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Regulation of cell fate by the stem cell factors Cdx2, Oct4 and Sox2 in the early mouse embryo

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY
in
Molecular, Cell and Developmental Biology

by

Tristan Frum

June 2014

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ABSTRACT

Regulation of cell fate by the stem cell factors *Cdx2*, *Oct4* and *Sox2* in the early mouse embryo

By

Tristan Frum

Pluripotent cells have the potential to create any cell type, and therefore represent a source of healthy cells by which to replace diseased cells in regenerative therapies. Thus, understanding how pluripotent cells are established and regulated is a major milestone in developing safe and effective regenerative therapies. In the mammalian embryo pluripotent cells emerge through two rounds of cell fate decisions that in addition to establishing pluripotent cells, establish two differentiated cell lineages. The molecules and signaling pathways that regulate these cell fate decisions represent key players in the establishment and regulation of pluripotent cells. In this thesis, I use the mouse embryo as a system in which to gain insight into how pluripotency is established and regulated. *Cdx2*, *Oct4* and *Sox2* are transcription factors that are important regulators of pluripotency *in vitro*, but their role in regulating the cell fate decisions that establish pluripotency *in vivo* is less clear. By using a loss-of-function approach, I define the roles of each of these factors in regulating the cell fate decisions that establish pluripotency in the early mouse embryo. First, by eliminating maternal and zygotic *Cdx2* I demonstrate that *Cdx2* is not a maternal determinant for pluripotency or differentiation during early embryo cell fate decisions. Second, I eliminate both maternal and zygotic *Oct4* and demonstrate that the initial specification of pluripotent and non-pluripotent lineages in the early mouse embryo is independent of *Oct4*, and that instead, *Oct4* cell-autonomously

regulates both formation of pluripotent cells, and differentiation of a non-pluripotent lineage, the primitive endoderm. Third, I demonstrate that in contrast to *Oct4*, *Sox2* promotes differentiation of primitive endoderm non-cell autonomously. These results support the model that pluripotency is acquired through these early embryonic cell fate decisions, rather than being determined during early embryogenesis. Furthermore, analysis of the roles of *Oct4* and *Sox2* in these early embryonic cell fate decisions highlights distinct roles for these factors in establishing pluripotency and suggests that although *in vitro Oct4* and *Sox2* appear to have overlapping roles, these master regulators of pluripotency have distinct cell type specific roles in regulating the cell fate decisions that establish pluripotency in the early mouse embryo. Taken together, the research presented in this thesis greatly increases our understanding of the regulation of the cell fate decisions that establish pluripotency in the embryo and provides important insights into the biology of key regulators of pluripotency *in vitro* and *in vivo.*

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CHAPTER 1: Introduction

1.1 Overview of preimplantation development in the mouse

The preimplantation period of mouse development comprises the first four days of the gestation period of the mouse embryo, from fertilization to implantation. During this period, the mouse zygote undergoes a series of cleavage divisions that generate a ball of approximately 100 cells. Concurrently, three distinct cell lineages are specified. Two of these lineages, the trophectoderm (TE) and the primitive endoderm (PE) are classified as extraembryonic. Extraembryonic lineages support development of the embryo, but do not contribute to the embryo itself, with the exception of minor contribution to embryonic endoderm by cells of the PE lineage (Kwon et al., 2008). The third lineage, the epiblast (EPI), is pluripotent and will give rise to the majority of the fetus.

Remarkably, three morphologically and molecularly distinct types of stem cells can be established from preimplantation mouse embryos (Fig. 1B). By definition, stem cells established from the preimplantation mouse embryo share the ability to self-renew, yet, when allowed to differentiate, each type of stem cell gives rise to a distinct repertoire of differentiated cell types. Thus, the preimplantion period of mouse development establishes three distinct cell states that share the functional property of self-renewal, but exhibit different potency. Importantly, stem cells derived from the EPI lineage of the preimplantation mouse embryo are pluripotent, meaning they have the capacity to differentiate to all cell types of the fetus. Due to this

remarkable developmental potential, human pluripotent stem cells are of great interest to medicine as a source of healthy cells to replace aged and diseased cells.

While the list of molecules essential for maintaining pluripotency and other stem cell states *in vitro* is rapidly expanding, little is known about the signaling and transcription factor networks that establish pluripotency and other stem cell states in development, or how these unique cell states are maintained *in vivo*. Because pluripotency and other stem cell states are established and maintained during the preimplantation period of mouse development, the preimplantation mouse embryo is an excellent system in which to study the signaling and transcription factor networks that establish and maintain pluripotent and other self-renewing cell states. It is my hope that insights from the studies presented here will significantly contribute to our understanding of pluripotency, both in human and mouse pluripotent cells.

In this chapter, I review models and mechanisms by which signaling and transcription factor networks orchestrate the establishment of pluripotent and other cell states in the preimplantation mouse embryo. Understanding these models and mechanisms is essential to the interpretation of preimplantation embryo phenotypes and elucidating gene function through reductionist paradigms.

Cells of the preimplantation mouse embryo are highly plastic

Underlying the establishment of pluripotent and other cell states in the mouse embryo is the remarkable plasticity of cells during the preimplantation period of mouse development. In contrast to the mosaic development observed in worms, frogs, flies and fish in which asymmetric localization of maternal determinants instructs development, the mouse embryo is highly adaptable to changes in context

and environment. For example, separation of the blastomeres of a two-cell stage embryo leads to the generation of two normal blastocysts, although with reduced cell number (Tarkowski et al., 1959). Likewise, aggregation of two 8-cell stage embryos generates a single, larger than normal embryo equally compromised of cells from each aggregated embryo (Mintz, 1964; Garner and McLaren, 1974).

Underlying the plasticity of cells of the preimplantation mouse embryo is the ability of individual cells to respond to particular signaling pathways that sense the environment and then act by activating or repressing transcription factors that regulate lineage identity. Thus defining the signaling pathways that underlie the plasticity of cells in the preimplantation mouse embryo will contribute greatly to our understanding of stem cell biology and provide new avenues with which to control pluripotent and other stem cell states both *in vivo* and *in vitro.*

Timeline of morphological changes and lineage establishment in the blastocyst

Lineage specification in the preimplantation mouse embryo occurs through a series of two lineage decisions. This series of lineage decisions occurs concurrently with other molecular and morphological milestones that provide a context by which to understand the environment that instructs lineage specification during preimplantation mouse development. In this section, I discuss the timing of developmental milestones in the preimplantation period of mouse development, including the establishment the EPI, PE and TE lineages.

After fertilization (E0.0), the mouse zygote begins development with series of cleavage divisions that result in the accumulation of progressively smaller cells. The

earliest cleavage division relies on proteins and transcripts deposited into the oocyte by the mother during oogenesis. At the 2-cell stage (E1.5), maternal transcripts are destabilized and the zygotic genome is activated (Schultz, 1993). At the 8-cell stage (E2.5), cells on the outside of the embryo epithelialize and form adherens and tight junctions with neighboring cells in a process called compaction. Compaction generates the first asymmetry in the mouse embryo along the apical-basal axis of epithelialized outside cells by the localization of apical polarity components to the apical side of outside blastomeres. At the 16-cell stage (E3.0), the second asymmetry of the mouse embryo is established along the inside-outside axis of the embryo. Together, these two asymmetries lead to establishment of the TE lineage from cells on the outside of the embryo and the ICM from cells on the inside of the embryo. Additional ICM cells are generated through two rounds of asymmetric cell divisions in outside cells, the first round occurring during the transition from the 8 to the 16-cell stage (E3.0 – E3.25), the second round occurring during the transition from the 16 to the 32-cell stage (E3.25 – E3.5). After E3.5, additional ICM cells are generated through symmetric divisions within the ICM. While symmetric cell divisions within the ICM also occur during the period in which ICM cells are predominantly generated from asymmetric divisions from outside cells, this transition in the mechanism by which ICM cells are generated reflects the progressive commitment of outside cells to TE cell fate. At the 32-cell stage (E3.5), TE cells pump ions into the inside of the embryo and the blastocoel cavity is generated by the flow of fluid across outside cells to balance ion concentrations inside and outside of the embryo. At this stage, after formation of the blastocoel cavity, the mouse embryo is referred to as a blastocyst. Subsequent to formation of the blastocyst, the ICM

begins the process of generating the EPI and PE cell lineages. EPI and PE cells emerge intermixed within the ICM, as evidenced by live imaging studies using fluorescent reporters of lineage identity (Plusa et al., 2008) and single cell transcriptome analysis (Kurimoto et al., 2006; Guo et al., 2010). After the embryo reaches the 64-cell stage EPI and PE cells undergo a process called cell sorting in which EPI and PE cells undergo rearrangement so that PE cells line the blastoceal cavity and surround the EPI. The blastocyst continues to expand both in size and cell number and at the >100 cell stage implants into the uterus of the mother. After implantation, the EPI undergoes gastrulation to form the endoderm, mesoderm and ectodermal cell lineages. The PE will give rise to parietal (ParE) and visceral endoderm (VE) and eventually the yolk sack while the TE will give rise to the ectoplacental cone and placenta. Molecular and morphological milestones in the preimplantation period of development are diagramed in Fig. 1A.

Isolation of stem cell lines from the mouse blastocyst

After completion of the developmental milestones of the preimplantation period of development described in the last section, cells of the EPI, PE and TE lineages are in a unique cell state in which they await environmental cues (e.g. implantation) to undergo further differentiation. Accordingly, under the appropriate environmental conditions, pluripotent and multipotent stem cells can be derived from the mouse blastocyst at this stage and these unique cell states can be extended indefinitely. (Fig. 1B). Trophoblast stem (TS) cells are derived from the trophectoderm (Tanaka et al., 1998). TS cells grow as flat colonies, required FGF4 for their maintenance and can self-renew or differentiate into specialized trophectoderm derivatives, such

as giant cells (Yan et al., 2001). Extraembryonic endoderm (XEN) cells are derived from the PE (Kunath et al., 2005). XEN cells grow as individual migratory cells and contribute to the parietal endoderm when injected into blastocyst stage embryos. Embryonic stem (ES) cells are derived from the EPI (Martin., 1981; Evans and Kaufman., 1981). ES cells are pluripotent, meaning they can give rise to any cell type in the fetus/adult, and require the cytokine LIF to maintain their pluripotency. Not only do TS, ES and XEN stem cell lines serve as important tools by which to investigate the establishment and maintenance of pluripotent and other stem cell states, the ability to derive each of these stem cell lines provides a functional readout of the developmental potential of mutant embryos. For example, ES and XEN cells cannot be derived from *Oct4* null, *Sox2* null or *Nanog* null embryos and this phenotype reflects the importance of OCT4, SOX2 and NANOG in establishing and regulating the ICM (Nichols et al., 1998; Avilion, 2003; Messerschmidt et al., 2010). This phenotypic commonality between *Oct4* null, *Sox2* null and *Nanog* null embryos has led to the model that OCT4, SOX2 and NANOG perform similar roles in the mouse embryo. In subsequent chapters of this thesis, we define the role of OCT4 and SOX2 in the preimplantation mouse embryo and find overlapping and distinct roles for these factors in establishing and maintaining all three cell lineages in the preimplantation mouse embryo.

To summarize, the preimplantation mouse embryo passes through a series of molecular and morphological milestones that establish three distinct cell lineages, the EPI, PE and TE. After establishment but prior to implantation, the EPI, PE and TE cell lineages are poised in a unique cellular state in which they retain developmental potency and await differentiation cues. Although EPI, PE and TE cell

states exist only transiently during development, cells resembling EPI, PE and TE can be extracted from the embryo and propagated indefinitely *in vitro*. Thus, by understanding how EPI, PE and TE cell fates are established and maintained *in vivo* we can gain insight into the stem cell state *in vitro*. Although the precise mechanisms and molecules that establish and maintain the EPI, PE and TE cell lineages are broadly unclear, it is well accepted that the establishment of these three preimplantation lineages occurs through two temporally separated lineage decisions. I will now discuss models and key molecular players in the two lineage decisions of the preimplantation period of mouse development, beginning with the first lineage decision that establishes the TE and the ICM.

1.2 The first preimplantation lineage decision establishes the TE and the ICM

The first lineage decision in mouse development establishes the TE and ICM cell lineages. This first lineage decision dramatically alters developmental potential of cells in the embryo; cells of the TE lineage will loose their potential to make cell types of the fetus, and likewise cells of the ICM will loose the potential to make cells of the TE lineage. Additionally, establishment of the ICM is the first step in setting aside a population of cells to become pluripotent. Thus, understanding how TE and ICM cell fates are established is essential to understanding how pluripotency is established.

While there is a general consensus that cell polarity and cell position are key determinants in ICM and TE cell fates, it is less well agreed upon what role, if any, maternal determinants regulate the first cell lineage decision. In this section, I will discuss the key models involved in the first cell lineage decision, followed by regulatory relationships between the molecules that regulate TE and ICM cell fate.

Models of TE and ICM cell fate specification

Understanding the cell extrinsic and cell intrinsic cues that regulate the first preimplantation cell lineage decision is essential to understanding the establishment and maintenance of EPI, PE and TE cell states. Three models exist for the molecular processes that act upstream of transcription factor activation to establish TE and ICM identity. The 'inside-outside' or positional model (Tarkowski and Wroblewska, 1967) proposes that TE and ICM cell fates are determined by the position of cells after embryo compaction. The 'inside-outside' model is supported by experiments in which blastomeres from a 32-cell stage (E3.5) embryo were repositioned from the inside of the embryo to the outside or vice-versa. Remarkably, repositioned blastomeres took on TE or ICM identities based on their new position (Suwinska et al., 2008). Additionally, after removal of the TE cells on the outside of the ICM by immunosurgery, ICM cells positioned on the outside of the ICM loose ICM identity and acquire TE identity (Spindle, 1978; Rossant and Lis, 1979). These experiments demonstrate that position along the inside-outside axis of the embryo is sufficient to determine lineage identity and that TE and ICM cells are constantly integrating positional information to establish TE identity on the outside of the embryo and ICM identity on the inside of the embryo.

The 'cell polarity' model (Johnson and Ziomek, 1981) proposes that asymmetric inheritance of cell polarity components controls TE and ICM cell fates. In this model, each blastomere of the 8-cell stage embryo is polarized such that the apical domain is facing the surface of the embryo and the basal domain is positioned within the embryo. In subsequent divisions, when the cell division plane is parallel to

the apical-basal axis of the embryo (conservative division), cell polarity components are divided equally amongst daughter cells and both cells become TE. When the cell division plane is perpendicular or skewed to the apical-basal axis of the embryo (differentiative division), the outside daughter cell inherits the majority of cell polarity components and becomes TE, while the inside daughter cell inherits less polarity components and becomes ICM. This model is supported by experiments in which disruption of apical-basal polarity by knockdown of the apical complex component *Par3* in one blastomere of a 4-cell stage embryo leads to *Par3* knockdown blastomeres preferentially contributing to the ICM (Plusa et al., 2005). Additionally, knockdown of the apical-basal polarity component *Pard6b* at the zygote stage leads to embryo collapse prior to the blastocyst stage (Alarcon et al., 2010).

It is important to note that the 'inside-outside' and 'cell polarity' models are not mutually exclusive and that it is widely accepted that both models contribute to the establishment of TE and ICM cell fates during the first preimplantation lineage decision. What is less agreed upon is the order in which cell polarity and position act to specify TE and ICM. The order in which cell polarity and cell position act to determine TE and ICM cell fates is a difficult issue to address experimentally, as outside cells always become polar.

Two recent studies suggest that the order in which cell position and cell polarity act to determine TE and ICM cell fate may be secondary to a third model in which cell fate is determined by maternally provided factors inherited asymmetrically in the first and subsequent cell divisions of the mouse embryo. The first study proposes that *Cdx2* mRNA is asymmetrically localized by the two-cell stage and subsequently asymmetrically inherited with apical polarity components in cell

divisions from the 8-cell to 16-cell stage (Skamagki et al., 2013, Jedrusik et al., 2008). The second study proposes that each blastomere at the 2-cell stage exhibits differential kinetics of nuclear export of the pluripotency factor OCT4 and that this difference determines lineage contribution, with cells exporting OCT4 quickly contributing to the TE, and blastomeres exporting OCT4 slowly contributing to the ICM (Plachta et al., 2011). Critical roles for CDX2 and OCT4 in TE and ICM cell fates prior to the establishment of positional asymmetry and polarity are supported by studies in which knockdown of CDX2 and OCT4 by RNA interference (RNAi) led to embryos that fail to form the blastocyst and in the case of *Cdx2* fail to establish cell polarity (Jedrusik et al., 2010; Foygel et al., 2008). However, many phenotypes in RNAi knowdown mouse embryos appear similar and it is difficult in preblastocyst embryos to distinguish between a specific role in TE and ICM cell fate and embryo arrest. Moreover, a model involving maternal determinants is in direct conflict with the observed plasticity of the mouse embryo. Resolving these two conflicting models is essential to understanding the events downstream of the first preimplantation cell lineage decision that establishes pluripotency.

To this end, in chapter 2 and 3 of this thesis I take an alternative approach to address the role of maternally provided *Cdx2* and *Oct4* without experimental manipulation of the embryo. In these chapters, based on my experimental evidence I argue that *Cdx2* and *Oct4* are not maternal determinants but instead play important, and in the case of *Oct4*, multiple important roles, in both the first and second cell lineage decision of the preimplantation period of mouse development.

Signaling pathways and transcription factors regulating TE and ICM cell fates

While the order of events that lead to TE and ICM cell fate is not yet resolved, much more is known about the signaling pathways and transcription factors that regulate the first preimplantation lineage decision. These signaling pathways and transcription factors set up relationships between master regulators of cell fate that are essential for subsequent lineage decisions and furthermore provide an excellent case study in how environmental cues are integrated to establish molecular differences amongst cells that ultimately regulate cell fate.

The transcription factors YAP and TEAD4 are essential for the establishment of TE identity (Yagi et al., 2007; Nishioka et al., 2008). Curiously, YAP and TEAD4 are present in both inside and outside cells (Nishioka et al., 2009) and therefore it is their regulation, and not their presence that determines TE cell fate. In fact, the HIPPO signaling pathway regulates YAP activity in inside and outside cells through Lats1/Lats2 kinase, which in response to elevated HIPPO signaling, phosphorylates YAP, which then causes YAP to be excluded from the nucleus. TEAD4 must partner with YAP to activate its transcriptional targets and thus in cells where HIPPO signaling is high, YAP is phosphorylated and exluded from the nucleus leaving TEAD4 unable to activate its transcriptional targets. Thus in the embryo, nuclear localized YAP provides a functional readout of HIPPO signaling activity. Downstream of TEAD4, CDX2 and GATA3 act in parallel to promote TE identity by activating common and independent targets (Ralston et al., 2010). Additionally, CDX2 represses ICM identity by repressing *Oct4* (Ralston and Rossant., 2008; Ralston et al., 2010; Strumpf et al., 2005). *Cdx2* null embryos form the blastocyst but collapse shortly there after (Strumpf et al., 2005), suggesting *Cdx2* is not required for specification of the TE, but instead required for TE maintenance. In

contrast, *Gata3* null embryos have no overt TE phenotype at the blastocyst stage (Ma et al., 1997), possibly owing to redundancy with *Gata2,* which is also expressed in the TE. Factors that act later to promote TE development include the T-box transcription factor *Eomes* (Russ et al., 2000) and the Ets-family transcription factor *Elf5* (Donnison et al., 2005).

The establishment of ICM identity is less well understood. The pluripotency factor OCT4 is initially expressed in inside and outside cells and then restricted to inside cells after blastocyst formation, where it represses *Cdx2* in the ICM (Ralston et al., 2010). Similar to OCT4, the pluripotency factor NANOG is also ubiquitous and then restricted to inside cells after blastocyst formation (Chambers et al., 2003). Restriction of OCT4 and NANOG to the ICM is dependent on repression by CDX2 in TE cells. Unlike OCT4 and NANOG, SOX2 is restricted to inside cells prior to blastocyst formation (Guo et al., 2010). Recently the Ralston Lab has shown that restriction of SOX2 to inside cells is dependent on HIPPO signaling activity and independent of *Cdx2* (Wicklow et al., *in revision*). Thus, at least two molecular mechanisms act to restrict pluripotency factors in the embryo, prior to blastocyst formation restriction of SOX2 by HIPPO signaling pathway components, and after blastocyst formation restriction of OCT4 and NANOG by CDX2.

