *Sox2* knockdown in ESCs may not be a direct result of loss of *Sox2*, but rather a result of loss of *Oct4*. These data also demonstrate that M *Sox2* is not required for development and that neither M nor Z *Sox2* is required for specification of TE vs ICM during the first lineage decision.

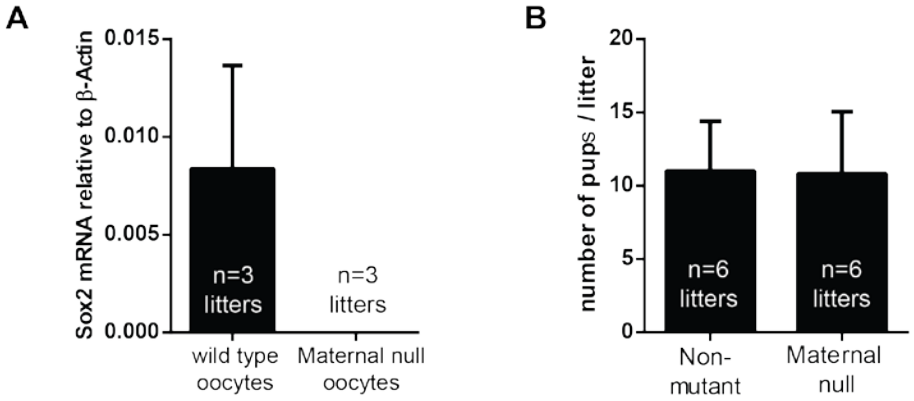


Figure 21. M *Sox2* is not required for normal development. A) qPCR analysis confirms that *Sox2* is deleted in oocytes from females carrying *Zp3Cre* and the floxed *Sox2* allele (bars represent standard deviation from the average of 3 replicate pools of ~10 oocytes each). B) M *Sox2* is not required for development because litter sizes did not significantly differ between nonmutant females and females in which *Sox2* had been deleted in the germ line.

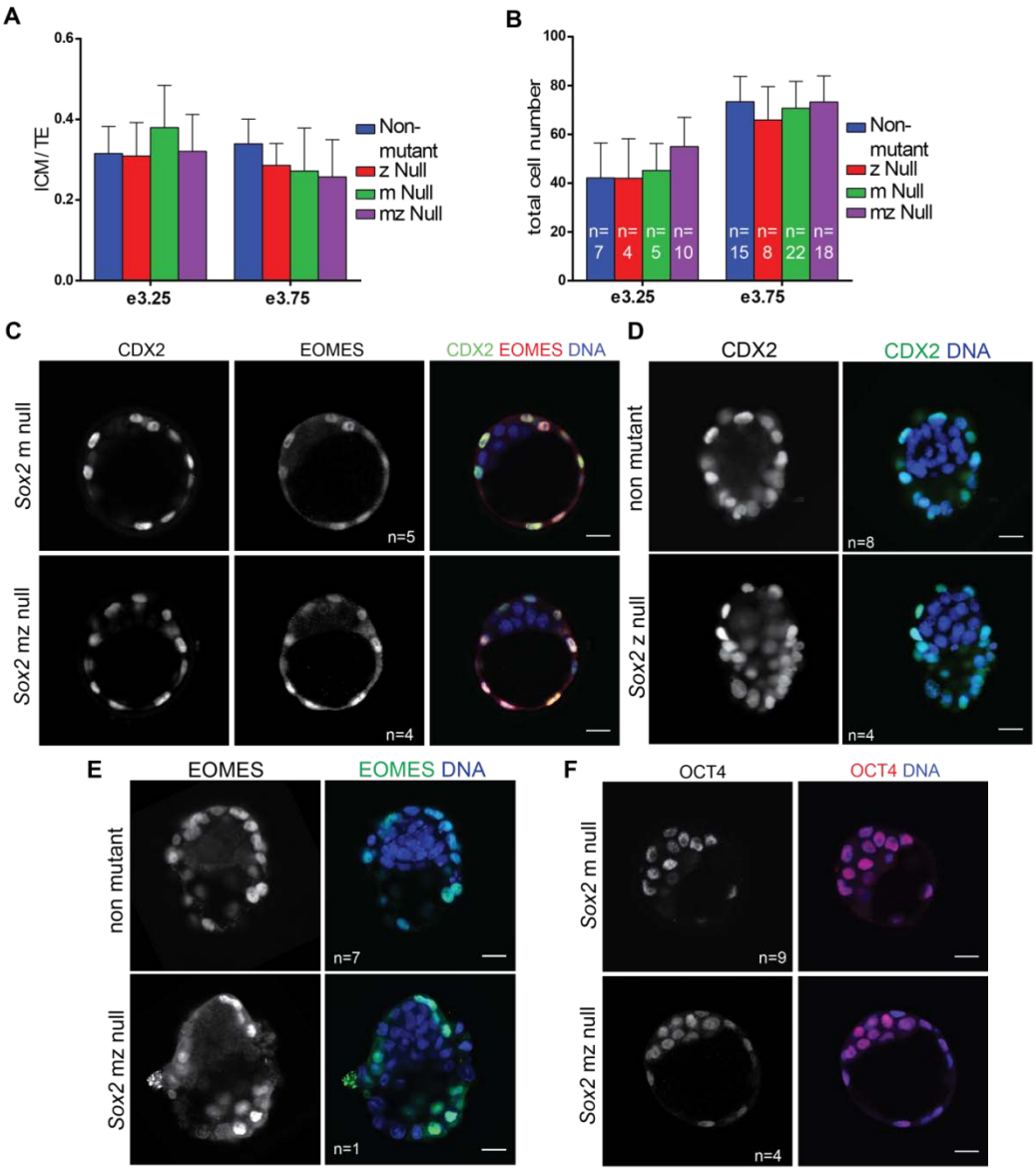


Figure 22. *Sox2* is not required for the first lineage decision: segregation of ICM and TE cell types. A-B) The number and proportion of total cells, inside (ICM) cells, and outside (TE) cells is normal in the absence of M, Z or MZ *Sox2*. C) The expression patterns of the TE markers CDX2 and EOMES are normal in E3.5 embryos lacking *Sox2*. D-E) The expression patterns of the TE markers CDX2 and EOMES are normal in E4.25 embryos lacking *Sox2*. F) The expression pattern of the ICM marker OCT4 is normal in embryos lacking *Sox2*. Bar = 20 µm, p calculated by T-test in A, B; ANOVA performed in A-B.

SOX2 is restricted to EPI cells by an FGF4/MAPK-dependent mechanism

Sox2 is not required for the first lineage decision in the embryo. However, as *Sox2* is expressed in the ICM at E3.5, we examined its expression and regulation during the second lineage decision: EPI vs PE. First we examined the expression pattern of SOX2 after E3.5, as the ICM begins to differentiate into PE and EPI lineages. At E3.75 when Nanog downregulation in the putative PE begins, SOX2 is expressed in the majority of ICM cells and the downregulation of SOX2 appears to follow the downregulation of NANOG (Fig. 23A,D). By late E3.75 SOX2 is restricted to putative EPI cells and is mutually exclusive with SOX17 (Fig. 23B). This pattern persists through E4.25, with SOX2 expressed in EPI cells and SOX17 in PE (Fig.23C). Although NANOG and SOX2 are not downregulated at precisely the same time, the dynamics of their expression in the ICM is quite similar, suggesting they may be regulated by similar mechanisms. NANOG downregulation in the ICM relies on FGF/MAPK signaling, and treatment with exogenous FGF4 or inhibitors of FGF/MAPK can repress or activate NANOG expression throughout the ICM, respectively (Frankenberg et al., 2011; Kang et al., 2013; Krawchuk et al., 2013; Nichols et al., 2009; Yamanaka et al., 2010). To determine if the patterning of SOX2 in the ICM relies on FGF/MAPK signaling, we first cultured wild-type embryos with FGF/MAPK inhibitors (FGFi/MAPKi) and examined the resulting expression of SOX2. Similar to NANOG, SOX2 was expressed in all cells of the ICM after FGFi/MAPKi treatment, while SOX17 was no longer detected (Fig. 23E). Next we cultured embryos in FGF4 and examined the resulting expression of SOX2. After FGF4 treatment SOX17 was expressed in all cells of the ICM, and SOX2 was no longer

detectable (Fig. 23F). These data suggest that SOX2 is restricted to EPI cells by FGF/MAPK signaling in a similar manner to NANOG.