The first lineage decision establishes relationships between master regulators of cell fate that are essential to the interpretation of phenotypes and gene function in the preimplantation mouse embryo. For example, the inability to outgrow ES and XEN cells from *Oct4* null, *Sox2* null and *Nanog* null embryos could either reflect genuine roles for OCT4, SOX2 and NANOG in EPI and PE cell fate, or alternatively

could be the consequence of the inability to establish ICM cell fate at the first lineage decision and thus only reflect a role for OCT4, SOX2 and NANOG in establishing ICM cell fate and not in establishing EPI and/or PE cell fate. In chapter 3 and 4 of this thesis, I examine the establishment of ICM cell identity in *Oct4* null and *Sox2* null embryos and based on my evidence argue that *Oct4* and *Sox2* are not required for ICM cell fate but instead have essential roles in promoting EPI and PE cell fates.

1.3 The second preimplantation lineage decision establishes the EPI and PE.

The second lineage decision in the preimplantation mouse embryo establishes the EPI and PE lineages from the ICM. Although when initially specified all ICM cells have the potential to become pluripotent, only a subset actually become pluripotent and retain the potential to make a diverse set of cell types of the ectoderm, endoderm and mesoderm cell lineages. The remaining ICM cells will become multipotent PE, which can only differentiate into a few cell types. Thus the mechanisms and molecules that control EPI and PE cell fate are of great interest to understanding how developmental potential is regulated, as well as understanding how pluripotency is established. Here I present current models for EPI and PE cell fate specification, followed by key molecules that regulate EPI and PE cell fates.

Models of EPI and PE cell fate specification

In contrast to specification of TE and ICM, cell position and cell-cell contact are not known to influence the segregation of the EPI and PE cell lineages. In fact, EPI and PE cell fates emerge within the ICM in an intermixed pattern that is unique from embryo to embryo. At blastocyst formation, ICM cells express markers of both the

EPI and PE lineage. Over time, overlapping EPI and PE marker expression resolves into mutually exclusive expression of EPI and PE markers. Two models have been proposed for how EPI and PE cells are specified.

In the first model, the level of FGF/MAPK signaling experienced by an individual ICM cell determines EPI and PE cell fates. ICM cells that experience a high level of FGF/MAPK signaling become PE, while cells that experience a low level of FGF/MAPK signaling become EPI. A critical role for FGF/MAPK signaling pathway in PE development is supported by the lack of PE in *Fgf4* null, *Fgfr2* null and in embryos null for the FGF/MAPK signaling transducer *Grb2* (Kang et al., 2013; Arman et al., 1998; Chazaud et al., 2006). Likewise, addition of FGF4 to embryos during the PE and EPI cell fate decision directs all ICM cells towards the PE lineage (Yamanaka et al., 2010). Additionally *Fgf4* null, *Grb2* null embryos (Kang et al., 2013, Chazaud et al., 2006) and FGF4 treated embryos (Yamanka et al., 2010) contain wild-type amounts of ICM cells, suggesting that gain or loss of function of the FGF/MAPK pathway influences EPI and PE cell fate rather than supporting EPI and/or PE cell survival.

Although the FGF/MAPK signaling pathway clearly plays an essential role in PE cell fate, it is less well understood how differential levels of FGF/MAPK signaling is established among ICM cells. Establishment of differential levels of FGF/MAPK signaling amongst ICM cells is essential to ensure the proper balance of EPI and PE cells are specified. The emergence of EPI and PE cells intermixed among the ICM argues against localization of FGF4 to any particular region of the ICM. One possible mechanism is that components of the FGF/MAPK signaling pathway are differentially distributed throughout ICM cells. Indeed, analysis of gene expression in

single E3.5 ICM cells has demonstrated that *Fgfr2* and *Fgf4* expression is heterogeneous amongst ICM cells (Guo et al., 2010). However, what acts upstream to establish differential *Fgfr2* and *Fgf4* expression is unknown. One possibility is that other signaling pathways that regulate EPI and PE cell fate are active in the ICM. In support of other EPI and PE regulating signaling pathways BMP signaling and NODAL signaling have been demonstrated to be active in the ICM during EPI and PE cell fate specification (Coucouvanis et al., 1999; James et al., 2005) and embryos lacking *Smad4,* a transcription factor activated by BMP signaling have a reduced number of PE cells (Sirard et al., 1998). In chapter 3, I present several lines of evidence that support additional signaling pathways in the ICM during the resolution of ICM cell fate.

An additional model for how PE and EPI identity is determined is that the timing of entry into the ICM determines EPI and PE identity, with cells that enter during the first round of ICM generating asymmetric divisions biased towards EPI cell fate, and cells generated during the second round of asymmetric cell divisions biased towards PE cell fates. Two studies have used live imaging to determine if timing of entry into the ICM biases cells towards a particular lineage and have formed contradictory conclusions with one study arguing there is no relationship between the timing of entry into the ICM and EPI or PE cell fate (Yamanaka et al., 2010) and the other arguing that timing of entry into the ICM does bias ICM cells towards EPI and PE cell fate (Morris et al., 2010). Regardless of whether timing of entry into the ICM biases ICM cells towards EPI or PE cell fate, because timing of entry into the ICM does not absolutely predict EPI or PE cell fate, other factors, such as FGF/MAPK signaling must be involved.

One intriguing possibility is that the timing of entry into the ICM influences the heterogeneity observed in levels of *Fgfr2* transcription throughout the ICM. For example, timing of entry into the ICM could determine inheritance of *Fgfr2* transcriptional potential or FGFR2 from parent TE cells, thereby biasing cells towards EPI and PE without absolutely determining cell fate. The model that timing of entry determines inheritance of *Fgfr2* transcriptional potential has recently been supported by studies that demonstrate *Fgfr2* expression levels in single E3.5 ICM cells correlates with timing of entry into the ICM (Morris et al., 2013; Krupa et al., 2014). How differential inheritance of *Fgfr2* transcripts or transcriptional potential is controlled during each round of asymmetric ICM generating cell divisions is an exciting area for future research the may bridge cell polarity mechanisms active at the first cell lineage decision with EPI and PE cell fate at the second cell lineage decision.

Clearly, multiple molecular events regulate the second preimplantation cell lineage decision, such as the potential for response to FGF/MAPK signaling of a single ICM cell and the actual FGF/MAPK signaling experienced by a single ICM cell. Because the second lineage decision controls the balance of pluripotency within the ICM it is essential to understand the molecules involved in the second lineage decision that promote or repress EPI and PE cell fate.

Molecular regulation of EPI and PE cell fate specification

NANOG, an essential factor for the EPI lineage (Chambers et al., 2003; Messerschmidt et al., 2010; Frankenberg et al., 2011) and GATA6, an essential

factor for the PE lineage (Morrisey et al., 1998) are initially coexpressed in all ICM cells in the E3.5 blastocyst. The first step in establishing EPI and PE cell identity within the ICM is repression of NANOG in approximately half of ICM cells, leading to a 'salt-and-pepper' pattern of NANOG. Salt-and-peppering of NANOG requires FGF/MAPK activity and accordingly, inhibition of FGFR and MAPK using small molecules during the period at which NANOG is salt-and-peppered leads to NANOG expression throughout the ICM (Nichols et al., 2009). Through an unknown process, NANOG acquires the ability to repress GATA6 between E3.5 and E3.75 (Frankenberg et al., 2011) leading to salt-and-peppering of GATA6 such that NANOG and GATA6 expression become mutually exclusive within the ICM by E3.75.

During the period in which NANOG and GATA6 are undergoing salt-and-peppering, PDGFRα and SOX17 expression is initiated within the ICM. PDGFRα is initially coexpressed with NANOG but after salt-and-peppering is only expressed in NANOG-negative cells (Plusa et al., 2008; Niakan et al., 2010). Relative to PDGFRα, SOX17 expression is initiated in fewer cells and the degree to which it overlaps with NANOG expression during the early phase of SOX17 expression has not been examined suggesting that PDGFRα and SOX17 are initiated by independent mechanisms. As PE and EPI development proceeds, the expression domain of PDGFRα and SOX17 expands into all NANOG-negative cells. Concurrently to the increase in PDGFRα and SOX17 in NANOG-negative cells, GATA4 expression is initiated in NANOG-negative cells. During EPI and PE sorting, SOX7 expression is initiated in PE cells correctly positioned at the blastocoel cavity (Artus et al., 2011). Thus, based on the expression patterns of currently known EPI and PE markers it appears that the EPI is specified through the restriction of NANOG

to the EPI, and the PE is specified through the restriction of GATA6 to the PE followed by the accumulation of multiple other PE specific factors.

Intriguingly, the pluripotency factor OCT4 is detected in both EPI and PE cells throughout this process, however, it is unknown to what degree *Oct4* is required in the EPI and the PE and why OCT4 is not restricted to the pluripotent compartment of the ICM like the other pluripotency factor NANOG. One intriguing possibility is that *Oct4* promotes EPI and PE cell fate through cell type specific roles. In support of this hypothesis EPI-like and PE-like cells can not be outgrown from *Oct4* null embryos (Nichols et al., 1998). Because *Oct4* is thought to be at the core of the transcriptional network that confers pluripotency, an essential role for *Oct4* in a non-pluripotent lineage would have profound implications for pluripotent cell biology. In chapter 3, I test the hypothesis that *Oct4* promotes PE cell fate and based on my evidence conclude that *Oct4* is required cell-autonomously for PE cell fate in the preimplantation mouse embryo.

A network of factors regulate PE development

The PE phenotypes of *Gata6* null, *Gata4* null, *Pdgfr*α null, *Sox17* null and *Sox7* null embryos vary in their severity indicating different requirements for *Gata6, Pdgfr*α*, Sox17 and Sox7* in PE cell fate. To properly infer gene function from null embryos with defects in the PE lineage it is essential to understand the relationships between factors in the network that promotes PE development. GATA6 and GATA4 are required for formation of the PE (Koutsourakis et al., 1999; Morrisey et al., 1998) suggesting that *Gata6* and *Gata4* are essential genes in the PE lineage.

Both *PDGFRα* null and *Sox17* null embryos specify normal numbers of PE cells indicating that neither gene alone is essential for PE development. However, XEN cells cannot be derived from *Sox17* null or *Pdgfr*α null embryos and both *Pdgfr*α null and *Sox17* null embryos fail to maintain normal amounts of PE cells when preimplantation development is extended by induction of diapause (Artus et al., 2010; Artus et al., 2011), suggesting that while not essential for PE specification *Sox17* and *Pdgfrα* contribute to the survival or maintenance of PE cells. Additionally, *Pdgfrα* is not required for *Sox17* expression and vice-versa, and therefore do not regulate each other.

Importantly, FGF/MAPK signaling does not appear to be required for the initiation of *Gata6, Gata4, Pdgfrα* or S*ox17* expression but instead is required for the expansion of *Gata4, Pdgfrα* and *Sox17* expression domains and the maintenance of *Gata6* expression (Kang et al., 2013; Krawchuk et al., 2013). This places FGF/MAPK signaling downstream of the initiation of these important PE regulators. But what is the source of FGF in the preimplantation mouse embryo? Insight into this question comes from studies of the *Nanog* null phenotype. *Nanog* null fail to give rise to both EPI-like and PE-like cells (Silva et al., 2009), functionally demonstrating that both EPI and PE development requires *Nanog.* However, NANOG is only expressed in the EPI during the formation of the PE leading to the hypothesis that NANOG must be regulating PE cell fate non-cell autonomously. Supporting a non-cell autonomous requirement for *Nanog* in PE cell fate, injection of 'EPI-like' ES cells into the blastocyst rescues *Nanog* null PE, demonstrating that *Nanog* promotes PE development non-cell autonomously and that EPI and ES cells secret a *Nanog*-dependent factor essential for PE cell fate (Messerschmidt et al.,

2010). Subsequent studies demonstrated that high levels of FGF4 alone could replace ES cells and still lead to rescue of *Nanog* null PE and thus it was likely EPI cells that are the source of FGF4 in the ICM (Frankenberg et al., 2011). This model has further been confirmed by single-cell studies demonstrating that high levels of *Fgf4* transcription is associated with high levels of *Nanog* transcription (Guo et al., 2010), and *in situ* hybridization studies in which *Fgf4* transcript localizes to NANOG-positive cells (Frankenberg et al., 2011). Thus, PE fate is dependent of establishment of FGF4-secreting EPI.

The dependence of PE on EPI establishment posits an intriguing model for how the relative balance of EPI and PE precursors is controlled during development. Because the amount of PE inducing FGF4 is linearly related to the number of FGF4-secreting EPI cells, and because EPI and PE cell fate is plastic, the amount of PE cells present will directly correlate to the number of EPI cells present. To gain insight into how the balance of EPI and PE cells is controlled in the embryo, evaluation of mutant embryos with defects in FGF4 regulation will be invaluable.

Additionally, in ES cells, an *in vitro* model of the EPI, *Fgf4* appears to be co-regulated by NANOG, SOX2 and OCT4 (Yuan et al., 1995; Ambrosetti et al., 1997, Shi et al., 2011), and thus examining PE development in the preimplantation mouse embryo provides an excellent opportunity to functionally validate a role for NANOG, SOX2 and OCT4 in promoting *Fgf4* in pluripotent cell types other than ES cells.

ICM cell plasticity and lineage commitment

Perturbation of the signaling environment during the resolution of EPI and PE cell fate has demonstrated that despite initial differences in NANOG and GATA6, as well

as *Fgfr2 and Fgf4* levels, ICM cell fate is highly plastic. For example, stimulation or inhibition of FGF/MAPK signaling alters ICM cell fate as late as E3.75 and even in some ICM cells as late as E4.0, demonstrating that EPI and PE cell fate commitment occurs gradually between E3.75 and E4.0 (Nichols et al., 2009; Yamanaka et al., 2010). Furthermore, manipulation of FGF/MAPK signaling prior to E3.75 can alter cell fate, but if embryos are returned to normal FGF/MAPK signaling conditions EPI and PE cell fate develop normally, suggesting that the mechanisms that balance the number of EPI and PE cells arising from the ICM are not affected by the history of FGF/MAPK signaling levels (Yamanaka et al., 2010). Finally, transplantation of older EPI and PE cells to younger blastocysts corroborates the model that EPI and PE cell fate commitment occurs gradually around E4.0 and also demonstrates that sustained FGF/MAPK signaling is required for the process of fate commitment (Grabarek et al., 2012). Despite the remarkable plasticity of ICM cells, lineage switching is a rare event (Plusa et al., 2008), thus the importance of ICM cell plasticity is currently not clear.

In conclusion, the preimplantation period of development is an excellent system in which to study mechanisms that regulate cell fate and lineage commitment in pluripotent and other cell types. The process of forming the blastocyst is rich with complex molecular and cell processes that coordinate to establish three molecularly distinct lineages, while concurrently conferring a remarkably robust ability to respond to changing environmental cues. There is no doubt that insight from the preimplantation period of mouse development will provide important insight into developmental biology and stem cell biology.

1.4 Contributions to research presented

Chapter 2 is a modification of the following published manuscript:

*Blij S., *Frum T., Akyol A., Fearon E., Ralston A, (2012). Maternal Cdx2 is dispensable for mouse development. *Development,* 139(21), 3969-72. (*equal contribution)

Conceptual framework for this study was the result of scientific discussion between AR, SB and TF. All experiments were preformed by SB and TF. AR, SB and TF made the figures. AR wrote the text of the original manuscript, which was edited by SB and TF. TF wrote the modified version of the manuscript presented here, which was edited by AR.

Chapter 3 is a modification of the following published manuscript:

Frum, T., Halbisen, M. A., Wang, C., Amiri, H., Robson, P., & Ralston, A. (2013). Oct4 cell-autonomously promotes primitive endoderm development in the mouse blastocyst. *Developmental cell*, *25*(6), 610-622.

Conceptual framework for this study was the result of scientific discussion between AR, MH and TF. All experiments were performed by TF with the exception of the RNA sequencing, in which embryos were collected by TF, RNA-seq was performed by CW under the guidance of PR and RNA-seq data was analyzed by MH. TF made

the figures, with the exception of Figure 8, which was made by MH, PR and AR and Figure 9, which was made by AR. AR wrote the text of the original manuscript, which was edited by TF. TF wrote the modified version of the manuscript presented here, which was edited by AR. The data presented in Figure 7L and Figure 10 and are not part of the published manuscript and have yet been published.

Chapter 4 is a modification of the following manuscript currently in revision for PLOS genetics:

*Wicklow E, *Blij S, Frum T, Hirate Y, Lang R, Sasaki H, Ralston A. (*in revision)* HIPPO pathway members restrict SOX2 to the inner cell mass where it promotes primitive endoderm development in the mouse blastocyst. *PLOS Genetics.* (*equal contribution)

Conceptual framework for this study was the result of scientific discussion between AR, EW, SB and TF. The majority of experiments performed by EW and SB are presented as background in the introduction section. TF performed all the experiments presented in the results section, with the exception of Chapter 4, Figure 1A – E, which provide context for my contribution. The original manuscript was written by AR and edited by EW, SB and TF. TF wrote the modified version of the manuscript presented here, which was edited by AR.

1.5: Figures and Legends

Figure 1: Developmental potential during the preimplantation period of mouse development

A) Time course of mouse preimplantation development showing early cleavage divisions and establishment of preimplantation cell lineages. Major developmental milestones are denoted by a filled black arrowhead and description of developmental milestone. Note: E denotes days after coitus. Example: E1.5 = 36 hrs after coitus.

B) Origin and derivation of stem cell lines from three preimplantation cell lineages.

C) Legend for A and B. Note: color refers to lineage potential.

Chapter 1, Figure 1

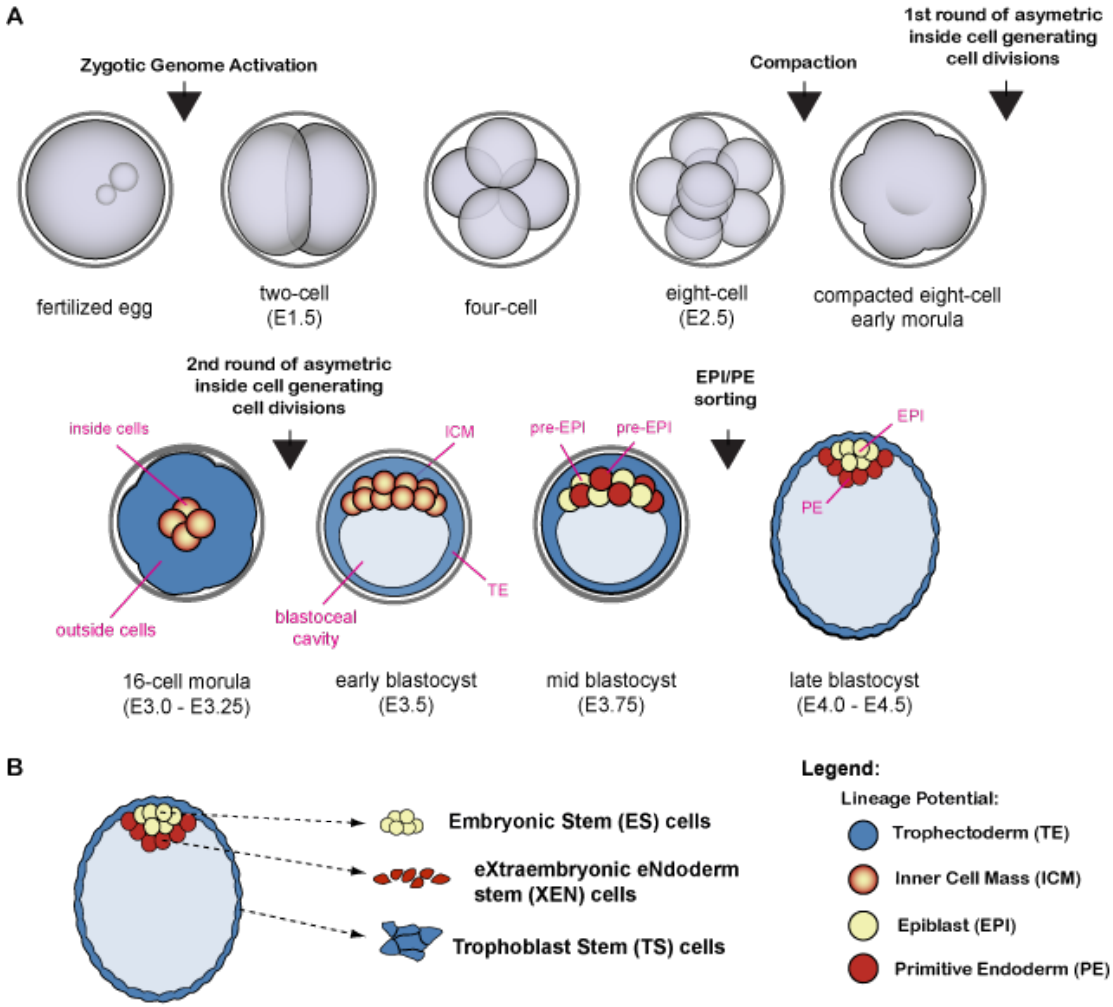


Figure 2: Expression patterns of TE, EPI and PE markers during the preimplantation period of mouse development.

Expression patterns of preimplantation lineage markers are shown. Grey = non-expressing cell, Amber = cell expressing indicated marker.

A) Expression of CDX2 is initiated in all cells at the 16-cell stage. CDX2 expression becomes restricted to TE lineage.

B) Expression of OCT4 is initiated in all cells at the 16-cell stage. OCT4 expression becomes restricted to the ICM.

C) Expression of SOX2 is initiated in inside cells at the 16-cell stage. SOX2 expression becomes restricted to the EPI.

D) Expression of NANOG is initiated in all cells at the 16-cell stage. NANOG expression becomes restricted to the EPI.

E) Expression of GATA6 is initiated in all cells at the 16-cell stage. GATA6 expression becomes restricted to the PE.

F) Expression of SOX17 is initiated at the early blastocyst stage. SOX17 expression is restricted to the PE.

G) Expression of GATA4 is initiated at the mid blastocyst stage. GATA4 expression is restricted to the PE.

H) Expression of SOX7 is initiated at the late blastocyst stage. SOX7 expression is restricted to PE cells lining the blastoceal cavity.

Chapter 1, Figure 2

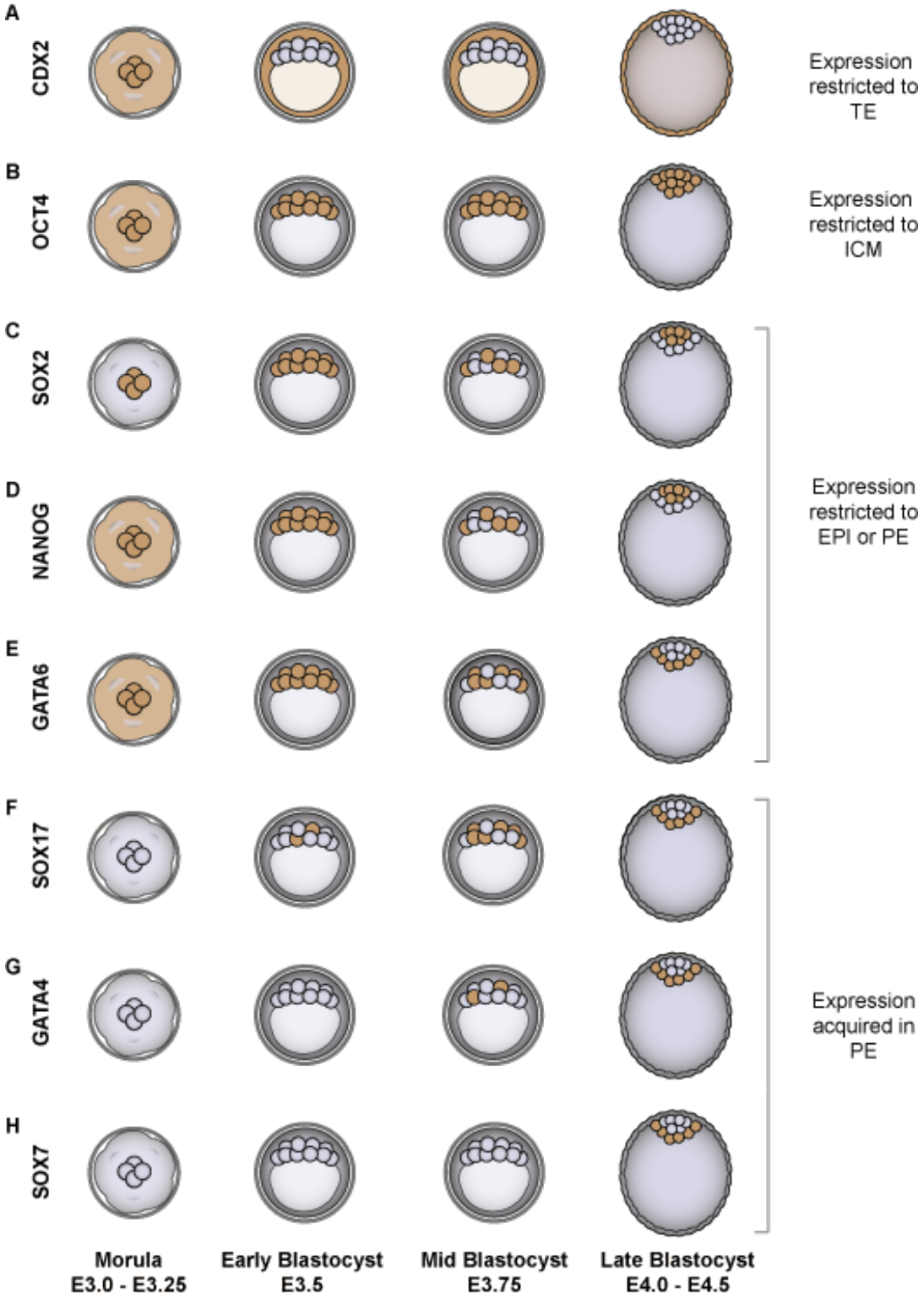
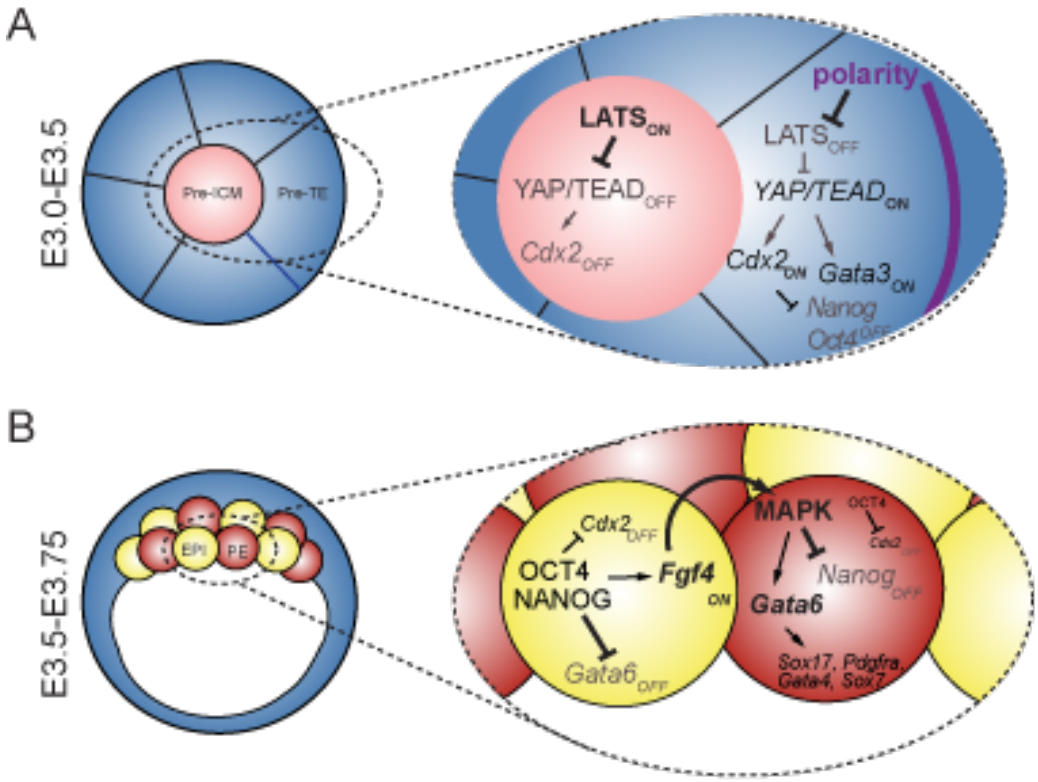


Figure 3: Models of cell fate specification in the preimplantation mouse embryo

A) The 1st lineage decision during mouse development establishes the TE and the ICM. In outside cells, cell polarity and/or cell-cell contact represses LATS1/2, relieving repression of YAP/TEAD activity. YAP/TEAD turns on *Cdx2* and *Gata3.* TE cell fate is reinforced through *Cdx2*-dependent repression of *Oct4.*

B) The 2nd lineage decision during mouse development establishes the EPI and PE. In the EPI, NANOG represses *Gata6* and, together with OCT4, promotes transcription of *Fgf4*. FGF4 is secreted from the EPI to the PE where it acts on through the FGF/MAPK signaling pathway to repress *Nanog* and promote *Gata6.* OCT4 represses transcription of *Cdx2* in the ICM.

Chapter 1, Figure 3



CHAPTER 2

Maternal *Cdx2* is not required for mouse development

2.1 Abstract

In many invertebrate and vertebrate species, cell fates in the early embryo 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-like family 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.

2.2 Introduction

Whether maternal factors regulate cell fate in the preimplantation mouse embryo is an active debate in mammalian developmental biology. Pre-patterning of the oocyte by maternal effect genes occurs in many metazoans including worms, frogs and insects and fish. For example, in the fly, *caudal* mRNA is asymmetrically localized to one pole of the syncitial embryo. When the syncitial embryo undergoes

cellularization, cells at one pole of the embryo inherit greater amounts of *caudal* mRNA than cells at the other end of the embryo. This gradient of *caudal* mRNA defines the anterior-posterior axis of the fly embryo through regulating posterior and anterior cell fate. Intriguingly, a mammalian homolog of *Caudal,* the caudal-related homeobox gene *Cdx2* is at the center of the debate about whether maternally provided factors regulate cell fate in the mammalian embryo. *Cdx2* null embryos form polarized outside cells and form the blastocyst but collapse shortly after blastocyst formation and fail to repress the pluripotency factors *Oct4* and *Nanog* in outside cells (Strumpf et al., 2005; Ralston and Rossant, 2008). These results suggest that *Cdx2* acts downstream of TE specification to maintain TE cell identity by repressing factors that promote ICM cell fate (Ralston and Rossant, 2008; Ralston et al., 2010). However, *Cdx2* null embryos are generated by mating two mice that are heterozygous for a loss-of-function *Cdx2* null allele and thus, in these experiments if *Cdx2* is maternally provided to the embryo maternally provided *Cdx2 could* still be present in *Cdx2* null embryos and compensate for loss of zygotic *Cdx2* thereby masking an earlier requirement for *Cdx2* in the TE. Thus, elimination of both maternal and zygotic *Cdx2* is essential to understanding when and how *Cdx2* promotes TE cell fate.

Two studies from different labs have addressed the role of maternal *Cdx2* during mouse development by knocking down both maternal and zygotic *Cdx2* by RNAi and have and have produced contradictory results. One study (Wu et al., 2010), found that embryos in which *Cdx2* is knocked down in the oocyte established a polarized layer of outside cells, formed the blastocyst and collapsed prior to implantation, essentially phenocopying embryos lacking only zygotic *Cdx2*. This

study supports the interpretation of the *Cdx2* null phenotype, that *Cdx2* is required after TE specification for TE maintenance. A separate study, in which *Cdx2* was knocked down in the zygote shortly after fertilization reported that in contrast to the results of Wu et al, a significant proportion of *Cdx2* knockdown embryos did not form the blastocyst relative to control embryos (Jedrusik et al., 2010). Additionally, *Cdx2* knockdown embryos failed to properly polarize and initiate expression of *keratin 8* and *E-cadherin*, genes that are normally expressed specifically in the TE. Based on these results the author to concluded that *Cdx2* establishes polarity in outside cells and is therefore required for TE specification and not simply TE maintenance.

Although attempts to address the role of maternal *Cdx2* by RNAi knockdown have failed to conclusively demonstrate when and how *Cdx2* regulates cell fate in the preimplantation mouse embryo, both studies agree that *Cdx2* promotes TE cell fate and therefore directs cells away from the pluripotent cell state. Therefore, to understand how pluripotency and other cell states are established and regulated *in vivo* it is essential to clearly distinguish if *Cdx2* represses pluripotent lineage potential by establishing cell polarity, and/or if *Cdx2* represses pluripotent lineage potential by repressing the pluripotency factors OCT4 and NANOG. We suspect that the conflicting results obtained by *Cdx2* knockdown are due to the experimental manipulation and culture of the early embryo, and therefore believe that an approach which does not require removal of embryos from the uterus or embryo manipulation is required to unambiguously resolve when and how *Cdx2* acts to direct cells away from pluripotency and towards the TE lineage. In this chapter, I use a conditional allele of *Cdx2* and a female germline specific Cre to remove maternally provided *Cdx2* in the oocyte without manipulation of the embryo and demonstrate that *Cdx2* is

not required for the TE specification and that maternal *Cdx2* is not required for development.

2.3 Materials and methods

Mouse strains

The following alleles or transgenes were maintained in an outbred (CD1) background: *Cdx2*^{tm1Fbe} (a null allele) (Chawengsaksophak 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.

Embryo genotyping

Embryo genotyping was performed blind, without prior knowledge of phenotypes. 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 (5'-3'): wild type and *Cdx2*^{tm1Fbe}, AGGGACTATTCAAACTACAGGAG, TAAAAGTCAACTGTGTTCGGATCC and ATATTGCTGAAGAGCTTGGCGGC; *Zp3Cre*, GCGGTCTGGCAGTAAAAACTATC and GTGAAACAGCATTGCTGTCACTT; and wild type, *Cdx2*^{fl} and *Cdx2*^{del}, GAATACGTCGTGTAATTAGCA and CAAAGCCAACAACTGGAC

Immunofluorescence, embryo culture and microscopy

Embryos were collected from timed natural matings by flushing dissected oviducts or uteri with M2 medium (Millipore). Embryos were fixed and stained as previously described (Ralston and Rossant, 2008) or cultured in KSOM (Millipore) at 37°C and 6% CO_2 to monitor morphological development. Primary antibodies included mouse anti-CDX2 1/200 (Biogenex) rabbit anti-NANOG 1/400 (Reprocell), rat anti-CDH1 1/100 (Sigma) and rat anti-KRT8 1/100 (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, USA). Flourescence conjugated secondary antibodies were all used at 1/400 (Jackson Labs). Nuclei were stained with Draq5 (Cell Signaling). Confocal microscopy was performed in 5 um sections using a Leica SP5 confocal microscope and 20X, 0.7 NA objective.

Oocyte collection and real-time PCR

To obtain oocytes, female mice were superovulated by subcutaneous injections of 5 IU each pregnant mare serum (PMS) and human chorionic gonadotropin (HCG) (Sigma), 46 hours apart. MII oocytes were collected 23 hours after HCG injection from dissected ampullae. Oocytes were then denuded of cumulus cells by incubation and gentle pipetting in 300 μg/ml bovine type IV-S hyaluronidase (Sigma). RNA was extracted from ~20 pooled oocytes or individual blastocysts using the PicoPure RNA Isolation Kit (Arcturus) according to the manufacturer's instructions. cDNA was prepared using the Quantitech Reverse Transcription Kit (Qiagen) and PCR amplification (50 cycles) was performed using SYBR Green I Master (Roche) on the Roche LightCycler 480. For each primer pair, the PCR efficiency was empirically determined from a standard curve, and this was used to calculate

measurements using the ΔC_T methods provided by the Roche Lightcyler software. qRT-PCR Primers used were (5'-3'): *Actb,* CTGAACCCTAAGGCCAACC and CCAGAGGCATACAGGGACAG; *Cdx2,* AAACCTGTGCGAGTGGATG and TCTGTGTACACCACCCGGTA.

2.4 Results

Cdx2 transcript levels in oocytes are below a threshold likely to be functionally relevant.

In the mouse embryo, the maternal to zygotic transition takes place between the first and second cleavage divisions (Flach et al., 1982). Therefore, CDX2 protein and *Cdx2* transcript present prior to the 2-cell stage is likely to be of maternal origin. To determine if CDX2 is present in the oocyte, several studies have examined whether CDX2 is detectable in mouse ovaries and oocytes and found that CDX2 is undetectable, either by immunohistochemistry (Beck et al., 1995) or by mass spectrometry (Wang et al., 2010). Similarly, CDX2 is not detectable 2-cell stage (Ralston and Rossant, 2008) suggesting that CDX2 is not maternally provided. In contrast, *Cdx2* transcript is detectable in oocytes (Jedrusik et al., 2010; Wang et al 2010a) but whether the level of *Cdx2* transcript in oocytes is similar to tissues in which *Cdx2* is required has not been examined.

Cdx2 is required to maintain TE cell fate in the E3.5 mouse blastocyst, but is not required in ES cells (Chawengsaksophak et al., 2000). We reasoned that ES cells would provide a relevant comparison to determine a threshold for functional relevance of *Cdx2* transcript levels. To determine the functional relevance of *Cdx2*

transcript in the mouse oocyte we measured the level of *Cdx2* transcript in oocytes, E3.5 blastocysts and ES cells by quantification real-time PCR (qPCR). If *Cdx2* transcript levels are lower in mouse oocytes than in ES cells then this would suggest that *Cdx2* transcript detected in oocytes is below the level of functional relevance. Conversely, if the *Cdx2* transcript level in oocytes is comparable to *Cdx2* transcript level in the blastocyst then this would suggest that *Cdx2* transcript is present at a functionally relevant level. We detected *Cdx2* transcript in both oocytes and ES cells (Fig. 1A), however the level of *Cdx2* transcript in oocytes is more than ten fold lower than *Cdx2* transcript level present in ES cells and more than 100 fold lower than the level of *Cdx2* transcript in E3.5 blastocysts. Because *Cdx2* transcript levels in oocytes are lower than the level of *Cdx2* transcript in ES cells, we conclude that while *Cdx2* is maternally provided to the oocyte level of *Cdx2* in oocytes is below a threshold likely to be functionally relevant.

Maternally provided *Cdx2* is not required for development

Our qPCR analysis of the level of *Cdx2* transcript in oocytes suggest that *Cdx2* is detectable but present below a level of functional relevance, we hypothesized that maternal *Cdx2* is not required for development. To test this hypothesis we first generated *Cdx2* germline null femalem mice by mating female mice homozygous for a conditional allele *Cdx2* ($Cdx2^{fl}$) to male mice also homozygous for $Cdx2^{fl}$ and hemizygous for a transgene in which the coding sequence of Cre-recombinase is under control of the promoter of *the Zp3* gene (*Zp3-Cre)* to generate female mice homozygous for $Cdx2^{fl}$ and hemizygous for the *Zp3-Cre transgene*. *Zp3* is expressed only in the female germline and thus in $Cdx2^{fl/fl}$;Zp3-Cre females both

copies of *Cdx2* will be excised from the female germline, eliminating maternal *Cdx2* from the oocyte (Fig 1D).

To test if maternal *Cdx2* is required for development we first confirmed the elimination of *Cdx2* mRNA from the oocytes of *Cdx2*$^{fl/fl}$*; Zp3-Cre* females by qPCR (Fig. 1B). We then mated *Cdx2*$^{fl/fl}$*; Zp3-Cre* females to wild-type males (Fig. 1D, mating scheme #1) with the expectation that if maternal *Cdx2* is required for development we would observe smaller average litter sizes when mating of *Cdx2* germline null females to wild-type males than from matings of wild-type animals. We obtained comparable numbers of viable offspring from *Cdx2*$^{fl/fl}$*; Zp3-Cre* females crossed to wild-type males as the number of viable offspring resulting crosses from crosses involving only wild-type mice. To confirm that Cre-mediated excision had occurred we genotyped pups and blastocysts from *Cdx2*-germline null crosses for the presence of *Cdx2*^{fl}*, Cdx2*^{del} and *Zp3-Cre*. Our results confirmed that excision of *Cdx2* by Cre recombinase was highly efficient as in 6/6 pups and 70/72 blastocysts had excised *Cdx2*. Furthermore, although only half of the progeny inherit the *Zp3-Cre* transgene (11/25 blastocysts), excision of *Cdx2* had occurred in all progeny (25/25 blastocysts), consistent with excision of *Cdx2* early during oogenesis. We conclude that maternal *Cdx2* is not required for development.

Cdx2 is not required for TE specification

Our results demonstrate that maternal *Cdx2* is not required for development, however elimination of maternal *Cdx2* does not address when or how *Cdx2* acts to promote TE cell fate, as zygotic *Cdx2* could compensate for loss of maternal *Cdx2* in *Cdx2* M null embryos. *Cdx2* knockdown embryos have been reported to have a

phenotype more severe than *Cdx2* null embryos (Jedrusik et al, 2010) suggesting that maternal *Cdx2* compensates for loss of zygotic *Cdx2* in *Cdx2* null embryos. Phenotypes of *Cdx2* knockdown embryos include failure to form the blastocyst in the majority of *Cdx2* knockdown embryos and a reduced number of outside cells in *Cdx2* knockdown embryos that reach the blastocyst stage. Molecular phenotypes reported in *Cdx2* null embryos include the failure to initiate expression of cell polarity genes *keratin 8* (KRT8) and E-cadherin (CDH1) genes and failure to initiate expression of NANOG. To examine the consequences of loss of both maternal and zygotic *Cdx2* we generated embryos lacking both maternal and zygotic *Cdx2* (*Cdx2* MZ null) by mating *Cdx2* germline null females to males heterozygous for a null allele of *Cdx2* ($Cdx2^{null)}$ (Chawengsaksophak et al., 1997) (Fig. 1D, mating scheme #2). First, we confirmed that *Cdx2* transcript was undetectable in *Cdx2* MZ null embryos at E3.5 by qPCR (Fig. 1C).