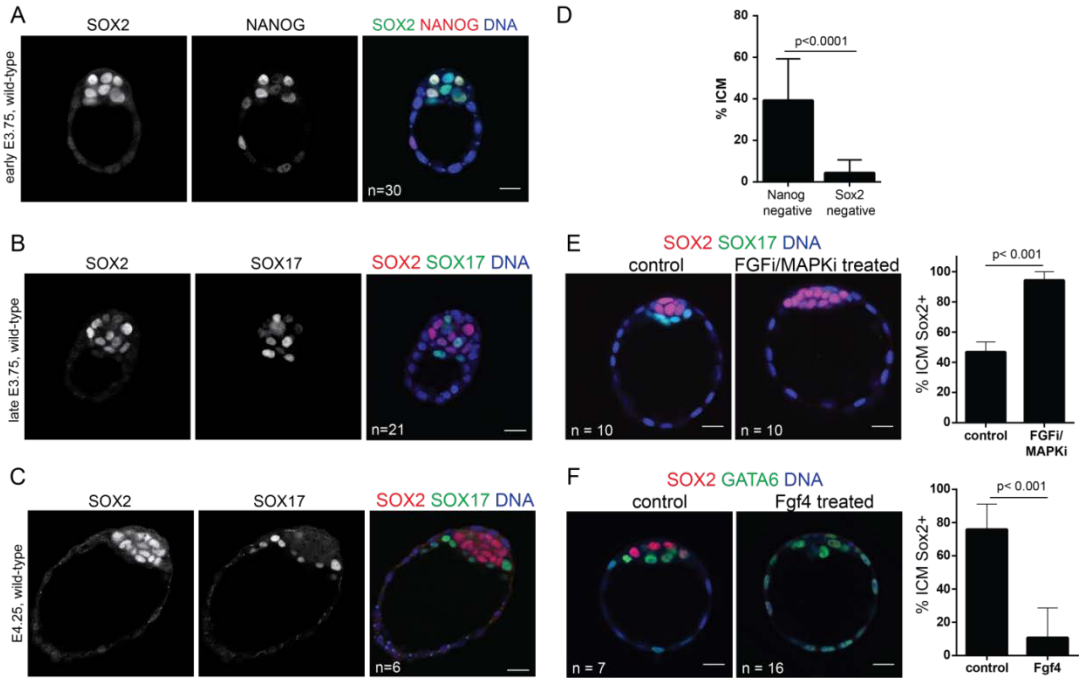


Figure 23. *Sox2* is restricted to EPI progenitors through an Fgf4/Mapk-dependent mechanism. A) At E3.75, NANOG is detected in a salt and pepper pattern in the ICM, while SOX2 is starting to become downregulated in PE cells. B) At E3.75, SOX2 and SOX17 are detected in a salt and pepper pattern in the ICM. C) At E4.25, SOX2 is exclusively detected in EPI and SOX17 in PE. D) At E3.75, SOX2 is detected in a larger proportion of ICM cells than is NANOG, indicating that NANOG is downregulated in the PE slightly before SOX2. E) The downregulation of SOX2 in PE cells is dependent on FGFR/MAPK, since the proportion of ICM cells expressing SOX2 is expanded (and the SOX17-expressing proportion concomitantly reduced) in wild-type embryos incubated in inhibitors of FGFR/MAPK. F) FGF4/HEPARIN (HEP) is sufficient to repress SOX2 expression in the ICM since the SOX2-expressing proportion of ICM cells is reduced (and GATA6-expressing proportion concomitantly expanded) in wild-type embryos incubated in FGF4/HEP. Bar = 20 µm, p calculated by T-test.

Sox2 regulates PE gene expression via FGF4

SOX2 is expressed exclusively in EPI cells beginning at E3.75, suggesting that it may have a role in the specification of EPI vs PE. To examine the role of Sox2 during the second lineage decision we examined the expression of the EPI marker NANOG and the PE marker SOX17 in *Sox2* null embryos. NANOG expression was unaffected in E3.75 *Sox2* null embryos (Fig. 24A,B). Interestingly, SOX17 expression was significantly reduced in *Sox2* null embryos (Fig. 24A,C). There was no difference in the number of NANOG or SOX17 expressing cells between non-mutant and M null embryos or between Z null and MZ null embryos, indicating that M *Sox2* is not required for the second lineage decision. The expression of other PE genes, GATA6, PDGRFA, and GATA4 was also reduced in *Sox2* null embryos (Fig.24 D-G). These data demonstrate that in the absence of *Sox2* the initiation of EPI cells is unaffected but the specification of PE cells is severely impaired.

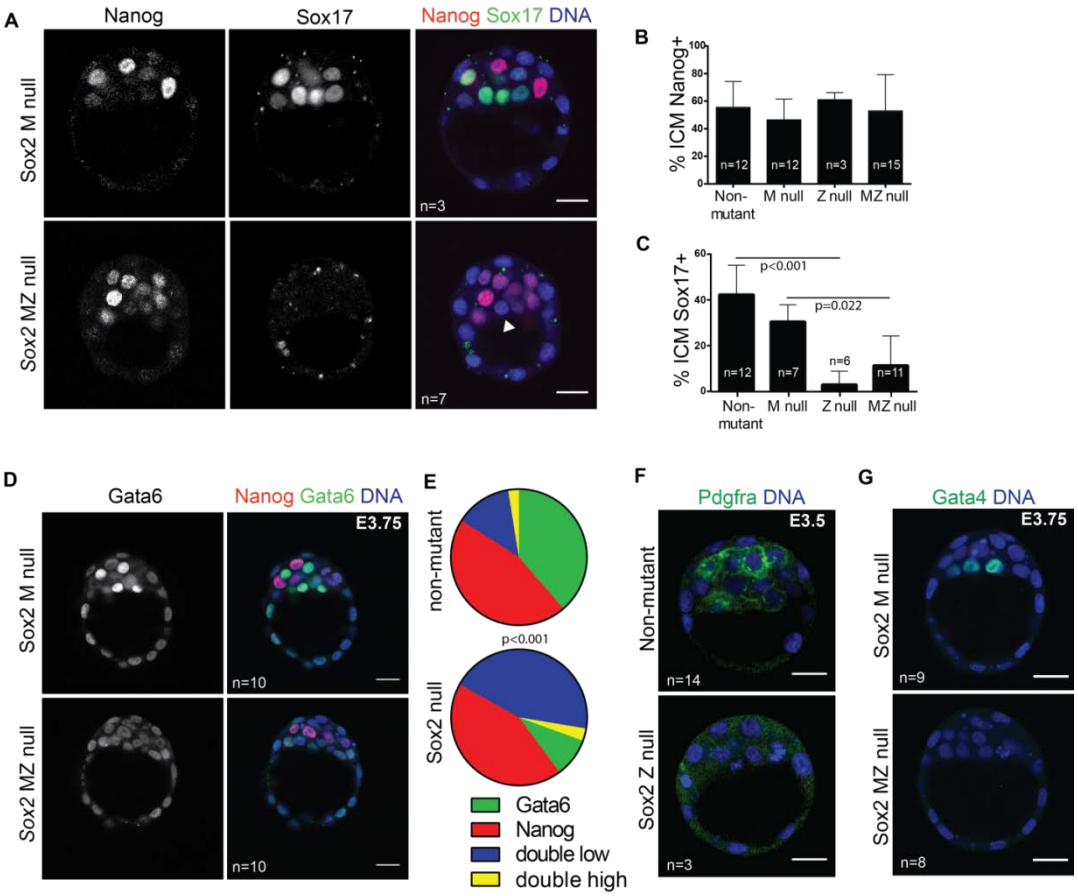


Figure 24. ***Sox2*** **is required for the initial expression of PE genes in the ICM.** A) At E3.75, NANOG is detected in *Sox2* null embryos, but SOX17 is not detected in most *Sox2* null embryos (arrowhead = ICM cell expressing neither NANOG nor SOX17). B) At E3.75, the average proportion of ICM cells in which NANOG is elevated is equivalent among all genotypes examined, indicating that *Sox2* is not required for expression of NANOG in the ICM. C) At E3.75, the average proportion of ICM cells in which SOX17 is detected is significantly reduced in the absence of either Z or MZ *Sox2*. There was no significant difference in the average proportion of ICM cells expressing SOX17 between between MZ and Z null, indicating that Z, but not M, *Sox2* is required for SOX17 expression. D) At E3.75, *Sox2* is required for elevated expression of GATA6 in the ICM. E) Quantification of immunofluorescent results showing that in *Sox2* null embryos at E3.75, the proportion of ICM cells in which GATA6 is elevated is reduced, while the proportion of cells expressing low levels of both GATA6 and NANOG (double low) is greatly increased, consistent with a role for *Sox2* in promoting PE gene expression in PE cells. F) Expression of PDGFRa in the ICM

depends on *Sox2*. G) Expression of GATA4 in the ICM depends on *Sox2*. Bar = 20 μm, p calculated by T-test in B and C, and by Chi-squared test in E.

SOX2 is not expressed in the PE, yet specification of PE is affected in the absence of *Sox2*. This suggests that *Sox2* regulates PE in a non-cell autonomous manner. It is known that FGF4 is necessary and sufficient for the induction of PE in the embryo, and in ESCs SOX2 and OCT4 act cooperatively to promote *Fgf4* expression (Ambrosetti et al., 1997; Yuan et al., 1995), suggesting that *Sox2* may promote *Fgf4* expression in the EPI. Moreover, in *Nanog* null embryos the specification of PE is also impaired in a non-cell autonomous manner via FGF4 (Frankenberg et al., 2011; Messerschmidt and Kemler, 2010). To determine if *Fgf4* expression is impaired in the absence of *Sox2*, we performed single blastocyst RT-qPCR on *Sox2* null embryos. The level of *Fgf4* was nearly 3x higher in *Sox2* M null embryos compared to MZ null, at E3.5 and E4.25 (Fig. 25A). However, *Fgf4* levels were indistinguishable between non-mutant and *Sox2* M null embryos (Fig. 25B), consistent with a lack of role for M *Sox2* in development. In addition, *Fgf4* levels were indistinguishable between *Sox2* Z null and MZ null embryos (Fig. 25B). Interestingly, we found a strong correlation between the level of *Sox2* and *Fgf4* in individual embryos from both Z null and MZ null crosses (Fig. 25C), suggesting that *Sox2* tightly regulates *Fgf4* expression levels.