To determine if maternal *Cdx2* compensates for loss of zygotic *Cdx2* we compares the phenotype of *Cdx2* MZ null embryos to *Cdx2* null embryos. *Cdx2* MZ null embryos reached the blastocyst stage (11/11 embryos) (Fig. 2A). Additionally *Cdx2* MZ null embryos collapsed at the same time as *Cdx2* Z null embryos (Fig. 2B). In fact, *Cdx2* MZ null embryos and *Cdx2* null embryos cultured side by side from the E0.5 to E4.25 underwent compaction, cavitation, blastocoel expansion and collapse within the same time frame (data not shown). Thus at the morphological level *Cdx2* MZ null and *Cdx2* null embryos are indistinguishable, suggesting that in *Cdx2* null embryos maternal *Cdx2* does not compensate for loss of zygotic *Cdx2* and that *Cdx2* is not required for blastocyst formation.

We next sought to examine if the phenotype of *Cdx2* MZ null embryos is more severe than phenotype of *Cdx2* null embryos at the molecular level by examining the expression of the KRT8, CDH1 and NANOG was reduced in *Cdx2* MZ null embryos compared to *Cdx2* null embryos. KRT8 and CDH1 were reduced in *Cdx2* MZ null embryos and *Cdx2* null embryos compared to non-mutant embryos but there was no noticible difference between *Cdx2* MZ null and *Cdx2* null embryos. In *Cdx2* MZ null embryos we detected NANOG expression at levels similar to *Cdx2* null embryos and non-mutant embryos at E3.75 and E4.25 (Fig. 2A and B). In non-mutant embryos at E3.75 and E4.25 NANOG expression was restricted to the ICM, however in *Cdx2* null and *Cdx2* MZ null embryos NANOG was detected in both the ICM and TE consistent with previous analysis of *Cdx2* null embryos.

Finally, to examine if loss of maternal and zygotic *Cdx2* regulates cell position as reported in *Cdx2* knockdown embryos that reach the blastocyst stage (Jedrusik et al., 2010) we counted the number of inside and outside cells in *Cdx2* null, *Cdx2* MZ null and non-mutant embryos at E3.5. We did not detect a significant difference in the average number of inside or outside cells in *Cdx2* MZ null, *Cdx2* null or control blastocysts (Fig. 2C and D). Thus because we observed no differences in the morphological or molecular phenotypes between *Cdx2* MZ null and *Cdx2* null embryos we conclude maternal *Cdx2* does not compensate for loss of zygotic *Cdx2* in *Cdx2* null embryos. These findings support the model that *Cdx2* does not participate in TE specification and instead is required for maintenance of TE and that maternal *Cdx2* does not regulate cell fate.

2.5 Discussion

In this chapter, we genetically ablated *Cdx2* from the female germline in order to clarify when and how *Cdx2* promotes TE cell fate during mouse development and whether maternal *Cdx2* is a maternal-effect gene. By comparing the phenotypes of *Cdx2* MZ null and *Cdx2* Z null embryos we demonstrate that embryos in which maternal and zygotic *Cdx2* has been eliminated phenocopy embryos in which only zygotic *Cdx2* has been eliminated, arguing against a role for *Cdx2* in specifying TE cells through regulating cell polarity. Additionally we show that embryos lacking maternal *Cdx2* develop normally and become viable pups. Therefore, *Cdx2* is not a maternal effect gene.

Do maternal determinants regulate cell fate in the preimplantation embryo

Whether maternal effect genes regulate cell fate in the mouse embryo is essential to understanding the establishment of pluripotency. In fact, pluripotency factors themselves may act as maternal determinants of cell fate in the preimplantation mouse embryo. For example, the transcription factor *Oct4* is required for the establishment of pluripotency in the embryo (Nichols et al., 1998) and in ESCs the level of *Oct4* expression regulates cell fate (Niwa et al., 2000). OCT4 is detected in embryos as early as the two-cell stage suggesting that *Oct4* may be maternally provided to the embryo (Palmeiri et al., 1994). Intriguingly, the kinetics of *Oct4* transport out of the nucleus has been demonstrated to predict contribution to the TE and ICM cell lineages (Plachta et al., 2011). Two studies have suggested that

maternal *Oct4* is required for early developmental milestones, including activation of the zygotic genome (Foygel et al., 2008, Kim et al., 2003). Also of interest is a putative role of maternal *Sox2* in promoting TE cell fate, supported by the observation that embryos in which *Sox2* has been knocked down at the 2-cell stage fail to form the blastocyst (Keramari et al., 2010). Of note, the knockdown studies that support a role for maternal *Oct4* and *Sox2* used RNAi to eliminate maternal *Oct4 and* maternal *Sox2,* which we have demonstrated in this chapter*,* can produce inconsistent results. It will be important for future studies to investigate the role of maternal *Oct4* and maternal *Sox2* by the non-invasive, genetic approach used here to clarify the role of *Cdx2* in mouse development.

2.6 Figures and Legends

Figure 1: Relative quantification of *Cdx2* transcript levels by qPCR and mating scheme for generating *Cdx2* germline null females

A) Average *Cdx2* transcript levels, normalized to *Actb*, in E3.5 blastocysts, oocytes and ES cells. Averages were calculated from three biological replicate measurments: three wild-type blastocysts, three ES cell lines (R1, E14 and G4), and pooled oocytes from three different mice per genotype.

B) Levels of *Cdx2* transcript, relative to *Actb*, in wild-type (wt) and *Cdx2* M null oocytes (average of three biological replicates per genotype).

C) Average levels of *Cdx2*, relative to *Actb*, in single wild-type (wt) blastocysts (n=4) and Cdx2 MZ null blastocysts (n=5) at E3.5. Error bars indicate standard deviation.

D) Mating scheme to generate *Cdx2* germline null females. *Cdx2* germline null females are mated with wild-type males to generate embryos lacking only maternal *Cdx2* (mating scheme #1). To generate embryos lacking maternal and zygotic *Cdx2*, *Cdx2* germline null females are mated with *Cdx2*$^{+/null}$ males (mating scheme #2) resulting in litters in which half the embryos lack maternal and zygotic *Cdx2,* and the other half lack maternal *Cdx2* only.

Chapter 2, Figure 1

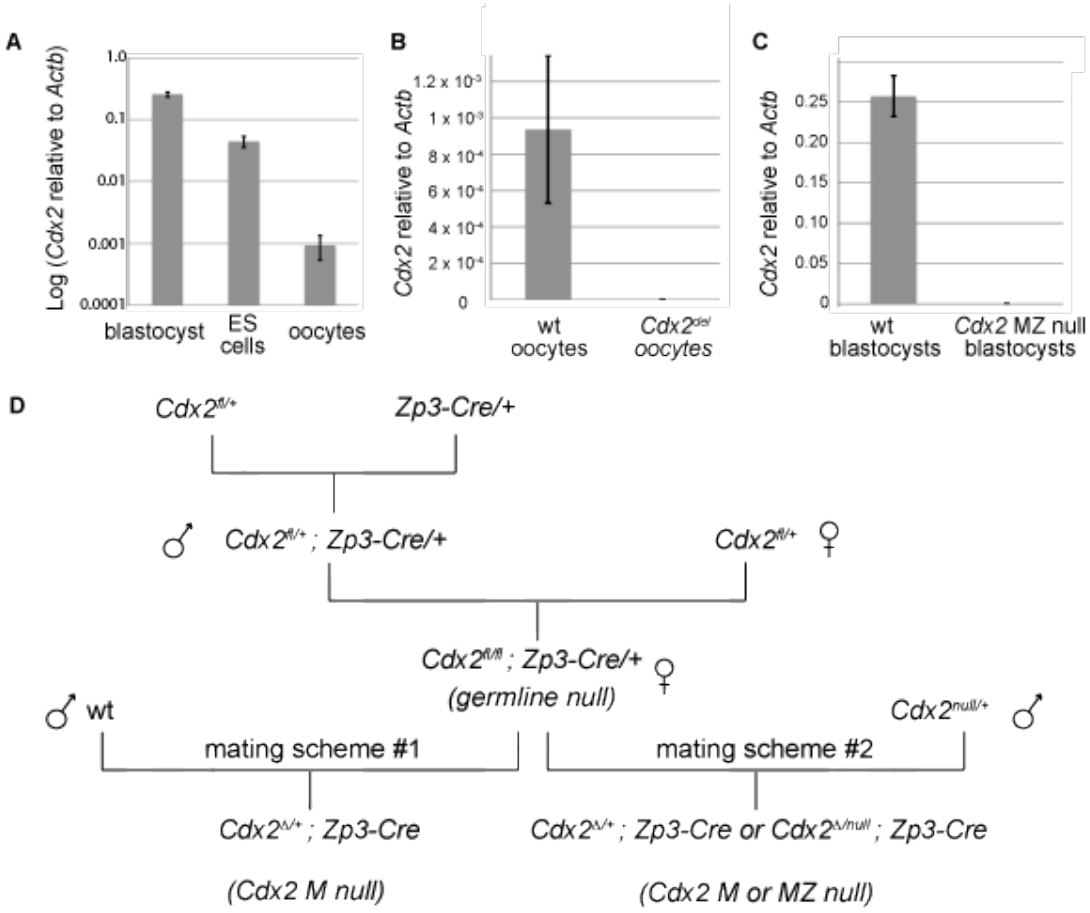


Figure 2: Elimination of maternal *Cdx2* does not alter the *Cdx2* null phenotype

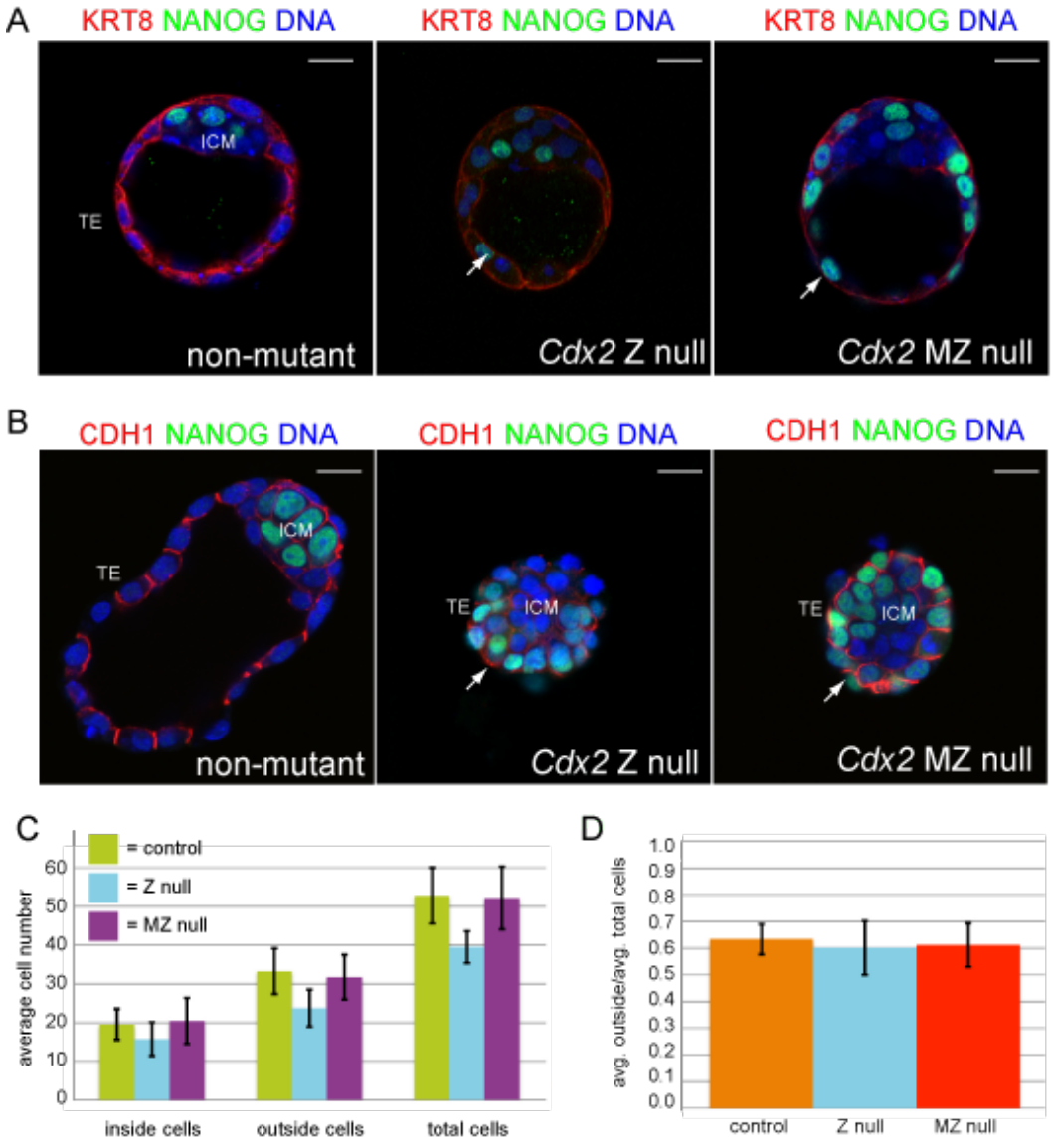
A) Expression of KRT8 (red) and NANOG (green) in confocal transverse sections of preimplantation mouse blastocysts at E3.75 (nuclei, blue). Images are representative of n = 20 control (*Cdx2*$^{+/-}$ or wild-type), n=6 zygotic null, n = 5 maternal/zygotic null blastocysts. In control blastocysts, KRT8 is restricted to the TE and NANOG is restricted to the ICM. In *Cdx2* zygotic null and *Cdx2* maternal/zygotic null blastocysts, KRT8 signal is reduced but still restricted to the TE and NANOG is misexpressed in the TE. white arrowheads = NANOG-positive TE cell. scale bar = 20 μm.

B) Expression of E-Cadherin/CDH1 (red) and NANOG (green) in impolanttion stage blastocysts at E4.25 (nuclei, blue). Control blastocysts are expanded and *Cdx2* zygotic null and maternal/zygotic null blastocysts are collapsed. CDH1 and NANOG are detectable in *Cdx2* zygotic and maternal/zygotic null blastocysts and Nanog is misexpressed in the RE. Confocal transverse sections representative of n = 7 control, n = 7 *Cdx2* zygotic null, and n = 9 *Cdx2* maternal/zygotic null blastocysts. white arrowheads = NANOG-positive TE cell. scale bar = 20 μm.

C) Average numbers of inside, outside and total cells in control (Zp3-Cre/+; n = 28), *Cdx2* zygotic null (n = 4) and *Cdx2* maternal/zygotic null (n = 22) blastocysts at E3.5. Inside and outside cells are identified on the basis of their morphological position in the blastocyst. There is no statistically significant different among the average number of inside, outside or total cells among genotypes examined. (student's t-test, $\alpha = 0.05$) Error bars indicate standard deviation.

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 examined. (student's t-test, $\alpha = 0.05$), Error bars indicate standard deviation.

Chapter 2, Figure 2



CHAPTER 3

Oct4 cell-autonomously promotes primitive endoderm development in the mouse blastocyst

3.1 Abstract

In embryonic stem (ES) cells and in early mouse embryos, the transcription factor OCT4 is an essential regulator of pluripotency. OCT4 transcriptional targets have been described in ES cell lines; however, the molecular mechanisms by which OCT4 regulates establishment of pluripotency in the epiblast (EPI) have not been fully elucidated. Here, we show that neither maternal nor zygotic *Oct4* is required for the formation of EPI cells in the blastocyst. Rather, *Oct4* is required for development of the primitive endoderm (PE), an extraembryonic lineage. We demonstrate that *Oct4* has dual roles in promoting PE cell fate, first through promoting transcription of *Fgf4* in the EPI and second by cell-autonomously promoting PE gene expression downstream of FGF/MAPK signaling. Finally, we show that Oct4 is required for the expression of multiple EPI and PE genes as well as multiple metabolic pathways essential for the continued growth of the preimplantation embryo.

3.2 Introduction

To understand how pluripotency is regulated, it is essential to understand how the pluripotent transcriptional network is established and maintained. The establishment of the pluripotent transcriptional network cannot be evaluated in ES cells, as ES cells are already pluripotent by their nature. Pluripotency is established during the preimplantation period of mouse development cells of the EPI lineage, thus the

preimplantation period mouse development is an excellent system in which to study the establishment of pluripotency. Furthermore, factors at the core of the pluripotency transcriptional network, such as *Oct4*, *Sox2* and *Nanog* are expressed in non-pluripotent cell types in the pre-implantation mouse embryo suggesting they might have additional roles beyond their roles in pluripotency. In this chapter, to better understand how pluripotency is established and to better understand how *Oct4* regulates pluripotentcy I examine the role of the pluripotency factor *Oct4* during the preimplantation phase of mouse development.

Oct4 is essential for pluripotency in ES cells and in the blastocyst

In the embryo and in ES cells, *Oct4* is widely appreciated as an essential pluripotency factor (Nichols et al., 1998. In ES cells, *Oct4* regulates a network of essential factors for pluripotency including *Sox2* and *Nanog* that together promote the transcription of pluripotency promoting genes and repress the transcription of genes that promote differentiation (Loh et al., 2006; Okumura-Nakanishi., 2005). Intriguingly, outgrowths from *Oct4* null embryos do not give rise to 'EPI-like" or 'PE-like' cells and instead only outgrow 'TE-like' cells (Nichols et al., 1998), suggesting that *Oct4* has is required for the establishment of EPI and PE cell fate. Intriguingly, in the embryo *Oct4* is required for maintaining ICM cell identity through repressing transcription of the TE lineage specific factor *Cdx2* (Ralston et al., 2010). Ectopic expression of *Cdx2* in *Oct4* null ICM cells could repress both EPI and PE and thereby suggest that the main role of *Oct4* in establishing pluripotency in the preimplantation mouse embryo is through repressing TE cell fate. However, expression of *Nanog* has been reported to be normal in *Oct4* null embryos shortly

after blastocyst formation (Chambers et al., 2003), suggesting that despite the role of *Oct4* in repressing PE cell fate, some aspects of ICM establishment are intact in *Oct4* null embryos and therefore in addition to repressing TE cell fate, *Oct4* also promotes EPI and PE cell fate. To distinguish between these two possibilities in this chapter I perform a thorough molecular analysis of TE, EPI and PE cell fate in the *Oct4* null embryo. Based on my molecular analysis TE, EPI and PE cell fate in the *Oct4* null embryo, I conclude that while *Oct4* is required to repress the TE cell fate in the ICM, ectopic expression of factors that promote TE cell fate within a subset of ICM cells does not account for the defects in EPI and PE establishment and therefore *Oct4* promotes EPI and PE cell fate in the preimplantion mouse embryo.

3.3 Materials and methods

Mouse Strains and Genotyping

The following alleles or transgenes were maintained in an outbred (CD1) background: *Pou5f1*^{tm1Scho} (Kehler et al., 2004) and *Tg(Zp3-cre)93Knw* (de Vries et al., 2000). Mice heterozygous for the Pou5f1 deletion allele (*Oct4*$^{del+}$) were generated by crossing mice carrying *Pou5f1*^{tm1Scho} with *129-Alpl*$^{tm1(cre)Nagy}$ (Lomelı´ et al., 2000). Mice were genotyped from ear punches using the following primers: (5'–3') *Oct4fl* and *Oct4wt* F: TTGTTACTGAAGAGGTTGGGTGTGACTGG; R: GGGGACTCCTGCTACAACAATCGCTAAG (449 bp fl and 415 bp wt), *Oct4del* F: AACTGGTTTGTGAGGTGTCCG; R: GTATCCACTCGCACCTTGTTC (245 bp), *Zp3-Cre* F: CTAGGCCACAGAATTGAAAGATCT; R: GTAGGTGGAAATTCTAGC ATCATCC. All animal research was conducted in accordance with the guidelines of

the University of California Santa Cruz Institutional Animal Care and Use Committee.

Embryo Collection and Culture

Mice were maintained on a 12 hr 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 37C and 6% CO_2 Embryos were cultured in 1 ug/ml Fgf4 recombinant human Fgf4 (R&D Systems) and 1 ug/ml heparin (Sigma) or in 100 nM of the FGF inhibitor PD173074 and 500 nM of the MAPK inhibitor PD0325901 (Stemgent). For outgrowth assays, embryos were flushed from the uterus at E3.5 and cultured for 24 hours and then subsequently transferred to individual wells of MEF- conditioned TS medium (Tanaka et al., 1998) with 1 ug/ml of FGF and Heparin. Final concentrations of FGFR/MAPK inhibitors were 100 nM PD173074 and 500 nM PD0325901 (Stemgent).