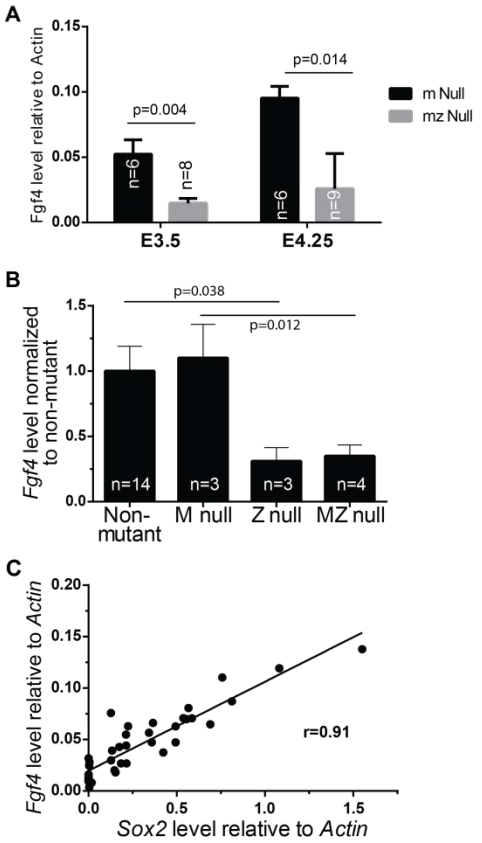


Figure 25. Sox2 promotes expression of Fgf4. The expression of *Fgf4* was measured by RT-qPCR in single blastocysts. A) *Fgf4* levels are reduced at E3.5 and E4.25 in the absence of *Sox2*. B) *Fgf4* levels are equivalent between non-mutant and *Sox2* M null embryos and between *Sox2* Z and MZ null embryos. C) The level of *Fgf4* in individual embryos from both Z null and MZ null crosses is strongly correlated to the level of *Sox2*.

To determine if *Sox2* regulates PE gene expression non-cell autonomously through FGF4, we attempted to restore PE gene expression by culturing *Sox2* null embryos in FGF4/HEP and examined the effect on EPI and PE gene expression (Fig. 26A). When embryos were cultured in FGF4/HEP until E3.75 we observed an increase in the number of $SOX17^+$ cells and a decrease in the number of $NANOG^+$ and unlabeled cells, relative to untreated controls, in both non-mutant and *Sox2* null embryos (Fig. 26B,C). However, after this FGF4/HEP treatment there were still

fewer SOX17^{+} cells in *Sox2* null embryos compared to non-mutants, therefore we repeated the experiment but allowed embryos to develop until E4.5 to determine if all cells in the ICM were capable of becoming PE in response to FGF4 treatment. With this extended treatment time all cells in the ICM were SOX17+ in *Sox2* null embryos, like wild-type (Fig. 26D,E). We conclude that *Sox2* is not required for ICM cells to respond to FGF4, and exogenous FGF4 can rescue PE gene expression. This suggests that the loss of PE cells in *Sox2* null embryos could be due to decreased FGF4.

Our observations that *Sox2* regulates PE gene expression via FGF4 predicted that *Sox2* promotes PE gene expression non-cell-autonomously. We tested this hypothesis by examining expression of PE genes in chimeric embryos containing a *Sox2* null PE and wild-type EPI. To generate chimeras, we aggregated wild-type, GFP-labeled ES cells with *Sox2* null or non-mutant host embryos, and then cultured these chimeras to E3.75 (Fig. 26F). In both control and *Sox2* null host embryos, expression of SOX17 was rescued by wild type ES cells (Fig. 26G,H). These results indicate that *Sox2* is required non-cell-autonomously to promote PE gene expression at E3.75.

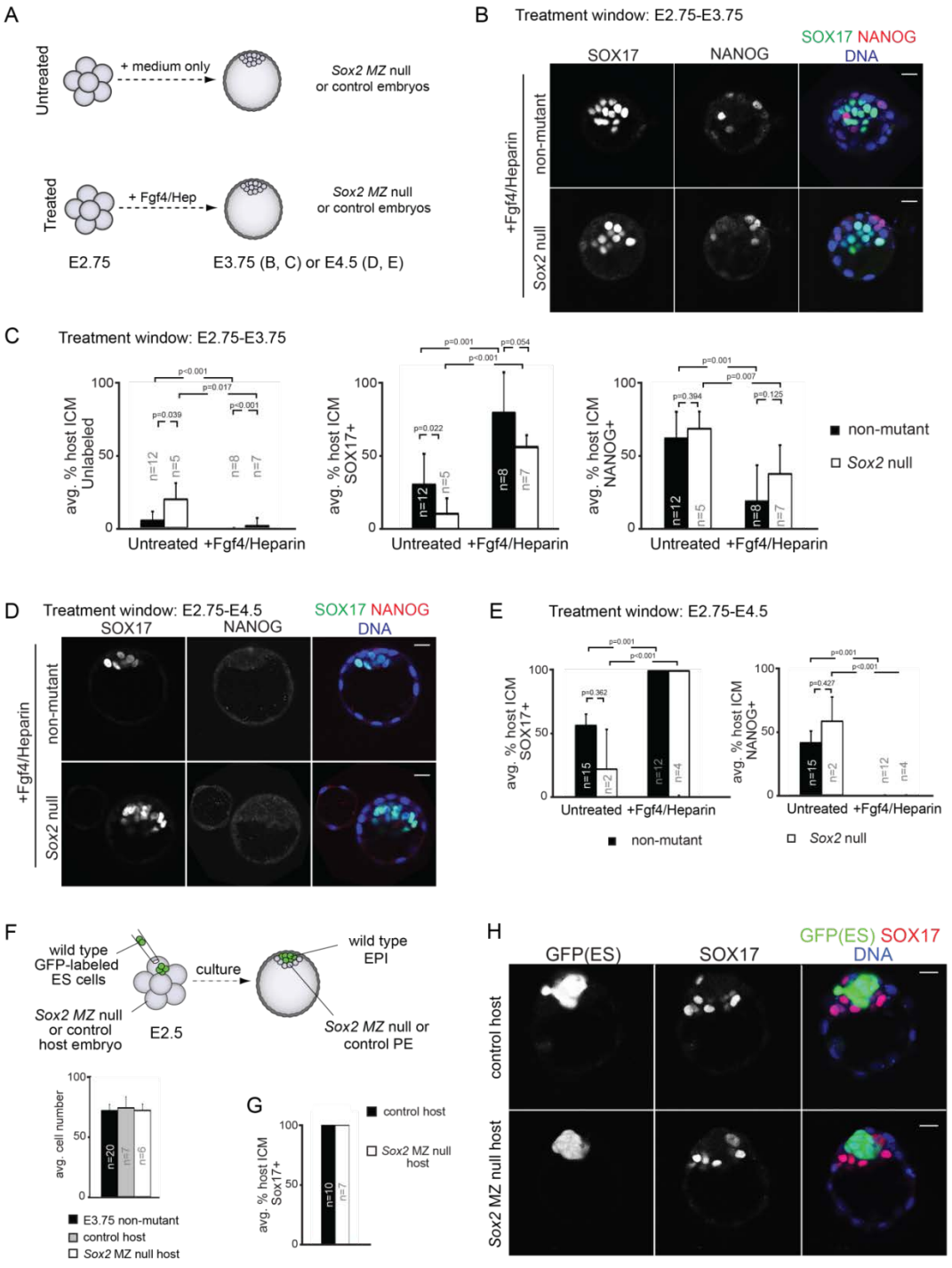


Figure 26. *Sox2* promotes PE development non cell-autonomously via FGF4. A) FGF/HEP treatment scheme B) FGF4/HEP treatment is sufficient to induce expression of SOX17 in E4.0 *Sox2* null embryos. C) Quantification of the experiment shown in panel B. D)

FGF4/HEP treatment is sufficient to induce expression of SOX17 in 100% of E4.5 *Sox2* null embryos. E) Quantification of the experiment shown in panel D. F) Overview of strategy to generate chimeric embryos and evidence that chimeras are equivalent in cell number to E3.75. G) Wild-type ES cells rescue expression of SOX17 in *Sox2* null embryos. H) Chimeras from panel G. Bar = 20 μm, p calculated by T-test.

Defects in late PE development in the absence of *Sox2*

We next examined whether PE gene expression is ever initiated in *Sox2* null embryos during preimplantation development. Surprisingly, by E4.25 all PE markers examined were expressed in *Sox2* null embryos at comparable levels to non-mutant embryos (Fig. 27A). The number of ICM cells expressing $GATA6^{+}$ and $SOX17^{+}$ was also comparable to non-mutant embryos (Fig. 27B,C). In fact, the number of $SOX17^{+}$ cells steadily increased over time until reaching wild-type levels at E4.25 (Fig. 27B). Since we found that *Fgf4* levels were still significantly reduced at E4.25, we next examined if the recovery of PE gene expression is dependent on FGF/MAPK signaling in *Sox2* null embryos. After culturing embryos in FGFi/MAPKi from E2.75-E4.5, there was a complete loss of SOX17 in *Sox2* null embryos, while mutant embryos cultured in control medium expressed similar levels of SOX17 (Fig. 27D). This demonstrates that the recovery of PE gene expression in *Sox2* null embryos relies on FGF/MAPK signaling.

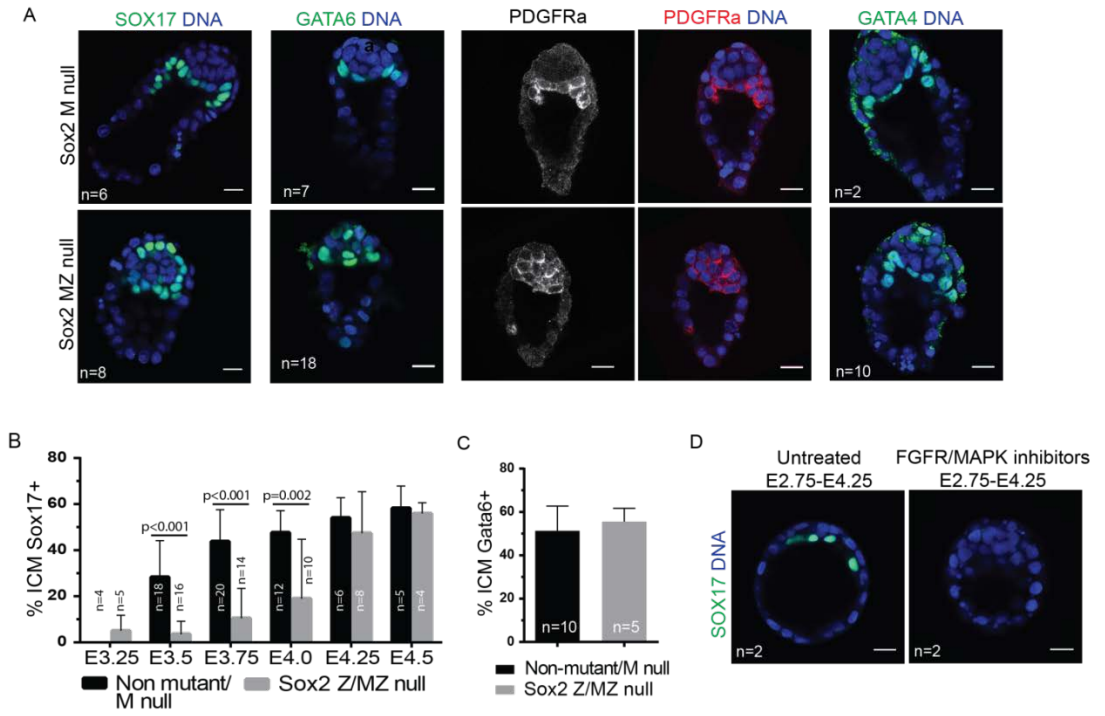


Figure 27. Recovery of PE gene expression by E4.25 A) By E4.25, expression of PE genes including SOX17, PDGFRA, and GATA4 is restored in *Sox2* Z and MZ null embryos, but the ICM appears disorganized relative to control embryos. B) In *Sox2* null embryos, the average proportion of ICM cells expressing SOX17 increases progressively, catching up with control embryos (here *Sox2* M null or nonmutant) by E4.25. C) At E4.25 the average proportion of ICM cells expressing GATA6 is not significantly different between non-mutant and *Sox2* mutant embryos D) At E4.25, the expression of SOX17 in *Sox2* null embryos depends on FGFR/MAPK.

Although PE gene expression recovers in *Sox2* mutants, we noticed that these PE cells failed to generate a cohesive PE layer at the blastocoel cavity (Fig. 27A). This could be due to lack of sufficient time for cells to migrate as PE specification occurs behind schedule. Alternatively, this could be due to misexpression of genes involved in sorting and migration of the PE. To test these hypotheses we first induced diapause, which delays implantation of embryos into the

utereus, to allow the late-forming PE in *Sox2* null embryos more time to develop. During diapause wild-type embryos essentially pause in development, however, some mutant phenotypes will manifest with this additional time, indicating that embryos can continue to develop if they have not reached a stable E4.5 state (Artus et al., 2010; Nichols et al., 2001). We induced diapause and harvested embryos at E5.5 and E6.5 and examined the subsequent expression of SOX17 and DAB2. At E5.5 *Sox2* null embryos in diapause exhibited increased DAB2 compared to E4.25 *Sox2* null embryos (Fig. 28A). It was difficult, however, to determine whether PE cells were sorted as there was a dramatic reduction in EPI cells (Fig. 28A,C). At E6.5 *Sox2* null embryos in diapause exhibited significantly reduced numbers of both EPI and PE cells (Fig. 28B,D). This demonstrates that *Sox2* is required for maintenance of the ICM during diapause, however, the early death of EPI cells precluded evaluation of the PE sorting phenotype. To examine the PE sorting phenotype in a different way we examined expression of SOX7, a marker of mature PE cells, to determine if PE cells are unsorted because they are immature (Artus et al., 2011). We observed that SOX7 is expressed in *Sox2* null PE cells by E4.25, suggesting that they have had sufficient time to reach a mature PE state (Fig. 28E). We also examined the expression of two extracellular proteins that are important for proper PE migration: LAMA1 and DAB2. The expression of both proteins was reduced in *Sox2* null embryos compared to non-mutants (Fig. Sox2 28F,G), suggesting that misexpression of genes important for PE migration may lead to a failure of EPI/PE sorting.

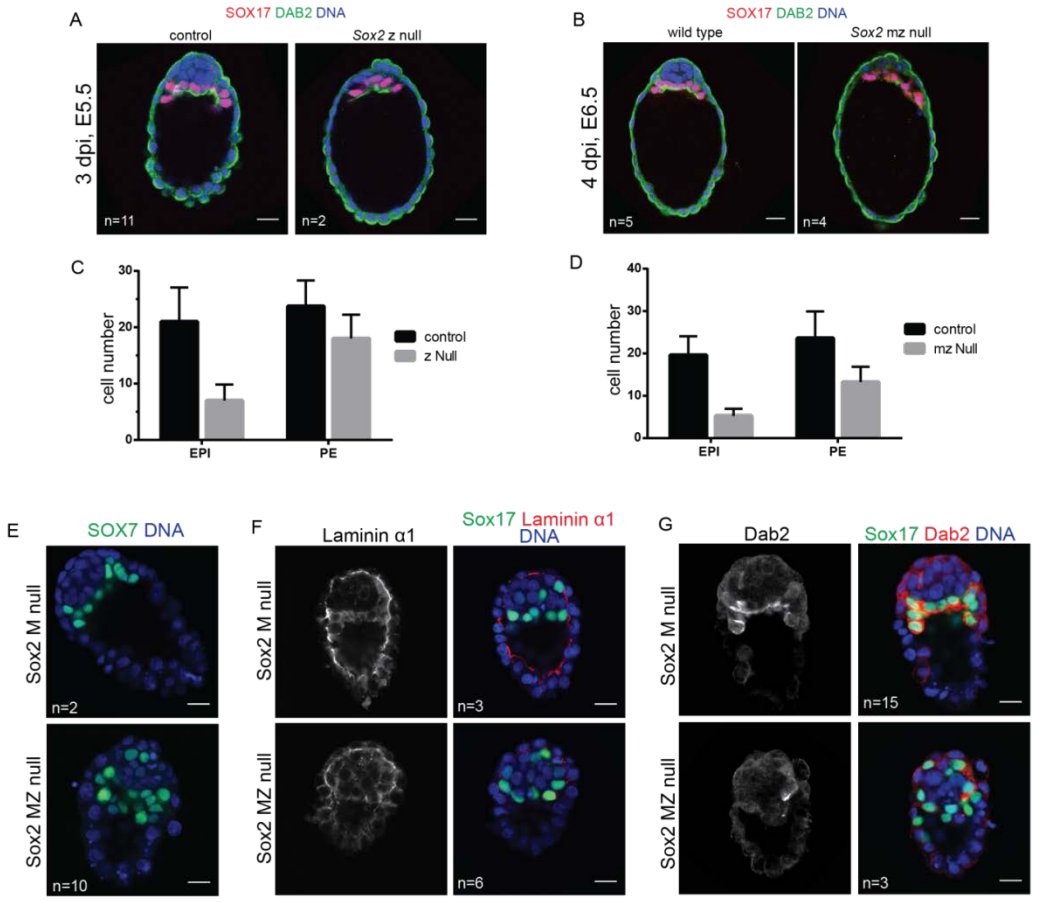


Figure 28. *Sox2* is not required to maintain expression of some PE genes A) Diapaused blastocysts harvested from hormone-primed mothers at E5.5 have increased levels of DAB2 and a decreased number of EPI cells. B) Quantification of results shown in A. C) Diapaused blastocysts harvested from hormone-primed mothers at E6.5 have reduced numbers of EPI and PE cells indicating death of the ICM. D) Quantification of results shown in C. E) SOX7, a marker of late PE fate, is detectable in the absence of *Sox2*, suggesting that *Sox2* null PE cells have matured in an age-appropriate manner. F) At E4.25, expression of LAMA1 is reduced in the absence of *Sox2.* G) At E4.25, expression of DAB2 is reduced in the absence of *Sox2*, consistent with observed defects in PE localization. Bar = 20 µm, p calculated by T-test.