Immunofluorescence and Confocal Microscopy

Embryos were fixed, stained, imaged, and recovered for genotyping as previously described (Ralston and Rossant, 2008). Optical sections 5 μm in thickness were collected as previously described (Blij et al., 2012). Nuclei were labeled with Draq5 (Cell Signaling Technology). Antibodies included mouse anti-Nanog (Reprocell) 1/400, rat anti-CD140 (PDGFRα) (eBioscience) 1/100, goat anti-Gata6 (R&D Systems) 1/500, rabbit anti-Oct4 (Abcam) 1/25,000, goat anti-Sox17 (R&D Systems) 1/2,000, goat anti-Sox7 (R&D Systems) 1/800, mouse anti-Cdx2 (BioGenex), 1/200, rabbit anti-Sox2 (Millipore) 1/500, and fluorescently-conjugated secondary antibodies (Jackson ImmunoResearch) 1/400. TUNEL assay was performed using the In Situ

Cell Death Detection Kit (Roche) according to the manufacturer's instructions with the following modifications: embryos were fixed in 4% formaldehyde for 1 hour and then incubated in 0.5% Triton X-100 for 20 minutes at room temperature prior to performing the TUNEL reaction.

Embryo and ES Cell Aggregation

YFP-expressing R1 ES cells (George et al., 2007) were cultured on MEFs in ES cell medium + 1 mM PD0325901 + 3 mM GSK3 inhibitor Chir99021 (Stemgent) (Nichols et al., 2009). Precompaction four- to eight-cell embryos were collected from *Oct4*$^{fl/del}$ intercrosses, zonas pellucida removed with Tyrode's solution, and embryos aggregated with groups of three to five ES cells in depression wells. Aggregations were cultured in KSOM under light mineral oil at 37C and 6% CO_2. Chimeras were subsequently genotyped by PCR using primers that could distinguish wild-type, floxed, and deleted *Oct4* alleles.

RNA Isolation and cDNA Preparation

Embryos were collected from natural timed matings and transferred to 50 ml Picopure extraction buffer (Acturus Biosciences). RNA isolation was carried out according to the manufacturer's instructions. Single-blastocyst qPCR was performed as previously described (Blij et al., 2012). For each embryo examined, approximately 1 kb transcripts were selectively amplified from the 3^0 ends to produce four independent complementary DNA libraries, which were then pooled prior to sequencing (Kurimoto et al., 2007). Sequencing was performed on an Applied Biosystems SOLiD4 sequencing platform.

Analysis of RNA-seq data

Analysis of RNA-seq data was performed by Michael Halbisen using the following methods. Transcripts were collapsed for gene-level analysis by selecting the most abundant transcript (total expression for all samples) for each detected gene at each developmental stage (E2.5, E3.75, and E4.5). For each of these stages, separate differential gene expression analyses were performed using edgeR (Robinson et al., 2010; Robinson and Smyth, 2008) in the R programming environment (version 2.15.1, http://www.r-project.org/). Transcripts were excluded from analyses unless they showed expression levels above 10 cpm for at least three of six individuals for each gene. For each gene in the filtered lists, exact tests were used to determine whether wild type and mutant gene expression differences were statistically significant. A multiple test correction was performed to reduce the number of false positives in the differentially expressed gene lists by rejecting tests with an FDR≥0.05. GSEA was performed using the GSEA Preranked tool (Mootha et al., 2003; Subramanian et al., 2005). At each developmental stage, preranked lists of genes (moderated *t*-statistic; *Oct4* null versus wild type) were generated from the filtered gene expression data using the RNA-seq data analysis option in the limma package in R (Robinson and Smyth, 2007; Smyth, 2004). In total, 217 BioCarta , 186 KEGG, and 687 Reactome gene sets were examined (MSigDB). Statistical significance of individual enrichment scores for each gene set was calculated using 1000 permutations over gene sets, and gene sets with fewer than 10 genes were excluded from the analysis. Gene sets were clustered using the Enrichment Map Plugin v1.2 for Cytoscape v2.8.3 (Cline et al., 2007; Merico et al., 2010; Smoot et al.,

2011), and similarity coefficients were calculated using recommended thresholds (0.5 gene set overlap, P<0.005; FDR<0.075). For EPI/PE/TE GSEA, query signatures for each lineage were established by compiling gene sets of lineage-specific genes from the literature. For many of these genes, single cell mRNA expression analysis (Guo et al., 2010) enabled their classification as EPI, PE, or TE-specific genes. For other genes, single cell microarray data (Kurimoto et al., 2006; Saitou et al., 2008) enabled their classification as EPI or PE genes, and we excluded any whose expression was TE-enriched using blastocyst and ICM RNA-seq data (Aksoy et al., 2013). Target signatures were established and query signature gene sets were tested for significant enrichment (FDR<0.05; 1000permutations over gene sets) in either tail of the target signature gene distribution (gene expression WT>NULL or NULL>WT).

Analysis of ChIP-seq data

Analysis of ChiP-seq data was performed by Paul Robson using the following methods: The genes with the top 10% highest enrichment values following Oct4 ChIP-seq with EC F9 or EC F9 treated with RA for 72 hr (Aksoy et al., 2013) were used for the analysis (MACS peak heights ≥ 65 or 50, respectively). ChIP-seq peaks were generated using Integrative Genomics Viewer software.

Statistical methods

For Fig. 1-5, p values were calculated by t-test, and ANOVA performed in cases were multiple pair wise comparisons were performed. In some cases (Fig. 1E, I and 2F), a Fischer's test was performed.

3.4 Results

Oct4 is required for cell proliferation in TE, EPI and PE at E4.0

Previous analysis of the role of *Oct4* during mouse development has demonstrated that *Oct4* null embryos form the blastocyst and specify normal amounts of TE and ICM cells and eventually collapse prior to implantation (Nichols et al., 1998). Because *Oct4* has a widely accepted role in promoting pluripotency, I hypothesized that the arrest of *Oct4* null embryos is due to selective loss of EPI cells and that TE cell numbers would be normal in *Oct4* null embryos. To test the hypothesis that *Oct4* null embryos arrest due to the selective loss of EPI cells I mated male and female mice heterozygous for a null allele of *Oct4* ($Oct4^{del}$) and compared the total number of inside (EPI + PE) and outside cells (TE) in *Oct4* null and non-mutant littermates at the early (E3.75) mid (E4.0) and late (E4.25) blastocyst stages.

Consistent with previous studies, *Oct4* null embryos had the same average number of inside and outside cells as non-mutant littermates at E3.75 (Fig. 1A,D; Nichols et al., 1998). *Oct4* null embryos grew at a similar rate as their non-mutant littermates and were indistinguishable from non-mutant littermates by average total cell number at E4.0 (Fig 1B,D), suggesting that cell proliferation and survival in both outside and inside cells is *Oct4*-independent until E4.0. At E4.25 *Oct4* null embryos contained significantly reduced numbers of both inside cells and outside cells compared to non-mutant littermates (Fig. 1C,D). The reduced numbers of inside cells and outside cells in *Oct4* null embryos demonstrate that both inside and outside cells require *Oct4* and suggests that *Oct4* has multiple roles in preimplantation

mouse development beyond the well characterize roll of *Oct4* in prompting pluripotency.

Although *Oct4* is required to maintain normal numbers of both inside and outside cells, we wondered if inside cells were disproportionately affected by the loss of *Oct4*, which would indicate a more critical role for *Oct4* in inside cells than in outside cells. To determine if inside or outside cells were disproportionately affected by the loss of *Oct4* we compared the ratio of inside and outside cells at E3.75, E4.0 and E4.25. The ratio of inside to outside cells in *Oct4* null embryos was not significantly different in *Oct4* null embryos compared to non-mutant littermates at E3.75 or E4.0 (Fig. 1E). However, by E4.25 the ratio of inside to outside cells in *Oct4* null embryos compared to non-mutant littermates had been significantly reduced (0.69 non-mutant/0.43 *Oct4* null) (Fig. 1E). My analysis of inside and outside cells in *Oct4* null embryos at E4.25 suggests that *Oct4* is required for maintaining inside and outside cell numbers and that *Oct4* is more critical for maintenance of inside cells rather than outside cells.

I next wanted to understand how *Oct4* is required for maintaining normal numbers of inside and outside cells in the preimplantation mouse embryo. *Oct4* could be maintaining the number of inside and outside cells during mouse pre-implantion development through either promoting proliferation or promoting cell survival. To distinguish between these possibilities I measured apoptosis in *Oct4* null embryos and non-mutant littermates at E4.0 and E4.25 by the TUNEL assay, which labels nicked-DNA, a hallmark of apoptosis. While apoptosis was detected at E4.0 and E4.25 in inside and outside cells in *Oct4* null embryos, there was no significant difference in the number of inside or outside cells undergoing apoptosis

between *Oct4* null embryos and non-mutant littermates at either E4.0 or E4.25 (Fig. 1J, K). Thus, *Oct4* is required for cell proliferation in both inside and outside cells between E4.0 and E4.25. This is an unexpected result as *Oct4* has generally been thought of as being required only for maintaining pluripotency, which would explain a role for *Oct4* in promoting proliferation of inside cells but not in outside cells. Because *Oct4* is not expressed in outside cells from E4.0 to E4.25, our results suggest that inside cells non-cell autonomously promote the proliferation of outside cells through an *Oct4*-depenent mechanism.

Expression of the pluripotency genes *Nanog and Sox2* is *Oct4*-independent

In ES cells, *Oct4* maintains pluripotency by promoting expression of multiple pluripotency factors, including *Nanog* and *Sox2.* Therefore, I hypothesized that the reduced number of inside cells in *Oct4* null embryos could result from a failure to initiate or maintain the expression of the pluripotency factors *Nanog and Sox2* in *Oct4* null embryos. To test whether *Oct4* is required to initiate or maintain the expression of *Nanog* and/or *Sox2* in the mouse blastocyst I evaluated the expression of NANOG and SOX2 in *Oct4* null embryos by immunoflourescence. Surprisingly, NANOG and SOX2 expression in *Oct4* null embryos was indistinguishable from NANOG and SOX2 expression in non-mutant littermates at this stage (Fig. 1A, L). Furthermore, robust expression of *Nanog* in *Oct4* null embryos at E4.25 suggests that *Oct4* is not required for maintenance or initiation of *Nanog.* Our results indicate that *Nanog* and *Sox2* expression is *Oct4*-independent during mouse development.

Oct4* is required to maintain expression of *Gata6

Our results indicate that *Oct4* null embryos specify the correct number of inside and outside cells and that *Oct4* null inside cells express markers of pluripotency. We next investigated the identity of *Oct4* null inside cells. Cells on the inside of the blastocyst make up the ICM, which then differentiates into the pluripotent EPI and the multipotent PE. Progenitors for the EPI and the PE are identified in the ICM by the expression of NANOG and GATA6, respectively. To examine whether EPI and PE progenitors are specified in *Oct4* null embryos, I compared the expression of NANOG and GATA6 by immunofluorescence in *Oct4* null and non-mutant littermates at E3.75, E4.0 and E4.25. In E3.75 non-mutant embryos, NANOG and GATA6 were expressed in a 'salt-and-pepper' pattern, as previously described (Chazaud et al., 2006). In E3.75 *Oct4* null embryos, NANOG and GATA6 were expressed in a salt-and-pepper pattern similar to non-mutant embryos (Fig. 1A). In *Oct4* null embryos at E4.0, NANOG was detected in the same number of cells as non-mutant embryos. In contrast, both the number of GATA6-positive cells and the intensity of GATA6 signal was significantly reduced in *Oct4* null embryos compared to non-mutant littermates (Fig. 1B, G). In *Oct4* null embryos at E4.0 there I detected a significant number of ICM cells in *Oct4* null embryos were negative for GATA6 and NANOG (Fig. 1H). Because the number of NANOG-positive cells was normal in *Oct4* null embryos at E4.0 I hypothesize that cells that were negative for GATA6 and NANOG in *Oct4* null embryos were cells that had initially been specified at PE and down regulated NANOG expression at E3.75 but had failed to maintain GATA6 expression at E4.0. Because these cells were initially specified as PE, I refer to them as presumptive PE throughout the rest of this chapter. Additionally, in non-mutant embryos at this stage NANOG and GATA6 expressing cells had sorted into their proper locations. In *Oct4*

null embryos NANOG-positive, GATA6-positive and unlabeled cells remained intermixed (Fig. 1B). In *Oct4* null embryos at E4.25, which had collapsed GATA6 was no longer detectable (Fig 1C, G). In contrast, NANOG was still detectable, however, NANOG-positive cells remained intermixed with unlabeled inside cells (Fig. 1C, F). Based on my analysis of GATA6 and NANOG expression in *Oct4* null embryos I conclude that while *Oct4* is not required for maintenance of NANOG expression, *Oct4* is required to maintain GATA6 expression by E4.0 and that *Oct4* is also required for sorting of EPI and PE cells at E4.0. The observation that NANOG and GATA6 undergo 'salt-and-peppering' at E3.75 suggests that the ICM lineage is properly specified and argues against the model that *Oct4* is required for ICM cell fate. Instead, the failure of *Oct4* null embryos to maintain GATA6 suggests that *Oct4* promotes PE cell fate, which is unexpected given that *Oct4* is mainly thought promote pluripotency and the PE is not pluripotent. Because of the novelty of this result, and the implications that a role for *Oct4* in a non-pluripotent cell type would have on stem cell biology, I next sought to more thoroughly characterize PE development in *Oct4* null embryos.

Oct4 is required for the expression of multiple PE fate markers

In addition to the expression of GATA6, development of the PE is characterized by the expression of the PE specific lineage markers, PDGFRα, SOX17, GATA4 and SOX7. SOX17 is first detectable at E3.5 (Niakan et al, 2010), followed by GATA4 at E3.75 and then finally SOX7 at E4.0 (Artus et al., 2011). To investigate whether *Oct4* is required for expression of multiple PE fate markers, or just the maintenance of GATA6 I investigated whether PDGFRα, SOX17, GATA4 or SOX7 were

detectable in *Oct4* null embryos at the developmental stages when their expression is initiated in non-mutant embryos. SOX17 and PDGFRa were never detected in *Oct4* null embryos (Fig. 2A,B). In contrast, GATA4 was detected in *Oct4* null embryos during its initiation phase at E3.75, but the intensity of GATA4 staining, as well as the number of cells expressing GATA4 was greatly reduced compared to non-mutant embryos (Fig. 2C). SOX7 was also not detected in *Oct4* null embryos during its initiation at E4.0 (Fig. 2D).

We conclude that *Oct4* is required for the initiation of PE development in regards as demonstrated by the absence of the PE cell fate markers PDGFRα and SOX17 in *Oct4* null embryos at E3.5 and the loss of GATA6 expression at E4.0.

Oct4 represses TE in some but not all ICM cells at E4.0

Oct4 is required to repress TE cell fate in the ICM, but the exact timing of *Oct4* null ICM differentiation to TE is not clear nor whether all *Oct4* null ICM cells differentiate into TE. I hypothesize that the defect in PE development observed in *Oct4* null embryos due to failure to repress TE cell fate. This hypothesis assumes that TE cell fate inhibits PE cell fate. My hypothesis predicts that because my analysis of multiple PE fate markers demonstrates that SOX17 and PDGFRα are never initiated in PE cells, all presumptive PE cells acquire TE cell fate. To test this hypothesis I labeled E4.0 *Oct4* null embryos for NANOG, to identify presumptive PE cells by the absence of NANOG expression, and for CDX2, a marker of TE cell fate. In non-mutant embryos at E4.0, CDX2 was not detected in any ICM cells. In contrast, CDX2 was detected in 27% of E4.0 *Oct4* null ICM cells (Fig. 3A, B). I detected no bias in CDX2 expression towards NANOG-positive EPI or NANOG-negative

presumptive PE cells in the ICM suggesting that *Oct4* is equally required in both the EPI and presumptive PE to repress TE cell fate. Importantly, the majority of presumptive PE cells were CDX2-negative. Because not all presumptive PE cells acquire PE cell fate this argues against my hypothesis that the defect in PE cell fate is due to ectopic expression of CDX2 in presumptive PE cells and suggests that *Oct4* promotes PE cell fate directly, rather than through repression of TE cell fate in the PE.

Maternal *Oct4* is dispensable for development

My morphological and molecular analysis of *Oct4* null embryos is that *Oct4* null embryos have no phenotype until E3.5, when PDGFRa and SOX17 expression fail to be initiated in *Oct4* null embryos. This suggests that *Oct4* is not required until after blastocyst formation. Alternatively, maternally provided *Oct4* could compensate for loss of zygotic *Oct4* and mask an earlier role for *Oct4* in the preimplantation mouse embryo. In support of the presence of maternally provided *Oct4, Oct4* transcript is highly abundant in the oocyte (Rosner et al., 1990) and OCT4 is present in the nucleus as early at the 2-cell stage (Palmieri et al., 1994). Two studies have investigated whether maternally provided *Oct4* is required for development by reducing both maternal and zygotic *Oct4* by RNAi. Both studies agree that maternally provided *Oct4* is required for preimplantation development prior to blastocyst formation however *Oct4* knockdown embryos have vastly different phenotypes (Kim et al., 2003; Foygel et al., 2008) Of note, both these studies use RNAi knock down *Oct4,* which may not be suitable for inferring gene function in the preimplantation mouse embryo, as I have previously shown (See Chapter 2; Blij et al., 2012). To

validate a requirement for *Oct4* prior to blastocyst formation, as suggested by *Oct4* knockdown experiments, I eliminated maternal *Oct4* through an alternative approach by specifically deleting *Oct4* in the female germline.

To determine if maternal *Oct4* is required for development, I used the female germline-expressed *Zp3-Cre* (de Vries et al., 2000) and a conditional allele of *Oct4* (*Oct4*^{fl}) (Kehler et al., 2004) to eliminate maternal *Oct4* during oogenesis (Fig. 4A). I first verified by qPCR that *Oct4* transcript levels were reduced to undetectable levels in oocytes from *Oct4* germline null females (Fig. 4B). demonstrating that embryos generated by *Oct4* germline null females (*Oct4 M null*) lack maternally provided *Oct4.* I then mated *Oct4*-germline null females to wild-type males and compared the average litter size to the average litter size of matings of wild-type males and females. *Oct4* germline null females gave birth to litters of viable pups and the number of pups from matings involving *Oct4* germline null females was not reduced compared to matings of wild-type males and females (*Oct4* germline null: 10.3 +/- 1.5, wild type: 7.0 +/- 0.8). Because *Oct4* M null embryos give rise to viable pups and I did not observe a reduction in litter size in matings from *Oct4* germline null females I conclude that maternal *Oct4* is not required for development.

To investigate if maternal *Oct4* compensates for zygotic *Oct4* we generated embryos lacking maternal and zygotic *Oct4* (*Oct4* MZ null) by crossing *Oct4* germline null females to Oct4$^{del/+}$ males *and* recorded the frequency of *Oct4* mz null embryos forming the blastocyst and the number GATA6-positive and NANOG-positive cells, with the expectation that if maternal *Oct4* is compensating for the loss of zygotic *Oct4* then the phenotype of *Oct4* MZ null embryos would be more severe than loss of zygotic *Oct4* alone. At E3.75, *Oct4* MZ null embryos had formed the blastocyst

structure (16/16 m null and 12/12 mz null), and were recovered at the expected frequency (Fig. 4D, p=0.592, Pearson's). Additionally, we observed no significant difference in the average numbers of inside and outside cells between non-mutant, *Oct4* null and *Oct4* M null and Oct4 MZ null embryos (Fig. 4C). Moreover, the average numbers of NANOG-positive and GATA6-positive expressing cells were not significantly different non-mutant, *Oct4* null and *Oct4* M null and Oct4 MZ null embryos (Fig. 4E). Because *Oct4* MZ null embryos essentially phenocopy *Oct4* null embryos I conclude that maternal *Oct4* does not compensate for loss of zygotic *Oct4* and that blastocyst formation and the initial expression and subsequent 'salt-and-peppering' of *Gata6* and *Nanog* are *Oct4*-independent.