Discussion

In ESCs OCT4, SOX2, and NANOG co-regulate pluripotency gene expression and have many overlapping functions (Boyer et al., 2005; Loh et al., 2006). The role of these genes in regulating pluripotency and the establishment of the embryonic lineage *in vivo* is not yet clear. One reason the *in vivo* roles are not clear is the ambiguity surrounding the specific role of SOX2 and the unconfirmed role of M Sox2 in development. Through detailed examination of the roles of M and Z *Sox2* in preimplantation development, the roles of OCT4, NANOG, and SOX2 can be compared. This comparison allows the unique and overlapping roles of these important pluripotentcy regulators to be established and provides new insight into the establishment of pluripotency *in vivo*.

Our analysis of Sox2 dynamics provides unique insight into the different ways in which OCT4, NANOG, and SOX2 are regulated during preimplantation development. For instance, SOX2 expression is restricted to inside cells of the embryo throughout preimplantation, while NANOG is not restricted to inside cells until the blastocyst stage and OCT4 is not restricted to inside cells until the late blastocyst stage. The restriction of SOX2 to inside cells is also not dependent on *Cdx2*, unlike the restriction of OCT4 and NANOG (Strumpf et al., 2005). Instead the restriction of SOX2 is downstream of the Hippo signaling pathway member LATS2. LATS2 is active in inside cells and prevents the expression of *Cdx2* by inhibiting YAP/TAZ (Nishioka et al., 2009). The initial ubiquitous expression of Nanog and Oct4 suggests that expression of pluripotency genes is the default developmental pathway and this default state is subsequently overridden by induction of TE cell fate through CDX2. However our study suggests that the specification of inside cells,

defined by SOX2 expression, is actively regulated in parallel to the specification of outside cell fate. Not only do these results provide new insight into inside cell fate specification, they also provide a previously unavailable tool for tracking the molecular identity of inside cells. At the morula stage inside cell fate was previously identified by a lack of CDX2, however the presence of SOX2 can now be used to identify cells which have an inside cell fate. For example, using SOX2 as a readout of inside cell fate will facilitate future studies to evaluate how position, polarity, and Hippo pathway components influence inside cell fate, as has been done for advancing our understanding of TE specification (Manzanares and Rodriguez, 2013; Stephenson et al., 2012) (Fig. 29A).

In addition, we also clarified how SOX2 expression is regulated within the ICM, showing that SOX2, like NANOG and unlike OCT4 (Chazaud et al., 2006; Guo et al., 2010; Palmieri et al., 1994), exhibits salt and pepper patterning in the ICM, and we show that the salt and pepper pattern of SOX2 depends on FGF4/MAPK signaling. We hypothesize that FGF4/MAPK represses expression of both *Sox2* and *Nanog* in PE cells in parallel (Fig. 29B), since we show that NANOG is not regulated by SOX2. However, it is not yet known whether expression of SOX2 is regulated by NANOG in the ICM. Resolving whether *Nanog* is required for expression of SOX2 in the ICM will reveal whether FGF4/MAPK regulates *Sox2* and *Nanog* in parallel or in sequence.

This analysis also provides new insight into the regulation of pluripotency in vivo. We demonstrated that neither M or Z *Sox2* is required for the expression of *Oct4* and *Nanog*, which differs from the role of *Sox2* in ESCs (Masui et al., 2007; Okumura-Nakanishi et al., 2005; Rodda et al., 2005). Interestingly, the role of *Oct4*

in regulation of pluripotency genes also differs between the embryo and ESCs (Chia Le Bin et al., 2014; Frum et al., 2013; Rodda et al., 2005; van den Berg et al., 2008; Wu et al., 2013). These observations suggest that OCT4 and SOX2 regulate target genes in parallel in the preimplantation embryo. One such target gene is *Fgf4*, whose expression is reduced to about 30% of wild type levels in the absence of either *Sox2* or *Oct4* (Frum et al., 2013). Thus OCT4 and SOX2 appear to promote expression of *Fgf4* synergistically in the embryo, as has been demonstrated in pluripotent stem cell lines (Ambrosetti et al., 1997; Yuan et al., 1995). The role of *Sox2* in repressing TE fate also differs between the embryo and ESCs. While knockdown of *Sox2* leads to upregulation of TE genes in ESCs (Li et al., 2007; Masui et al., 2007), we found that in the absence of *Sox2*, TE fate is still restricted to outside cells in the embryo. However, this may be due to the continued presence of OCT4 in the embryo, as it was also shown that overexpression of *Oct4* after *Sox2* knockdown in ESCs prevents the misexpression of TE genes (Masui et al., 2007). Thus pluripotent stem cell lines bear molecular similarities, as well as differences, to the blastocyst, underscoring the importance of both models for understanding pluripotency.

Finally, we show that although Sox2 does not appear to be required for specification of EPI cells, it is required for the proper specification of PE cells. During early blastocyst development of *Sox2* null embryos PE genes fail to be expressed, despite the downregulation of NANOG in presumptive PE cells. We showed that *Sox2* null embryos have reduced *Fgf4* expression, which may be responsible for the failure to initiate PE genes. However, we also showed that late in blastocyst development the expression of many PE genes recovers and that this recovery is

dependent on FGF4. These observations suggest that the duration of FGF4 treatment can compensate for reduced levels of FGF4. While FGF4 has been shown to regulate ICM fate in a dose-dependent manner (Kang et al., 2013; Krawchuk et al., 2013), the effect of the duration of FGF4 treatment has not yet been examined in *Fgf4* null embryos, but will be an interesting topic for future exploration. In addition to the delay in PE formation, we also found a defect in the sorting of PE cells. This defect is evidenced by reduced expression of LAMININα1 and DAB2, genes involved in PE migration and cohesion, in *Sox2* null embryos. Notably, *Oct4* is also required for the sorting of PE cells (Frum et al., 2013), suggesting that this is an *Fgf4*-dependent process. In postimplantation *Sox2* mutants, some PE derivatives have been observed, surrounding abnormal EPI (Avilion et al., 2003). Identification of direct and indirect targets of SOX2 and OCT4 in the postimplantation EPI will be the next step toward understanding the regulation of pluripotency *in vivo*.

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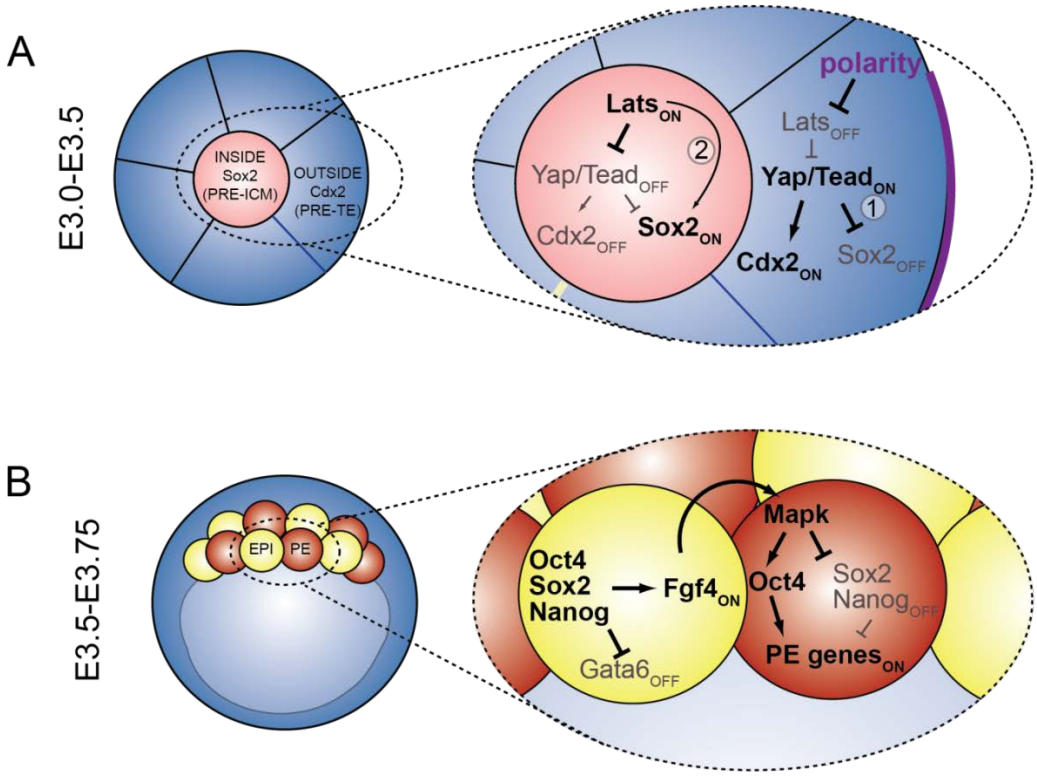

Figure 29. Model of the regulation and roles of Sox2 during blastocyst formation. A) At the 16-cell stage, when ICM progenitors first arise, HIPPO pathway members regulate expression of TE (*Cdx2*) and ICM (*Sox2*) genes in parallel. B) In the blastocyst, *Sox2* expression is restricted to EPI cells by FGFR/MAPK signaling and in EPI cells, SOX2 promotes expression of *Fgf4*, which signals to neighboring cells to promote PE development. In PE cells, MAPK promotes PE gene expression in an *Oct4*-dependent manner (Aksoy et al., 2013; Chia Le Bin et al., 2014; Frum et al., 2013), and represses expression of *Sox2* and *Nanog*.

CHAPTER 5. Exploration of additional mechanisms of preimplantation lineage regulation

Abstract

Many aspects of early lineage development in the mammalian embryo are poorly understood. Transcription factors typically regulate many genes within a cell, including those producing secreted proteins which can signal to neighboring cells, and therefore loss of a single transcription factor can lead to many developmental defects. Several preliminary studies addressing the role of particular transcription factors expressed in the TE and EPI are presented here.

Loss of *Cdx2* leads to reduced nutrient uptake

Cdx2 is required for proper specification and maintenance of the TE, and many tight and adherens junction proteins are absent or mislocalized in *Cdx2*$^{-/-}$ embryos (Chawengsaksophak et al., 2004; Ralston et al., 2005; Strumpf et al., 2005). However, *Cdx2*$^{-/-}$ embryos initially form a blastocyst with a polarized TE, and the mechanisms leading to the failure of TE in *Cdx2*$^{-/-}$ embryos are unclear. RNA-Seq analysis of wild type and *Cdx2*$^{-/-}$ embryos showed reduced expression of several genes with known roles in endocytosis and nutrient uptake in *Cdx2*$^{-/-}$ embryos (Robson lab, Genome Institute of Singapore, unpublished). The dipeptide transporters *Slc15a1 and Slc15a2* are both expressed in the CDX2^{+} intestinal epithelium and *Slc15a1* has shown to be under the direct transcriptional control of *Cdx2* (Shimakura et al., 2006). The expression of *Slc15a2* was also greatly reduced in *Cdx2*$^{-/-}$ embryos (done in collaboration with Robson lab, Genome Institute of Singapore, unpublished). This suggests that in addition to promoting proper epithelial polarization, *Cdx2* may also promote nutrient uptake in the TE.

To test whether *Cdx2* is required for nutrient uptake we utilized a fluorescently tagged dipeptide, d-Ala-Lys-AMCA, which has been shown to be taken up through SLC15A2 (Dieck et al., 1999). We first performed a time course to determine the optimal incubation time for detection of D-Ala-Lys-AMCA in non-mutant embryos. After a 2-hour incubation in 50 µM D-Ala-Lys-AMCA, fluorescence was weakly and variably detectable in non-mutant embryos, but not in *Cdx2* null embryos (Table 2). After a 4-hour incubation, fluorescence was detected in all non-mutants, but not in *Cdx2* null embryos (Fig. 30 and Table 2). We next examined whether fluorescence was detectable in *Cdx2* null embryos, following incubation in a higher concentration of D-Ala-Lys-AMCA. After a 4-hour incubation in 100 µM D-Ala-Lys-AMCA, higher levels of fluorescence were detected in both non-mutant and *Cdx2* null embryos compared to the 50 µM condition (Table 2). However, fluorescence levels were higher in non-mutant than in null (Fig. 30 and Table 2). These results show that *Cdx2* is required for maximal dipeptide uptake, and suggest a role for *Cdx2* in promoting nutrient absorption in the TE. Further research will uncover the extent to which *Cdx2* is required for uptake of additional types of nutrients.

		2 h incubation		**4 h incubation**	
Conc.	Genotype	**N**	**Fluorescence**	**N**	**fluorescence**
50 µM	Non-mutant	15	+	12	++
50 µM	*Cdx2* null	1	-	4	-
100 µM	Non-mutant	n.d.	n.d.	10	+++
100 µM	*Cdx2* null	n.d.	n.d.	8	+

n.d. = not done

Tabe 2. Summary of D-Ala-Lys(AMCA) assay

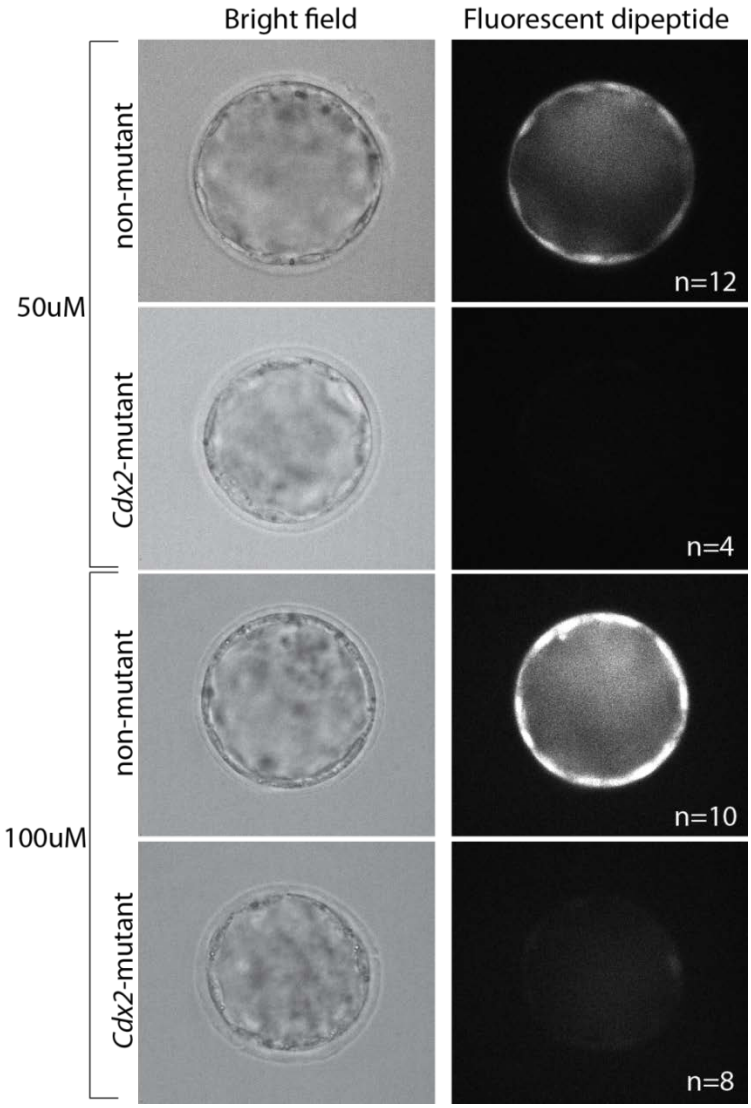

Figure 30. *Cdx2* mutant embryos exhibit deficient uptake of D-Ala-Lys(AMCA)

Single sections from confocal images of blastocysts cultured in either 50 µM or 100 µM AMCA-conjugated D-Ala-Lys dipeptide. *Cdx2* null embryos exhibit a dramatic reduction in AMCA fluorescence compared to non-mutants.

Dipeptide uptake assay methods:

Embryos were harvested from maternal null *Cdx2* heterozygous intercrosses at E3.5, and cultured in KSOM (Millipore) containing 50 or 100 µM D-Ala-Lys-AMCA (Biotrend BP0410) for 2 or 4 hours at 37ºC and 5% CO_2. Embryos were then fixed

for 10 minutes in 4% EM-grade formaldehyde at room temperature. Z-series confocal images were collected at 4 µM intervals on a Perkin Elmer Volocity spinning disc confocal.

Promotion of trophoblast maintenance and differentiation by *Eomes*

The T-box transcription factor *Eomesodermin* (*Eomes*) is expressed specifically in TE cells at the blastocyst stage (Russ et al., 2000). It is also expressed in trophoblast stem (TS) cells, which are stable cell lines derived from the trophectoderm that serve as an *in vitro* equivelant of undifferentiated trophoblast (Tanaka, *et al*., 1998). Loss of *Eomes* causes embryonic lethality shortly after implantation, and although embryonic stem (ES) cells can be derived from *Eomes* null embryos, TS cells cannot (Russ, *et al*., 2000). Null embryos appear to arrest in a blastocyst-like stage and express early trophoblast markers but not later markers (Russ, *et al*., 2000; Strumpf, *et al*., 2005), consistent with impaired trophoblast differentiation. However, this interpretation is inconsistent with data demonstrating that *Eomes* is expressed in undifferentiated TS cells and is rapidly downregulated upon their differentiation (Tanaka, *et al*., 1998). The expression of *Eomes* in undifferentiated stem cells suggests that *Eomes* has a role in maintaining the undifferentiated state of trophoblast, potentially inhibiting differentiation.