Oct4 is not required for FGF/MAPK-dependent regulation of Nanog

Because FGF/MAPK signaling is required for PE cell fate, (Kang et al., 2013; Chazaud et al., 2006; Frankenberg et al., 2010) in order to infer the role of *Oct4* in PE development from the phenotype of the *Oct4* null embryos it is essential to know whether *Oct4* null ICM cells are capable of responding to FGF/MAPK signaling. The 'salt-and-pepper' pattern of NANOG in the E3.75 is established through FGF/MAPK-dependent repression of NANOG. Because NANOG is salt and peppered in *Oct4* null embryos at E3.75 this suggests that FGF/MAPK signaling is intact in *Oct4* null embryos. Alternatively, NANOG could be 'salt-and-peppered' through an FGF/MAPK independent mechanism in *Oct4* null embryos mechanism. For example, *Oct4* could be required to maintain NANOG in a subset of ICM cells, creating the appearance of a 'salt-and-pepper' NANOG pattern independent of FGF/MAPK signaling.

To test whether FGF/MAPK signaling is active in *Oct4* null embryos I treated *Oct4* null embryos and non-mutant littermates with inhibitors of FGFR and MAPK from E2.75 to E4.0 with the expectation that if NANOG is 'salt-and-peppered' through an FGF/MAPK dependent mechanism, then inhibition of FGF/MAPK signaling would inhibit 'salt-and-peppering' of NANOG and NANOG would be detected in all ICM cells (Nichols et al., 2009). Treatment of non-mutant embryos with inhibitors of FGFR and MAPK inhibited 'salt-and-peppering' of NANOG and led to embryos in which NANOG was detected throughout the ICM at E4.0, as expected (Fig. 5A,B) In *Oct4* null embryos treated with FGFR and MAPK inhibitors NANOG was also expressed throughout the entire ICM (Fig 5A,B). Based on these results, I conclude that regulation of NANOG by FGF/MAPK signaling does not require *Oct4* and FGF/MAPK signaling is intact and active at sufficient levels in *Oct4* null embryos to 'salt-and-pepper' NANOG.

PE specification is indirectly regulated by FGF/MAPK signaling through NANOG mediated repression of GATA6 (Frankenberg et al., 2011). For example, in embryos treated with FGFR and MAPK inhibitors GATA6 is repressed by ectopic NANOG throughout the entire ICM. Surprisingly, FGF and MAPK inhibition did not lead to a loss of GATA6 in *Oct4* null embryos (Fig 5A,C), inspite of the expression of NANOG in all ICM cells. In fact, both the intensity of GATA6 immunostaining and the average number of GATA6-positive cells was increased in *Oct4* null FGFR/MAPK inhibitor treated embryos compared to untreated *Oct4* null embryos. We conclude that *Oct4* is required for NANOG-dependent repression of GATA6. This result is intriguing and conflicts with my previous observation that *Oct4* promotes GATA6 under normal conditions (See Fig 1. B, C, G and I). I interpret this conflict in roles for

Oct4 in regards to promoting and repressing *Gata6* expression as evidence that *Oct4* can either promote EPI cell fate by repressing GATA6 or PE cell fate by maintaining GATA6. This interpretation is consistent with cell type specific roles for *Oct4* in promoting EPI and PE cell fate.

Because inhibition of FGFR and MAPK appears to alleviate the requirement for *Oct4* in maintaining GATA6 expression in *Oct4* null embryos until at least E4.0, a later stage than GATA6 is robustly detected in untreated *Oct4* null embryos, I wanted to test the hypothesis that persistence of GATA6 in *Oct4* null embryos treated with FGFR/MAPK inhibitors is sufficient to rescue SOX17 or SOX7 in *Oct4* null embryos. To test this hypothesis I cultured *Oct4* null and non-mutant embryos from E2.75 to E4.0 or E4.5 and then labeled the embryos for SOX17 or SOX7. As expected, SOX17 and SOX7 were not detected in non-mutant embryos treated with FGFR and MAPK inhibitors. SOX17 and SOX7 were also not detected in *Oct4* null embryos treated with FGFR and MAPK inhibitors (Fig. 5D – G). Therefore, expression of GATA6 is not sufficient to rescue SOX17 and SOX7 expression in the absence of *Oct4* suggesting that *Oct4* is required for the expression of multiple genes that regulate PE cell fate and not just GATA6.

I also examined NANOG in *Oct4* null embryos treated by FGFR/MAPK inhibitors from E2.75 to E4.5. Surprisingly, and in contrast to FGFR/MAPK inhibitor treated *Oct4* null embryos at E4.0, NANOG was detected in only half of ICM cells of FGFR/MAPK inhibitor treated *Oct4* null embryos at E4.5 (Fig. 5E, H). Therefore it appears that ‘salt-and-peppering’ of NANOG eventually becomes FGF/MAPK-independent in *Oct4* null embryos. Because ‘salt-and-peppering’ of NANOG is FGF/MAPK-dependent at E4.0 in *Oct4* null embryos (Fig. 4A, B, D) and NANOG is

detectable in the appropriate proportion of ICM cells as late as E4.25 in untreated *Oct4* null embryos (Fig. 1C), I conclude that EPI and presumptive PE cells have different requirements for *Oct4* in maintaining NANOG expression. In presumptive PE cells (or cells that ectopically express NANOG in response to FGFR/MAPK inhibition) NANOG becomes *Oct4*-dependent at E4.5 while in presumptive EPI, maintenance of NANOG is *Oct4*-independent during the preimplantation period of mouse development.

Oct4 is required for FGF4-induced expression of PE genes

Our results demonstrate that FGF/MAPK signaling is active in *Oct4* null embryos at sufficient levels to 'salt-and-pepper' NANOG expression. Intriguingly *Fgf4* transcription is greatly reduced in *Oct4* null embryos (Nichols et al., 1998). I hypothesize that the failure of *Oct4* null embryos to express PE markers is due to reduced FGF4 and that because the FGF/MAPK signaling pathway is active in *Oct4* null embryos, providing FGF4 would rescue PE cell fate in *Oct4* null embryos. To test this hypothesis I cultured *Oct4* null embryos and non-mutant littermates in recombinant FGF4 and Heparin (FGF4/Heparin) from E2.75 to E4.5, FGF4/Heparin treatment led to GATA6 and SOX17 throughout the ICM, as expected (Yamanaka et al., 2010, Frankenberg et al., 2011, Kang et al., 2013). In *Oct4* null embryos treated with FGF4/Heparin GATA6 was not detected (Fig. 6A, D). In contrast, significantly more SOX17-positive cells were detected in FGF4/Heparin treated *Oct4* null embryos than untreated *Oct4* null embryos, however the percentage of SOX17-positive cells in the ICM of Fgf4/Heparin treated *Oct4* null embryos was greatly reduced compared to non-mutant Fgf4/Heparin treated embryos (100% non-mutant

v. 6% *Oct4* null) (Fig 6B, E). Additionally, I was unable to establish PE-like cells in outgrowths from FGF4/Heparin treated *Oct4* null embryos, even in the continued presence of high-levels of FGF4/Heparin suggesting that the slight increase of SOX17-positive cells in FGF/Heparin treated embryos does not lead to rescue of the PE (Fig. 6H).

Because *Oct4* null embryos still receive maternally provided *Oct4*, I hypothesized that the slight increase in SOX17-positive cells in response to FGF4/Heparin treatment in *Oct4* null was due to the presence of maternal *Oct4* in *Oct4* null embryos. To test this hypothesis I treated *Oct4* MZ null embryos with FGF4 and Heparin and compared the percentage of SOX17-positive cells in *Oct4* MZ null embryos treated with FGF4/Heparin to the percentage of SOX17-positive cells in *Oct4* null embryos treated with FGF4/Heparin. If the presence of maternal *Oct4* is leading to the slight rescue of SOX17 expression in *Oct4* null embryos treated with FGF4/Heparin then I expect that the percentage of SOX17-positive cells in *Oct4* MZ null treated with FGF4/Heparin will be reduced compared to the percentage of SOX17-positive cells in *Oct4* null embryos. I found no difference in the amount of SOX17-positive cells induced in response to FGF4/Heparin treatment between *Oct4* MZ null embryos and *Oct4 null* embryos, suggesting that FGF4/Heparin treatment can induce a slight increase in SOX17-expression, but that *Oct4* is required for maximal response to FGF4/Heparin.

I have demonstrated that the FGF/MAPK pathway regulates NANOG 'salt-and-peppering' in *Oct4* null embryos and in non-mutant embryos treatment with FGF4/Heparin represses NANOG in the entire ICM (Yamanaka et al., 2010) I hypothesize that while *Oct4* is required for maximal response of PE genes to

FGF4/Heparin, *Oct4* is not required for maximal repression of NANOG in response to FGF4/Heparin. As expected, in non-mutant embryos treated with FGF4/Heparin, NANOG was not detected, confirming the efficacy of our FGF4/Heparin treatment conditions. Surprisingly, and in spite of the fact that 'salt-and-peppering' of NANOG is regulated by FGF/MAPK signaling in *Oct4* null embryos, NANOG was not repressed in the entire ICM of *Oct4* null embryos treated with FGF4/Heparin. In fact, the percentage of NANOG-positive ICM cells in *Oct4* null embryos treated with FGF4 and Heparin was not significantly reduced compared to untreated *Oct4* null embryos (Fig. 6C, F). These results suggest that while *Oct4* is not required for FGF/MAPK dependent salt-and-peppering, *Oct4* is required for maximal repression of NANOG cell fate in response to ectopic FGF4/Heparin.

Taken together, poor response of *Oct4* null embryos to FGF4 in promoting PE cell fate (SOX17) and repressing EPI cell fate (NANOG) demonstrates that *Oct4* is required for maximal response to FGF4 in the preimplantation mouse embryo. Because FGF4 does not rescue the PE lineage in *Oct4* null embryos, *Oct4* must be required for multiple aspects of PE development and not just the production of FGF4. Therefore, in addition to being required for expression of *Fgf4* (Nichols et al., 1998; Fig 8B) *Oct4* could promote PE development non-cell autonomously through other signaling pathways that promote PE development, and/or *Oct4* could be required cell-autonomously in the PE to promote expression of *Sox17*, and other PE promoting genes.

Oct4 **is required cell-autonomously for PE gene expression**

Oct4 is required for maximal response to FGF4/Heparin treatment suggesting the possibility that *Oct4* is required downstream of FGF/MAPK signaling to cell-autonomously promote PE cell fate. Alternatively, *Oct4* could be required non-cell autonomously for PE cell fate by regulating signaling pathways that promote PE cell fate. To define where *Oct4* is required in PE development, I generated chimeric embryos containing a wild-type ES cell derived EPI and *Oct4* null PE by aggregating wild-type ES cells with 8-cell precompaction stage (E2.5) *Oct4* null embryos and then asked whether the presence of a wild-type EPI could rescue SOX17 in *Oct4* null embryos. If *Oct4* is required cell-autonomously for PE cell fate, then SOX17/SOX7 expression will not be rescued in *Oct4* null embryos aggregated with wild-type ES cells. Conversely, if *Oct4* is required non-cell autonomously for PE cell fate, wild-type ES cells will rescue *Oct4's* non-cell autonomous role in promoting PE cell fate from EPI and rescue PE development. First, we confirmed that host ICM cells are induced towards PE cell fate in response to ES cells by immunostaining for SOX17 and SOX7 in non-mutant embryos aggregated with wild-type ES cells. ES cells were permanently labeled by YFP and host-derived ICM cells were identified by the absence of YFP expression and their location on the inside of the embryo. We observed that in non-mutant chimeras the vast majority (>95%, Fig. 7C - F) of host ICM cells expressed PE markers. In contrast, *Oct4* null embryos aggregated with wild-type ES cells did not robustly express PE markers (Fig 7C - F). In some *Oct4* null chimeras, we detected one or two SOX17-positive or SOX7-positive (6/10 Sox17 and 1/6 Sox7) but in all cases, the percentage of SOX17-positive or SOX7-positive host ICM was greatly and significantly reduced compared to non-mutant embryos. Importantly, the low level of SOX17-positive and SOX7-positive cells was not

correlated with the number of ES cells present in the chimera (Fig 7J, K) nor was the low level of Sox17 expression dependent on maternal *Oct4*, since Sox17 was also induced in *Oct4* maternal/zygotic null ES cell chimeras (Fig. 7I).

The low levels of PE gene expression observed in *Oct4* null ES cell chimeras are consistent with my finding that FGF4 can induce modest PE gene expression in *Oct4* null embryos. Intriguingly, comparison of the frequency of rescue between our FGF4/Heparin rescue and ES cell rescue experiments reveals that ES cells are more capable of rescuing SOX17 than FGF4 alone, suggesting that ES cells rescue additional *Oct4*-dependent PE promoting pathways beyond FGF4. BMP and NODAL signaling are active during preimplantation mouse development (Roelen et al., 1997; Granier et al., 2011) and ligands that stimulate BMP and NODAL signaling are secreted by ES cells (Galvin et al., 2010), making BMP and NODAL signaling attractive candidates for additional signaling pathways that promote PE development in addition to FGF/MAPK signaling.

Whole transcriptome analysis of non-mutant and *Oct4* null embryos

To gain an unbiased, global view of role of *Oct4* in the preimplantation mouse development we performed RNA sequencing on individual *Oct4* null and non-mutant embryos at E2.75, E3.75 and E4.25. We first performed multidimensional scaling on *Oct4* null and non-mutant transcriptomes to confirm that transcriptional profiles were separated by genotype, as would be expected if our data represented consistent differences among genotypes. Indeed, we found that embryos were separated by genotype, however, at E4.5, the stage when *Oct4* null embryos are undergoing arrest, this trend was weaker (Fig. 8A). As expected, *Oct4* was the most reduced

transcript at each stage examined. Additionally, *Sox17, Gata4* and *Pdgfra* were also significantly reduced in *Oct4* null embryos at E3.75, consistent with our immunofluorescence results. Consistent with a role for *Oct4* in promoting multiple pathways required for PE development, other known PE genes such as *Sparc, Lama1, Lamb1,* and *Lamc1* (Miner et al., 2004; Niakan et al., 2010) were significantly reduced in *Oct4* null embryos. Consistent with a role for *Oct4* in promoting EPI development we detected a reduction in transcripts from the pluriopotency genes, *Essrb, Klf2* and *Zscan10* (Ivanova et al., 2006; Jiang et al., 2008; Martello et al., 2012., Wang et al., 2007., Zhang et al., 2006).

To discriminate lineage-specific differences in gene expression between *Oct4* null embryos and non-mutant littermates we compared our list of differentially expressed (DE) genes in *Oct4* null embryos to a list of known lineage-specific genes in the blastocyst and looked for lineage-specific enrichment among differentially expressed genes at each stage examined (Fig. 8E). At E2.5, EPI and PE genes were not enriched among the list of DE transcripts in *Oct4* null embryos. In contrast, TE genes were slightly upregulated in *Oct4* null embryos at E2.5. However, by E3.75 and E4.5, TE genes were no longer upregulated and *Oct4* null embryos showed a significant enrichment in the number of reduced EPI and PE genes. These results are consistent with a role for *Oct4* in all lineages in the blastocyst and inconsistent with the conversion of the entire *Oct4* null embryo to any one lineage, as has been proposed (Nichols et al., 1998; Chia Le Bin et al., 2014)

To determine which DE genes in *Oct4* null blastocysts are direct targets of OCT4 we compared the list of DE genes with known OCT4 binding sites in pluripotent F9 embryonyl carcinoma (EC) cells and in F9 EC cells that have been

differentiated into PE-like cells by treatment with retinoic acid (RA). (Askoy et al., 2013). 19% DE genes in *Oct4* null embryos at E3.75 were bound by OCT4 in F9 EC cells before and/or after differentiation to PE-like cells (Fig. 8C). Notably, OCT4 was detected binding near important PE genes such as *Pdgfrα, Gata4* and *Gata6* in differentiated F9 cells into PE-like cells, but not in pluripotent F9 EC cells (Fig. 8D), suggesting *Oct4* might directly regulate *Pdgfrα, Gata4 and Gata6* in the preimplantation mouse embryo. We also noted that OCT4 bound near *Bmp4* and *Tdgfr1*, genes encoding BMP and NODAL signaling molecules that are expressed in both ES cells and preimplantation mouse embryos.

Finally, we addressed the functional pathways regulated by *Oct4* in the blastocyst by performing Gene Set Enrichment Analysis (GSEA) of functional pathways in the KEGG, Reactome and Biocarta databases. At E2.5 we failed to identify any pathways that were enriched within the list of genes differentially expressed in *Oct4* null embryos. In contrast, at E3.75 and E4.25 several pathways were significantly enriched among differentially expressed transcripts in *Oct4* null embryos, including pathways such as oxidative phosphorylation, glycolysis and other metabolic pathways (Fig 8G, H), suggesting *Oct4* null embryos acquire major defects in cellular metabolism prior to embryo collapse, but after blastocyst formation.

Our RNA-seq analysis of *Oct4* null and non-mutant embryos demonstrates that *Oct4* directly and indirectly regulates genes required for EPI, PE and TE cell fate, in addition to pathways required for normal cellular metabolism and continued proliferation of the embryo. Because these trends are weak prior to E3.75, this supports a model where the *Oct4* is required in preimplantation mouse embryo after blastocyst formation.

3.5 Discussion

In this chapter, I have confirmed that *Oct4* is required to repress TE cell fate in the ICM, but only in a minority of ICM cells at E4.0. Prior to E4.0, *Oct4* is required cell-autonomously to promote PE cell fate. *Oct4* is a well-appreciated master regulator of pluripotency. Therefore, a role for *Oct4* in PE cells, which are not pluripotent, is surprising. I hypothesize that the role of *Oct4* in PE cells and the role of *Oct4* in pluripotent cells are distinct and regulated in a cell type specific manner. Identification of *Oct4* binding partners in different cellular contexts demonstrates that OCT4 can bind to different SRY-family transcription factors including SOX2 and SOX17 (Stephanovic et al., 2009; Ng et al., 2012). Intriguingly, shortly after blastocyst formation SOX2 is specific to the EPI and SOX17 is specific to the PE suggesting that in EPI cells OCT4 interacts with SOX2 to promote EPI cell fate and in PE cells OCT4 interacts with SOX17 to promote PE cell fate. The mechanisms that regulate whether OCT4 interacts with SOX2 or SOX17 are of great importance to stem cell biology, as they could regulate the choice between pluripotency and differentiation in ICM cells and ES cells.

Oct4 acts upstream of multiple parallel pathways in PE development

Although PE development proceeds through accumulation of endoderm specific transcription factors, these factors appear to be regulated by distinct pathways. For example, *Sox17* null embryos contain the same number of GATA4 and SOX7-positive ICM cells as wild-type embryos (Artus et al., 2011), indicating that *Sox17* is

not required for *Gata4* or *Sox7* expression. Therefore, the reduction in GATA4 and loss of Gata6 expression and failure to initiate *Sox7* expression, observed in *Oct4* null embryos, is not simply a consequence the failure to initiate expression of a single PE transcription factor, such as *Sox17*. Curiously, one of the earliest defects in *Oct4* null embryos is the failure to initiate expression of *Pdgfr*α, which promotes survival of PE cell number and for derivation of XEN cells from the PE of preimplantation mouse embryos (Kunath et al., 2005; Artus et al., 2010). *Pdgfr*α null embryos contain normal amounts of GATA4-positive cells and SOX17-positive cells as wild-type embryos, suggesting *Pdgfr*α *and Sox17 expression* is initiated by distinct mechanisms and that because *Pdgfr*α and *Sox17* expression is not initiated in *Oct4 null embryos, Oct4* is required in multiple parallel pathways that promote PE development.

When is *Oct4* required in PE development?

A recent study used a drug-inducible *Oct4* deletion allele to investigate the timing of the requirement for *Oct4* in PE development (Le Bin G et al., 2014). First, the authors confirmed my results that PE was disrupted in *Oct4* null embryos and that neither FGF4/Heparin treatment nor aggregation of wild-type ES cells rescued *Oct4* null PE. The authors then compared *Oct4* null embryos to embryos in which *Oct4* deletion had been induced at the 8-cell stage. Unlike *Oct4* null embryos, *Oct4* deletion embryos contained SOX17-positive cells, although the number of SOX17-positive cells was greatly reduced compared to non-mutant embryos, suggesting that in *Oct4* deletion embryos, residual OCT4 is present at the time when SOX17 is initiated. Furthermore, *Oct4* deletion at the 8-cell stage followed by addition of

ectopic FGF4/Heparin 24-hours after *Oct4* deletion rescued SOX17 expression. However, despite the presence of SOX17-positive cells in FGF4/Heparin treated *Oct4* deletion embryos, XEN cells could not be established from FGF4/Heparin treated *Oct4* deletion embryos indicating that *Oct4* promotes the expression of genes essential for PE development in addition to promoting *Sox17* expression. Finally, the authors aggregated wild-type ES cells prior to induction of *Oct4* deletion and after 24-hours of culture transferred ES cell aggregated *Oct4* deletion embryos to pseudopregnant females. Remarkably, *in utero,* embryos in which *Oct4* deletion was induced after aggregation with wild-type ES cell derived EPI contained normal PE. The authors interpreted rescue of PE in ES cell aggregated *Oct4* deletion embryos as evidence that *Oct4* is not required for PE development directly, but instead is required prior to PE development to establish ICM cell fate by repressing TE cell fate. This interpretation is not consistent with our data, which demonstrates that aspects of EPI and PE development are intact in *Oct4* null embryos and that TE identity is not acquired in all *Oct4* null ICM cells. An alternative explanation for the rescue of the PE lineage in embryos in which ES cell aggregation occurs prior to induction of *Oct4* deletion is that in embryos in which ES cell aggregation occurs prior to induction of *Oct4* deletion, OCT4 perdures and is present during the period when aggregated ES cells induce PE cell fate in *Oct4* deletion embryos. The perdurance of OCT4 in *Oct4* deletion embryos is supported by the presence of SOX17-positive cells in unaggregated, non-FGF4/Heparin treated *Oct4* deletion embryos, which is not observed in *Oct4* null embryos. In order to accurately interpret the results presented by Le Bin G et al. it is essential to define when OCT4 is eliminated from *Oct4* deletion embryos. Never the less, because I demonstrate that

multiple aspects of ICM development are intact, such as NANOG and GATA6 'salt-and-peppering' and repression of TE identity in most ICM cells of *Oct4* null embryos, it is unlikely that the defects in the PE of *Oct4* null embryos are due to an earlier role for *Oct4* in establishing the ICM.