Defective trophectoderm differentiation and proliferation in *Eomes* null embryos

We first sought to characterize the morphology of *Eomes* null embryos after implantation to determine if embryos arrest in a blastocyst-like state. Embryos were dissected at E5.5 from *Eomes*$^{+/-}$ crosses, stained with a nuclear marker and imaged

by confocal microscopy. At E5.5 *Eomes* null embryos ranged from a near wild-type morphology to a blastocyst-like morphology, (Fig. 31), demonstrating partial penetrance of the blastocyst arrest phenotype. We next examined E7.5 *Eomes* null embryos and found either embryos which resembled E5.5 embryos or empty decidua, indicating an embryos which have been reabsorbed (Fig. 31). The E7.5 *Eomes* null embryos appear to have a grossly normal embryonic egg cylinder, both parietal and visceral endoderm layers, and a very small group of cells where the tissues generated from the TE, extraemryonic ectoderm (ExE) and ectoplacental cone (EPC), should be. This indicates that development of the EPI and PE after implantation is largely unaffected, while development of the TE is impaired. Overall this embryo analysis shows that some embryos do arrest in a blastocyst-like state, suggesting that the TE fails to differentiate and/or proliferate, leading to embryo arrest. However, in some embryos the EPI and PE lineages continue to develop until around E5.5, with very limited development of TE-derived cells. Embryos which arrest in a blastocyst-like state appear to have defective differentiation of the TE. However, clearly the TE of some *Eomes* null embryos can further differentiate and generate structures resembling the ExE and EPC. The ExE is the source of many important developmental signals for embryonic development and maintains a TSC population until E7.5 (Arnold and Robertson, 2009; Tanaka et al., 1998). *Eomes* is required for the derivation of TSCs (Russ et al., 2000), therefore it may be required for maintenance of the TSC population in the ExE. Loss of *Eomes* may also affect the expression of important developmental signals from the ExE which would lead to pleiotropic effects on embryo development.

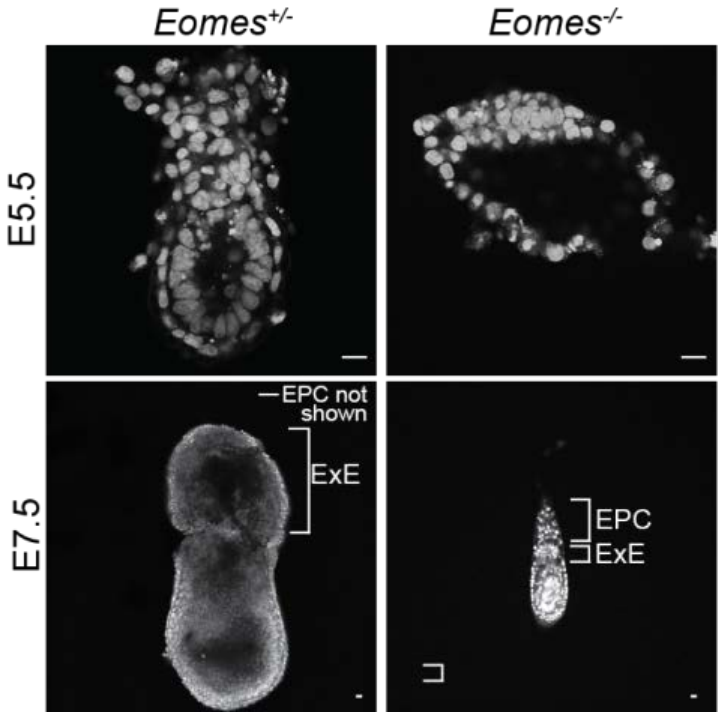

Figure 31. Variable developmental arrest of post-implantation *Eomes* null embryos Single sections from confocal images of embryos harvested from Eomes$^{+/-}$ crosses at E5.5 and E7.5 and stained with a DNA marker.

Although *Eomes* null embryos do not have obvious TE defects during preimplantation, our data show that *Eomes* null embryo arrest between E4.5-E5.5 and this may be the result of defects in either proliferation or differentiation of the TE. To distinguish between these possibilities we examined E4.5 embryos and counted cells to determine if any subtle defects in TE proliferation exist just prior to embryo arrest. Although the morphology and expression pattern of CDX2 of OCT4 are normal in *Eomes* null preimplantation embryos, the previous analysis did not evaluate either total or TE cell number to evaluate deficiencies in proliferation (Strumpf et al., 2005). Embryos from *Eomes*$^{+/-}$ crosses were harvested and immunostained with anti-CDX2 and OCT4 antibodies to label the TE and ICM, and then all cells were counted. As previously reported, the expression of CDX2 and

OCT4 were not affected in *Eomes* null embryos (Fig. 32A). There was also no difference in total cell number or TE cell number between control and *Eomes* null embryos (Fig. 32B,C). These results confirm that TE development prior to implantation does not require *Eomes*, and support the idea that *Eomes* is not required for development of the TE until after implantation. These data also suggest that *Eomes* does not promote proliferation of the trophoblast and that the arrest of *Eomes* null embryos is likely due to differentiation and signaling defects rather than proliferation defects.

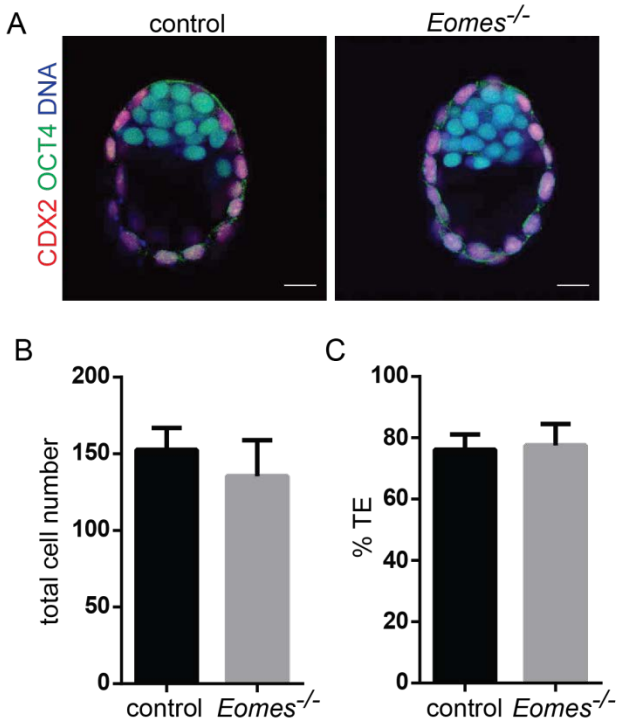

Figure 32. TE cell number is not affected in E4.5 *Eomes* null embryos (A) CDX2 and OCT4 expression are unaffected in E4.5 *Eomes* null embryos. (B) Quantification of total cell number from embryos represented in panel A reveals no difference in total cell number (C) Quantification of TE cell number from embryos represented in panel A reveals no difference in the percent of embryo comprised of TE. Bar = 20 µm, T-test was performed for data in panels B and C and $p>0.05$.

Eomes is required for the maintenance of TSC identity

In the embryo Eomes appears to have a role in trophoblast differentiation, however, TSCs cannot be derived from *Eomes* null embryos, suggesting that *Eomes* is required for maintenance of undifferentiated trophoblast. It is not yet clear whether there are unknown defects in the establishment of the TE in Eomes null embryos, or if Eomes is in fact not required for proper establishment of the TE, but is only required for maintenance of the undifferentiated stem cell state in TSCs. To test the requirement for *Eomes* in the establishment of the TSC state we utilized the *Cdx2*-overexpression assay (outlined in Fig. 6), which forces ESCs to adopt a TSC fate, in both wild type and Eomes null ESCs. At the end of 6 days of *Cdx2* overexpression the morphology of wild type and *Eomes* null ESCs resembled that of TSCs (Fig. 33A-B), however, after several passages in standard TSC conditions wild-type cells had a robust TSC morphology while *Eomes* null cells no longer resembled TSCs (Fig. 33C-D). Gene expression analysis revealed that after 6 days of *Cdx2* overexpression *Eomes* null cells expressed the TSC genes *Fgfr2* and *Gata3* at similar levels to the wild-type cells, although they did not express *Rhox4b* (Fig. 33E-G). After additional passages the expression of these TSCs genes increased in the wild-type cells to the same level as in TSCs, while *Eomes* null cells failed to express these TSC genes (Fig. 33E-G). Together the morphology and gene expression indicate that *Cdx2* overexpression is sufficient to induce TSC characteristics in *Eomes* null ESCs, however, these TSC characteristics cannot be maintained in the absence of *Eomes*. These results demonstrate that *Eomes* is not only required for trophoblast differentiation, it is also required for the maintenance of the undifferentiated TSC state. This further supports the idea that defects in the developing ExE, which

maintains undifferentiated trophoblast cells, may contribute to the *Eomes* null embryo phenotype.