Is *Oct4* essential in other non-pluripotent cell types?

The finding that *Oct4* cell-autonomously promotes PE development suggests that *Oct4* may play other roles in other non-pluripotent cell types. Indeed, *Oct4* transcript has been detected in the visceral endoderm (VE), which is derived from the PE lineage after implantation (Downs, 2008). *Oct4* is not required in the VE after E6.5 (DeVeale et al., 2013), but the VE appears shortly after implantation, therefore I hypothesized that *Oct4* may be required earlier than E6.5 in the VE. To test if *Oct4* is required in the VE earlier that E6.5 I mated mice with one conditional allele of *Oct4* (*Oct4*$^{fl/del)}$) to mice carrying an allele of *Cre-recombinase* under control of the VE specific *transthyretin* (*ttr-Cre)* promoter (Kwon and Hadjantonakis, 2009) to generate embryos in which at E5.5 *Oct4* is deleted in the VE only, and asked if development proceeded normally in the absence of *Oct4* in the VE. I first defined the timing and specificity of *ttr-Cre* expression by crossing *ttr-Cre* male mice to female mice carrying a *tomato* reporter that is activated in response to *Cre* expression and characterized the domain and timing of tomato expression at E5.5 and E6.5 (Fig. 10). At E5.5 and E6.5, TOMATO was absent from the EPI and TE lineages, and the parietal endoderm (ParE), which, like VE, is also derived from the PE lineage. TOMATO expression was detected specifically in the VE at E5.5 confirming that *ttr-Cre* expression is highly VE-specific and occurs as early as E5.5. I then asked if deletion

of *Oct4* from the VE affects normal development. Embryos in which *Oct4* was deleted specifically from the VE at E5.5 developed normally and were recovered at the expected frequency. Therefore, *Oct4* is not required in the VE as early as E5.5. Future studies should address the role of *Oct4* in other non-pluripotent tissues in which OCT4 has been detected, such as the embryonic endoderm lineage during gastrulation in the mouse embryo.

Implications for regenerative medicine

Forced expression of OCT4 with the pluripotency factors SOX2 and KLF4 in somatic cells is sufficient to induce somatic cells to become pluripotent, a technique called reprogramming. Only a minor fraction of the starting population of cells become pluripotent during reprogramming, it is not known what happens to the remaining cells. One intriguing possibility given the cell-autonomous requirement for *Oct4* is that in cells that do not become pluripotent during reprogramming, OCT4 instructs these cells away from pluriptency and instead towards becoming PE. Future studies should investigate whether reprogramming induces PE cell fate, and what OCT4's role in this process is.

A common practice in stem cell biology is to use an *Oct4-GFP reporter* to identify pluripotent cells. My results suggest that *Oct4* is required in non-pluripotent cells, and thus *Oct4* expression is not a reliable indicator of pluripotency. I suggest that *Nanog* expression is a more specific marker of pluripotency (Chambers et al., 2009) and a *Nanog-GFP* reporter would make a more specific reporter of pluripotency than *Oct4-GFP.*

3.6 Figures and Legends

Figure 1: Cell proliferation and cell lineage defects in *Oct4* null embryos

A – C) GATA6 and NANOG immunostaining in transverse confocal cross-sections of *Oct4* null and non-mutant embryos at indicated stages. White arrowhead = NANOG-negative and GATA6-negative cells in *Oct4* null embryos. Dotted line = ICM. White bar = 20 μm.

D) Average numbers of inside and outside cells in *Oct4* null and non-mutant embryos at indicated stages. N = number of embryos examined.

E) Average proportions of inside and outside cells in *Oct4* null and non-mutant embryos at indicated stages.

F – H) Average numbers of NANOG-positive, GATA6-positive and NANOG-negative/GATA6-negative (unlabeled) inside cells in *Oct4* null and non-mutant embryos at indicated stages.

I) Average proportions of NANOG-positive, GATA6-positive and NANOG-negative/GATA6-negative (unlabeled) inside cells in *Oct4* null and non-mutant embryos at indicated stages.

J) Average number of TUNEL-positive inside and outside cells in *Oct4* null and non-mutant embryos at indicated stages.

K) Representative TUNEL staining in *Oct4* null a non-mutant embryos at indicated stages.

L) SOX2 immunostaining in *Oct4* maternal/zygotic null and non-mutant embryos at E3.5.

Chapter 3, Figure 1

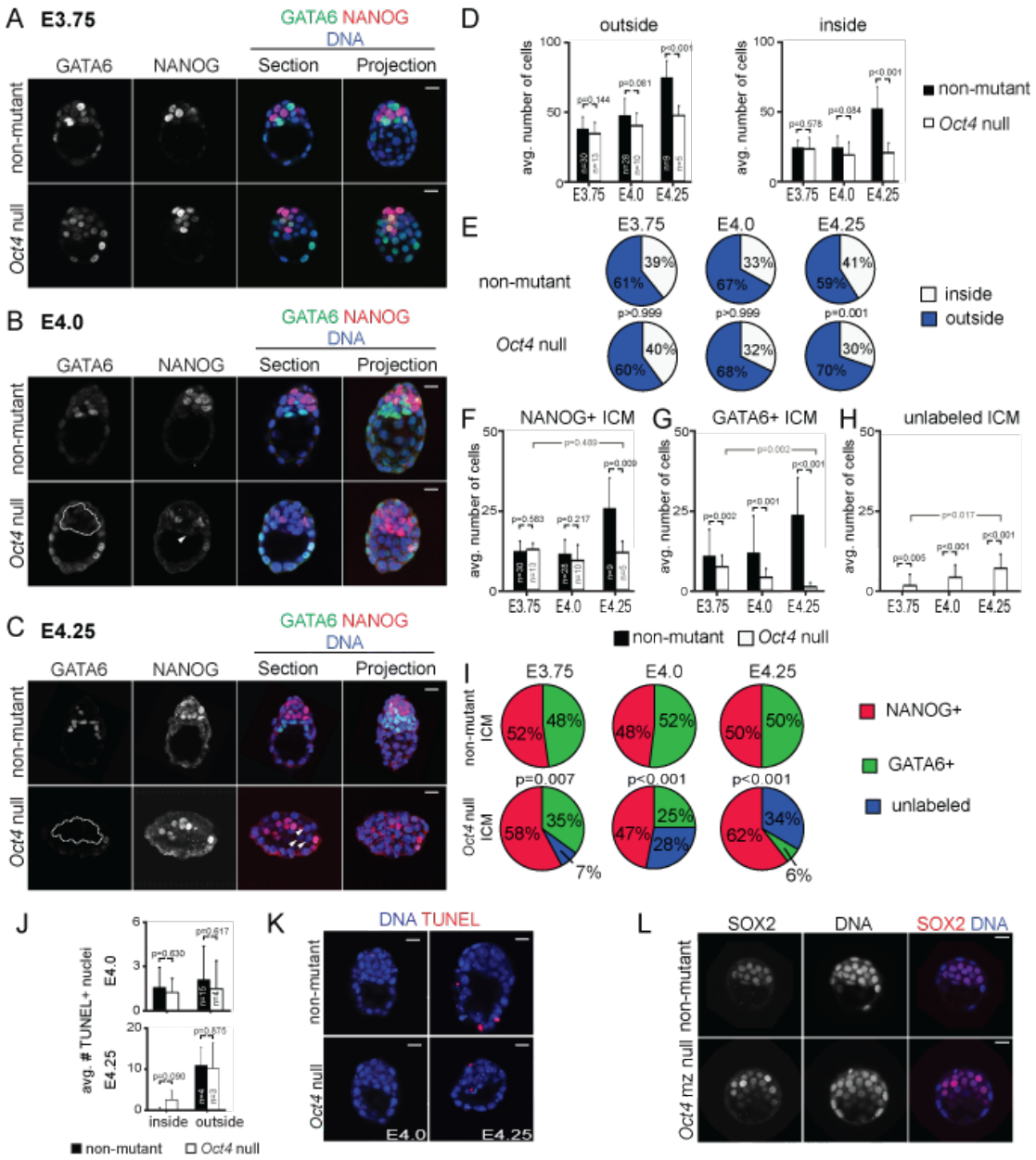


Figure 2: Expression of PE genes is disrupted in *Oct4* null embryos

A) PDGFRα immunostaining in *Oct4* null and non-mutant embryos at E3.5 and E3.75. N = number of embryos examined. White bar = 20 μm.

B) SOX17 immunostaining in *Oct4* null and non-mutant embryos at E3.5 and E3.75. N = number of embryos examined. White bar = 20 μm.

C) GATA4 immunostaining in *Oct4* null and non-mutant embryos at E3.75. N = number of embryos examined. White bar = 20 μm.

D) SOX7 immunostaining in *Oct4* null and non-mutant embryos at E4.0. N = number of embryos examined. White bar = 20 μm.

Chapter 3, Figure 2

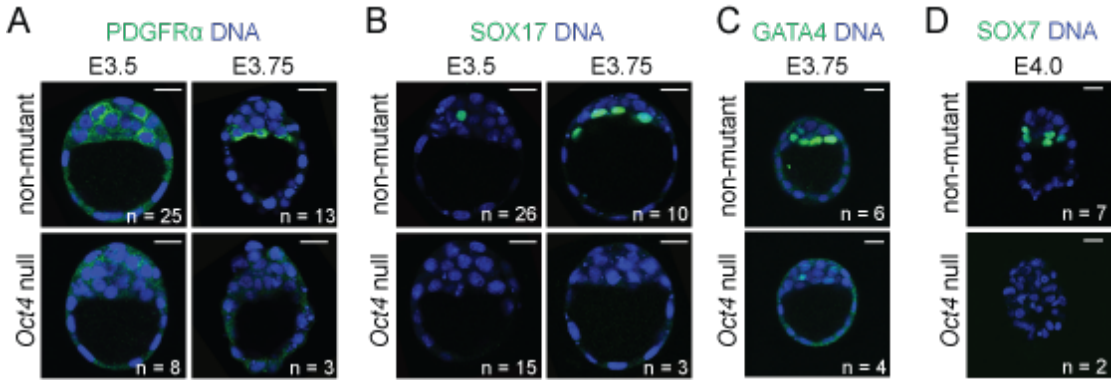


Figure 3: CDX2 is ectopically expressed in some, but not all, ICM cells in *Oct4* null embryos

A) NANOG and CDX2 immunostaining in *Oct4* null embryos and non-mutant embryos at E4.0. Dotted line denotes ICM. White arrow = CDX2-negative and NANOG-negative ICM cell. White bar = 20 μm.

B) Average proportion of *Oct4* null ICM cells expressing CDX2 or NANOG at E4.0 (n = 10 embryos). In *Oct4* null embryos at E4.0 CDX2 is detected in 27% of ICM cells. Of the remaining 73% CDX2-negative cells, 40% of CDX2-negative cells are also NANOG-negative. Based on NANOG expression, these cells are pre-PE cells in which failure to repress CDX2 expression cannot explain absence of PE markers.

Chapter 3, Figure 3

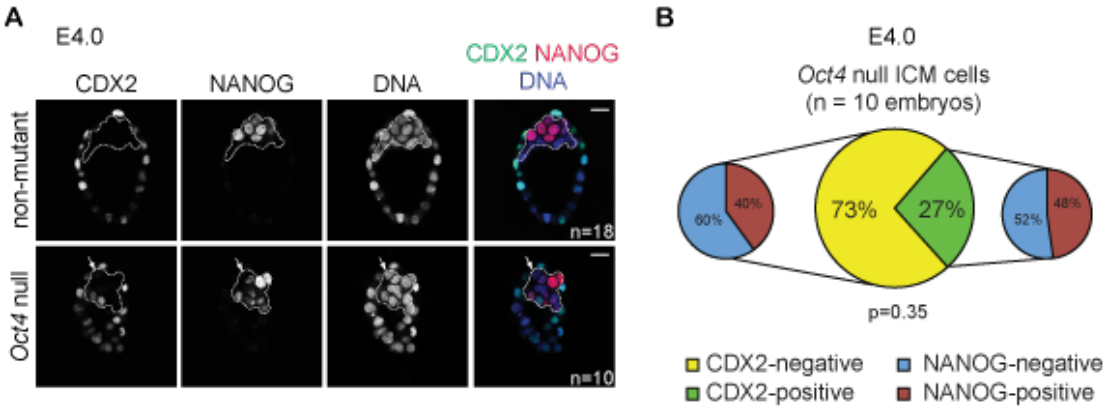


Figure 4. Maternally provided *Oct4* is not required for development and does not participate in preimplantation lineage decisions.

A) Breeding strategy for generation of *Oct4* M null and *Oct4* MZ null embryos and offspring. del = deleted; fl = floxed.

B) qPCR analysis of average *Oct4* transcript levels in wild-type and *Oct4* maternal null oocytes (n = 3 pools of 10 oocytes) Error bars = standard deviation.

C) Average numbers of inside and outside cells in control, *Oct4* maternal null, *Oct4* zygotic null and *Oct4* maternal/zygotic null blastocysts.

D) Representative confocal transverse sections of GATA6 and NANOG immunostaining in *Oct4* maternal and *Oct4* maternal/zygotic null embryos at E3.75. White bar = 20 μm.

E) Average numbers of GATA6-positive or NANOG-positive ICM cells in control, *Oct4* maternal null, *Oct4* zygotic null and *Oct4* maternal/zygotic null blastocysts.

Chapter 3 Figure 4

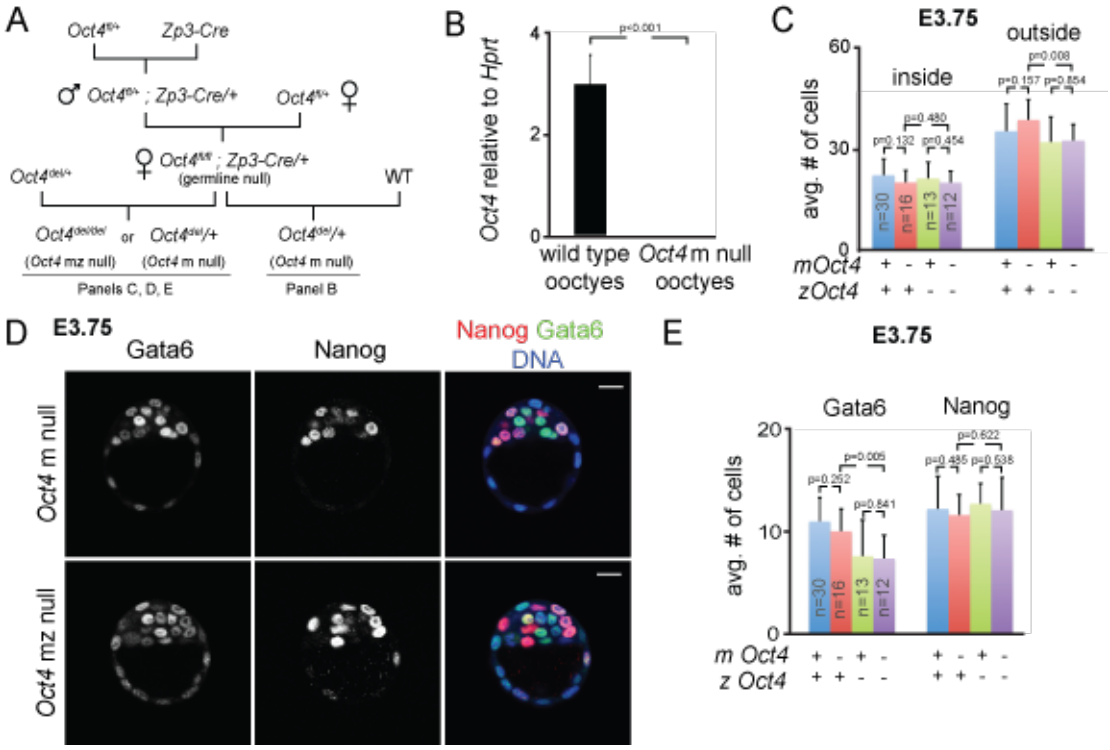


Figure 5: *Oct4* is not required for FGF/MAPK dependent repression of *Nanog*

A) NANOG and GATA6 immunostaining in *Oct4* null and non-mutant embryos cultured from E2.75 to E4.25 in control medium or medium containing FGFR/MAPK inhibitors.

B) Average proportions of inside cells expressing NANOG in *Oct4* null and non-mutant embryos following culture in control medium of FGFR/MAPK inhibitors.

C) Average proportions of inside cells expressing GATA6 in *Oct4* null and non-mutant embryos following culture in control medium or medium containing FGFR/MAPK inhibitors.

D) SOX17 immunostaining in *Oct4* null and non-mutant embryos cultured from E2.75 to E4.0 in control medium or medium containing FGFR/MAPK inhibitors.

E) SOX7 immunostaining in *Oct4* null and non-mutant embryos cultured from E2.75 to E4.5 in control medium or medium containing FGFR/MAPK inhibitors.

F) Average proportion of inside cells expressing SOX17 in *Oct4* null and non-mutant embryos cultured from E2.75 to E4.0 in control medium or medium containing FGFR/MAPK inhibitors.

G) Average proportion of iinside cells expressing SOX7 in *Oct4* null and non-mutant embryos cultured from E2.75 to E4.5 in control medium or medium containing FGFR/MAPK inhibitors.

H) Average proportion of inside cells expressing NANOG in *Oct4* null and non-mutant embryos cultured from E2.75 to E4.5 in control medium or medium containing FGFR/MAPK inhibitors.

Chapter 3, Figure 5

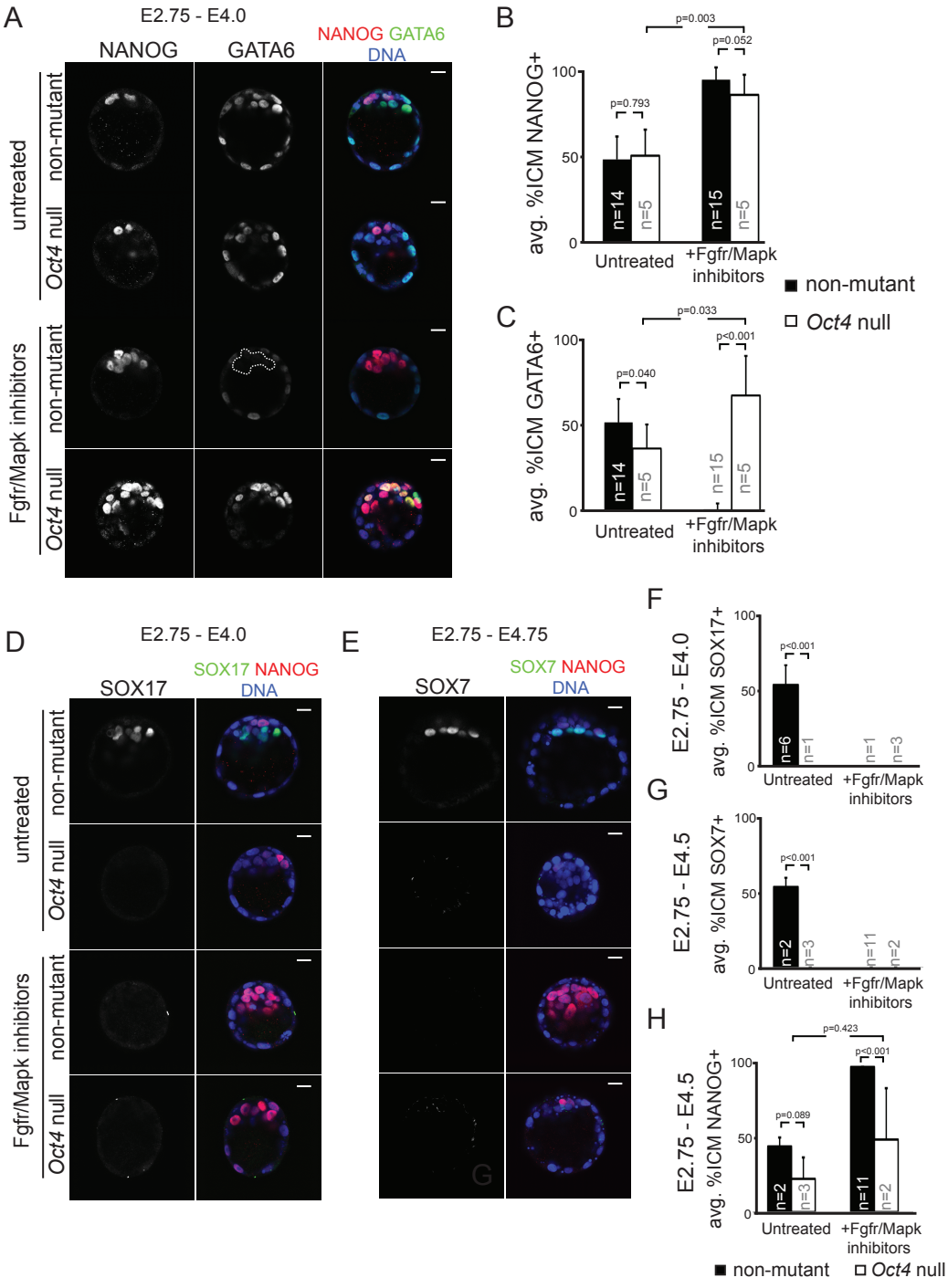


Figure 6: *Oct4* is required for FGF/MAPK signaling dependent expression of PE genes

A) GATA6 immunostaining in *Oct4* null embryos and wild-type embryos cultured from E2.75 to E4.5 in FGF4 and Heparin.