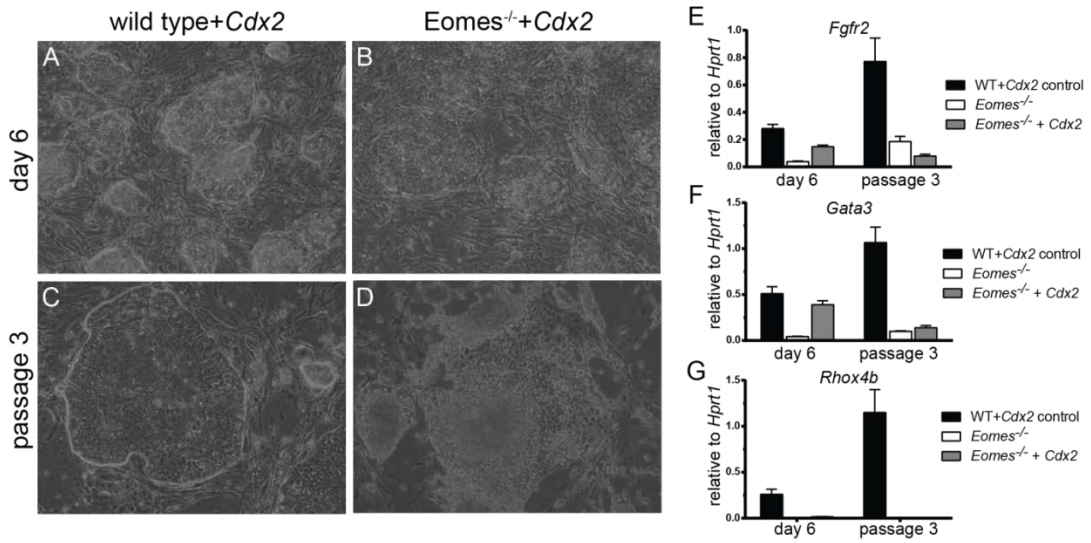

Figure 33. *Eomes* null ESCs do not generate stable TSCs after *Cdx2* overexpression (A) Wild type (R1) ESCs after 6 days of *Cdx2* overexpression in TSC medium. (B) *Eomes* null ESCs after 6 days of *Cdx2* overexpression in TSC medium. (C) Wild type (R1) ESC-derived TSCs 3 passages after 6 days of *Cdx2* overexpression in TSC medium. (D) *Eomes* null ESCs 3 passages after 6 days of *Cdx2* overexpression in TSC medium. (E-G) qPCR determination of gene expression levels relative to *Hprt1* of one representative ESC subclone and parental control for both wild type and *Eomes* null ESCs after 6 days *Cdx2* overexpression in TSC medium and after 3 additional passages for the TSC genes (E) *Fgfr2* (F) *Gata3* (G) *Rhox4b*. Error bars = standard error among qPCR replicates. Scale bar = 500uM

Cooperation among pluripotentcy genes during preimplantation development

The loss of either *Nanog*, *Oct4*, or *Sox2* does not impair the initial specification of ICM versus TE in the embryo. In fact, the loss of *Oct4* or *Sox2* seems to largely impair specification of the PE but not the EPI. As these transcription factors have been shown to regulate many of the same target genes in ESCs, it stands to reason that there may be some functional redundancy among

them during ICM/EPI specification. If Oct4 and Sox2 regulate many of the same genes independently of each other during preimplantation, than disruption of EPI development may not occur with loss of a single gene. The future of research into pluripotency specification *in vivo* will require examination of EPI specification upon loss of multiple pluripotency genes. Therefore we generated mice heterozygous for both *Oct4* and *Sox2* in order to examine ICM/EPI specification in embryos from $Oct4^{\Delta/+};Sox2^{\Delta/+}$ heterozygous crosses. *Oct4;Sox2* null embryos appeared grossly normal and closer analysis of cell number indicates that the number of ICM versus TE cells is not affected in mutant embryos (Fig. last A,B). In addition, NANOG was expressed in *Oct4;Sox2* null embryos from early stages through E4.25 (Fig. last C,E), further supporting the idea that pluripotency genes do not regulate each other in the embryo. Interestingly, CDX2 was also expressed in mutant embryos and restricted to the TE at early stages (Fig. last C, D), however, by E4.25 CDX2 is expressed in nearly all cells of the embryo (Fig. last E). This phenotype is more severe than loss of either *Sox2* or *Oct4* alone (Frum et al., 2013) (Fig. 22), suggesting that these genes have overlapping roles in maintaining the repression of TE gene expression in the ICM. However, this also demonstrates that in the absence of both *Oct4* and *Sox2*, the ICM is initially established. Continued analysis of these embryos along with examination of additional double mutants, such as *Oct4* and *Nanog*, will begin to reveal the requirement for the pluripotency network *in vivo* and allow us to decipher unique versus overlapping functions of these pluripotency genes.

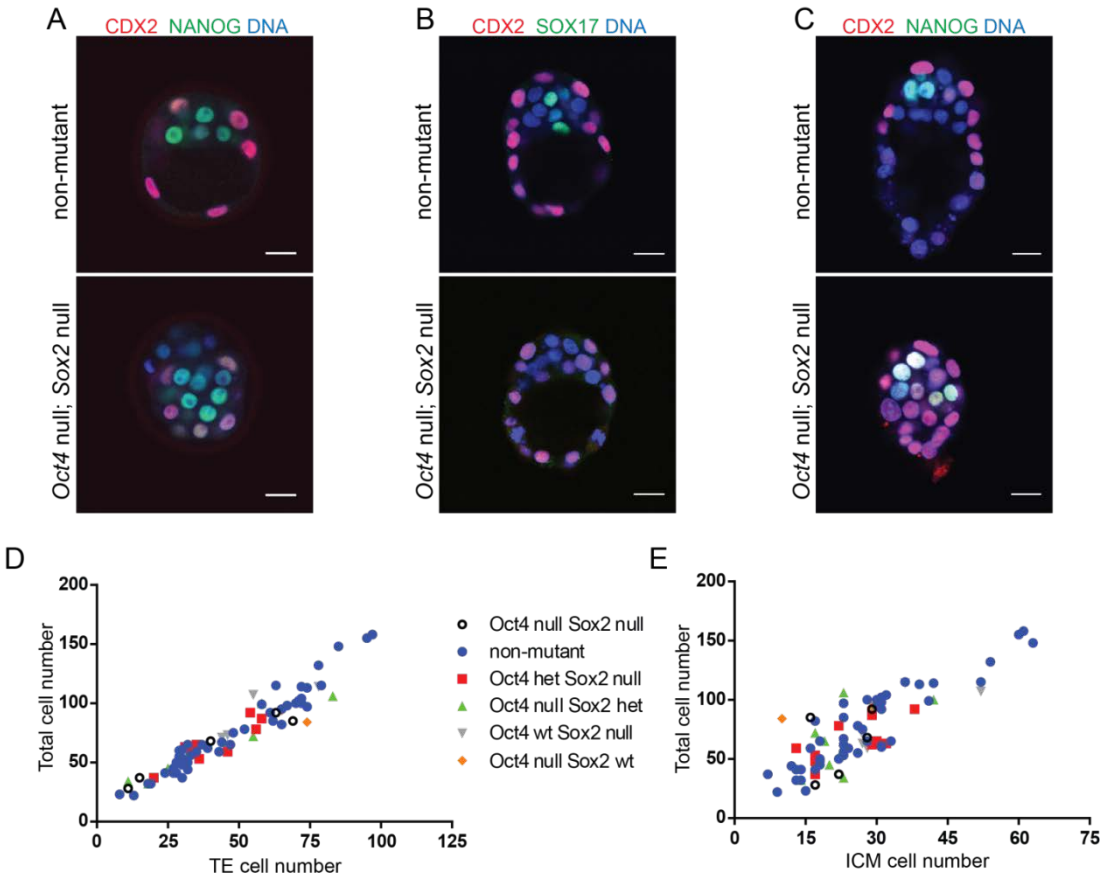

Figure 34. Establishment of ICM and TE in Oct4/Sox2 double mutant embryos (A-C) Representative single sections from confocal images of immunofluorescently labeled embryos from Oct4$^{\Delta/+}$;Sox2$^{\Delta/+}$ crosses at A) E3.25 B) E3.75 C) E4.25. (D-E) Quantification of total cell number for all embryos represented in A-C versus D) TE cell number and E) ICM cell number. Scale bar = 20uM

Conclusion

The work presented here addresses many previously outstanding questions in the fields of preimplantation development and stem cell biology. The role of maternal factors in early lineage specification has remained controversial for some time, and here I demonstrate than neither maternal *Cdx2* nor maternal *Sox2* are required for development. To date, no other maternally provided factors have been reported to directly contribute to lineage specification in mammals. I have demonstrated that zygotic *Sox2* has a non-cell autonomous role in development of the primitive endoderm. This work also shows that *Sox2* is the first known factor to be restricted to the embryonic lineage during development and that the *Sox2* expression pattern is independent of *Cdx2* but dependent on Hippo signaling. This exciting discovery broadens our understanding of how the pluripotent embryonic lineage develops and paves the way for future studies into the mechanisms of pluripotent lineage establishment in the embryo. I have also demonstrated how embryo development can be used to inform stem cell biology. My examination of pluripotent stem cell developmental potential revealed that naïve pluripotent stem cells generate TSCs more efficiently than primed pluripotent stem cells. This type of assay can be used as a read out for additional studies examining the way different signaling pathways and transcription factors impact the pluripotent state and developmental potential of stem cell lines.

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