B) SOX17 immunostaining in *Oct4* null embryos and wild-type embryos cultured from E2.75 to E4.5 in FGF4 and Heparin.

C) NANOG immunostaining in *Oct4* null embryos and wild-type embryos cultured from E2.75 to E4.5 in FGF4 and Heparin.

D) Average proportions of ICM cells expressing GATA6 in *Oct4* null and wild-type embryos cultured from E2.75 to E4.5 in FGF4 and Heparin. Error bars = standard deviation.

E) Average proportions of ICM cells expressing SOX17 in *Oct4* null and wild-type embryos cultured from E2.75 to E4.5 in FGF4 and Heparin. Error bars = standard deviation.

F) Average proportions of ICM cells expressing NANOG in *Oct4* null and wild-type embryos cultured from E2.75 to E4.5 in FGF4 and Heparin. Error bars = standard deviation.

G) Comparison of average proportions of ICM cells expressing SOX17 in *Oct4* zygotic null and *Oct4* maternal/zygotic null embryos. Error bars = standard deviation.

J) Representative images of *Oct4* null and non-mutant outgrowths in normal conditions or supplemented with FGF4 and Heparin. Only non-mutant embryos outgrew PE-like cells, regardless of culture conditions.

Chapter 3, Figure 6

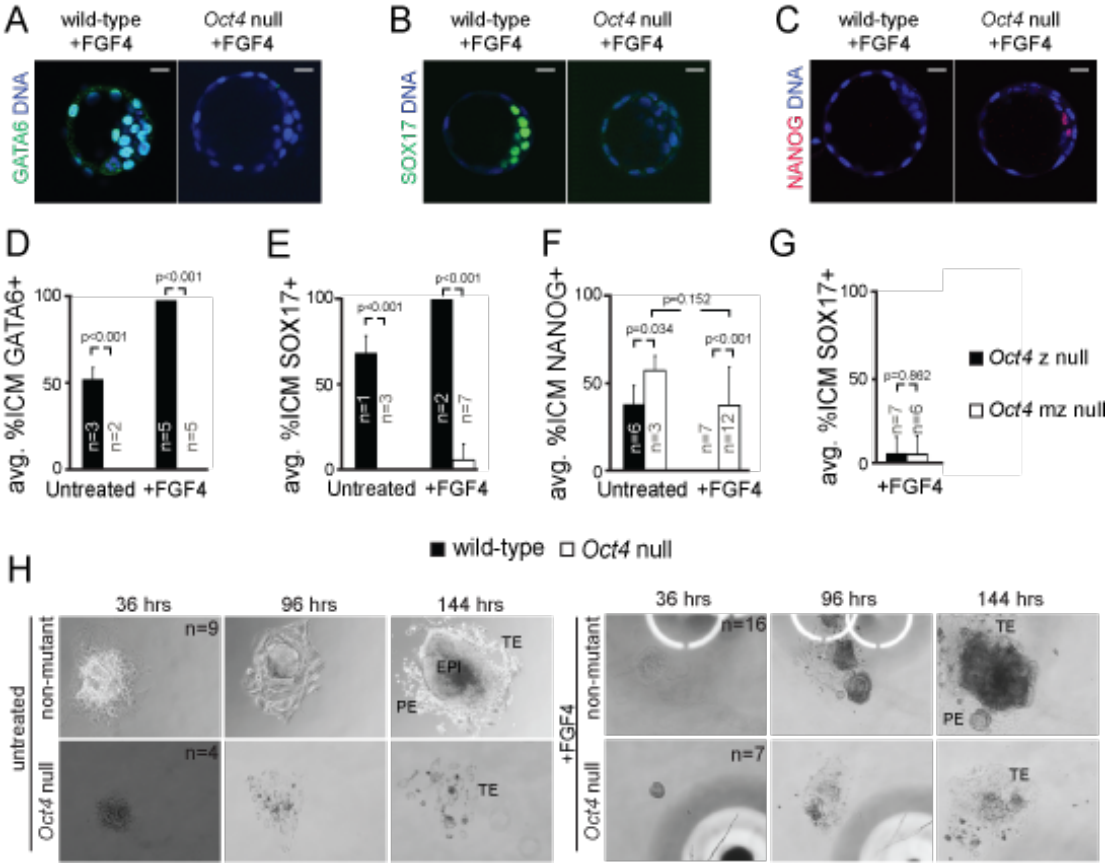


Figure 7: Oct4 cell-autonomously promotes expression of PE genes

A) Schematic of method used to generate chimeric embryos.

B) ES cell contribution to *Oct4* null and non-mutant embryo hosts. Error bars = standard deviation.

C) SOX17 immunostaining and YFP signal in *Oct4* null and non-mutant chimeras. White bar = 20 μm.

D) SOX7 immunostaining and YFP signal in *Oct4* null and non-mutant chimeras. Note the presence of a rare SOX7-positive cell in the *Oct4* null chimera. White bar = 20 μm.

E) Average proportions of SOX17-positive host-derived ICM cells in *Oct4* null and non-mutant chimeras. Error bars = standard deviation.

F) Average proportions of SOX7-positive host-derived ICM cells in *Oct4* null and non-mutant chimeras. Error bars = standard deviation.

G) NANOG immunostaining and YFP signal of *Oct4* null and non-mutant chimeras. White bar = 20 μm.

H) Average proportions of NANOG-positive host-derived ICM cells in *Oct4* null and non-mutant chimeras. Error bars = standard deviation.

I) Average proportions of SOX17-positive host-derived ICM cells in *Oct4* maternal null and *Oct4* maternal/zygotic chimeras. Error bars = standard deviation.

J) Example of rare SOX17-positive *Oct4* null ICM cell. SOX17 immunostaining and YFP signal in *Oct4* null and non-mutant chimeras. White bar = 20 μm.

K) ES cell contribution to *Oct4* null chimeras with no PE gene expression and *Oct4* null chimeras with weak PE gene expression. PE gene expression = SOX17 or SOX7. Error bars = standard deviation.

L) Comparison of SOX17-positive cells treated with FGF4/Heparin and ES cells. ES cells provide a rare, but significant increase in SOX17 expression compared to FGF4/Heparin treatment. Error bars = standard deviation.

Chapter 3, Figure 7

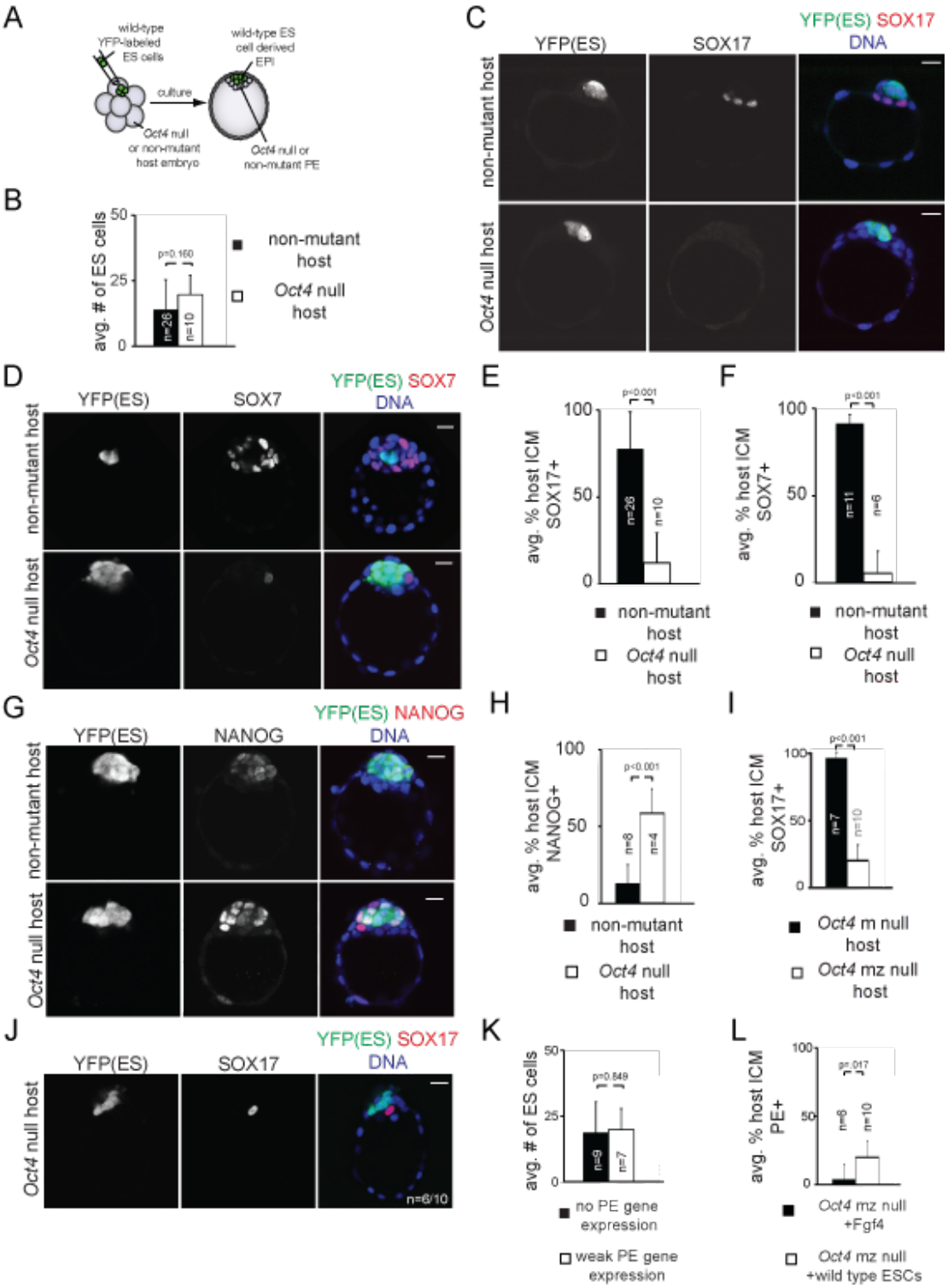


Figure 8: Whole transcriptome analysis of *Oct4* null and non-mutant embryos

A) MDS plots of *Oct4* null and wild-type embryos generated from the top 500 differentially expressed genes.

B) Volcano plots for each developmental stage examined by RNA-seq. White dots = Refseq gene entry. Items to the left of the Y-axis are greater are reduced in *Oct4* null embryos. Y-axis = $-\log_{10}$(FDR). X-axis = Log_2 (*Oct4* null/wild-type). Red line = false discovery rate threshold < 0.05.

C) Venn diagram showing degree of overlap between E3.75 *Oct4*-dependent genes identified in this study and genes identified as bound by OCT4 in F9 EC cells before and/or after differentiation to PE-like cells (Askoy et al., 2013).

D) Examples of ChIP-seq data (Askoy et al., 2013) showing locations of OCT4 binding sites (boxed regions) near *Pdgfra, Gata4* and *Gata6*. SOX17 ChIP-seq data is also included to show overlapping OCT4/SOX17 peaks at *Pdgfra* and *Gata4* loci in F9 EC cells differentiated to PE-like cells.

E) Gene signature analysis showing enrichment scores for lineage-specific query signature gene sets evaluated against target gene signature distributions at each stage (*FDR < 0.05).

F) Representative GSEA plots for major and minor networks shown in (G).

G and H) Enrichment maps of *Oct4*-dependent functional networks at E3.75 and E4.25. Nodes represent individual gene sets, and node color corresponds to direction of gene expression difference between the genotypes (red = down in null, blue = up in null). Green lines indicate shared genes and line thickness is proportional to number of shared genes.

Chapter 3, Figure 8

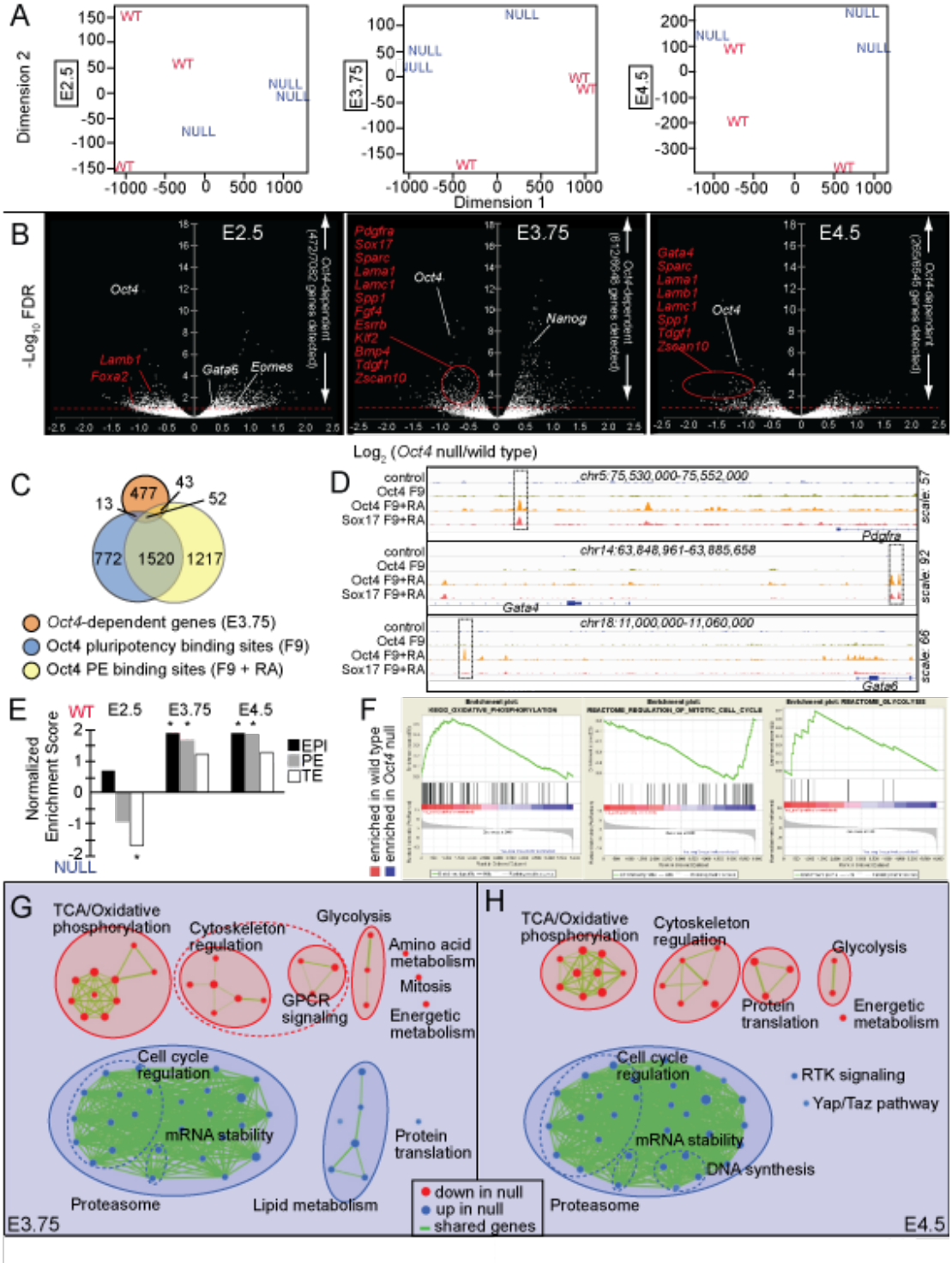
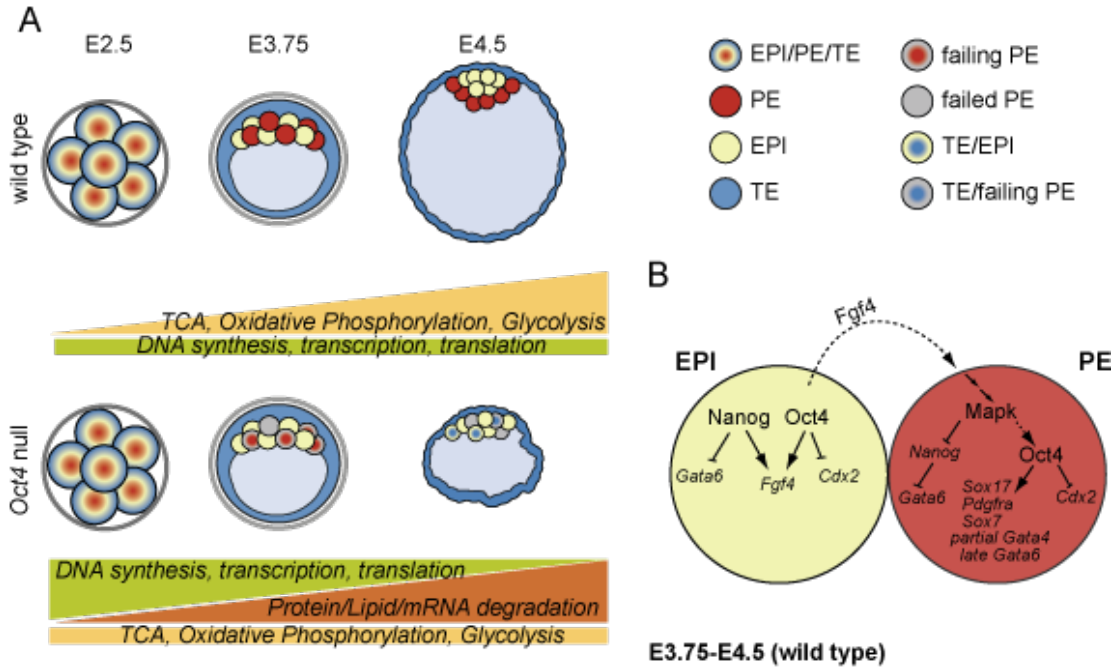


Figure 9: Summary of *Oct4* null phenotype and working model

(A) In normal embryos, Oct4 promotes survival of all three blastocyst lineages and facilitates the transcription of genes involved in multiple metabolic pathways, as well as the transcription of lineage-specific, cell fate determining transcription factors. (B) In EPI cells, Oct4 induces expression of multiple genes, including signaling molecules such as *Fgf4* that induce PE cell fate non-cell-autonomously. In PE cells, Oct4 normally acts downstream of MAPK to promote expression of *Sox17*, *Pdgfr*α, *Gata4*, *Sox7*, and eventually *Gata6*. In parallel, MAPK represses *Nanog* in an *Oct4*-independent manner in PE cells. Also in parallel, OCT4 represses expression of *Cdx2* and other TE genes in ICM cells.

Chapter 3, Figure 9



E3.75-E4.5 (wild type)

Figure 10: *Ttr-Cre* is active specifically in the visceral endoderm prior to E5.5

A) TOMATO reporter signal in a single transverse section of ttr-Cre/+; tdtomato/+ mouse embryo at E5.5. VE and EPI are labeled. ParE has been removed from this embryo. White bar = 20 microns.

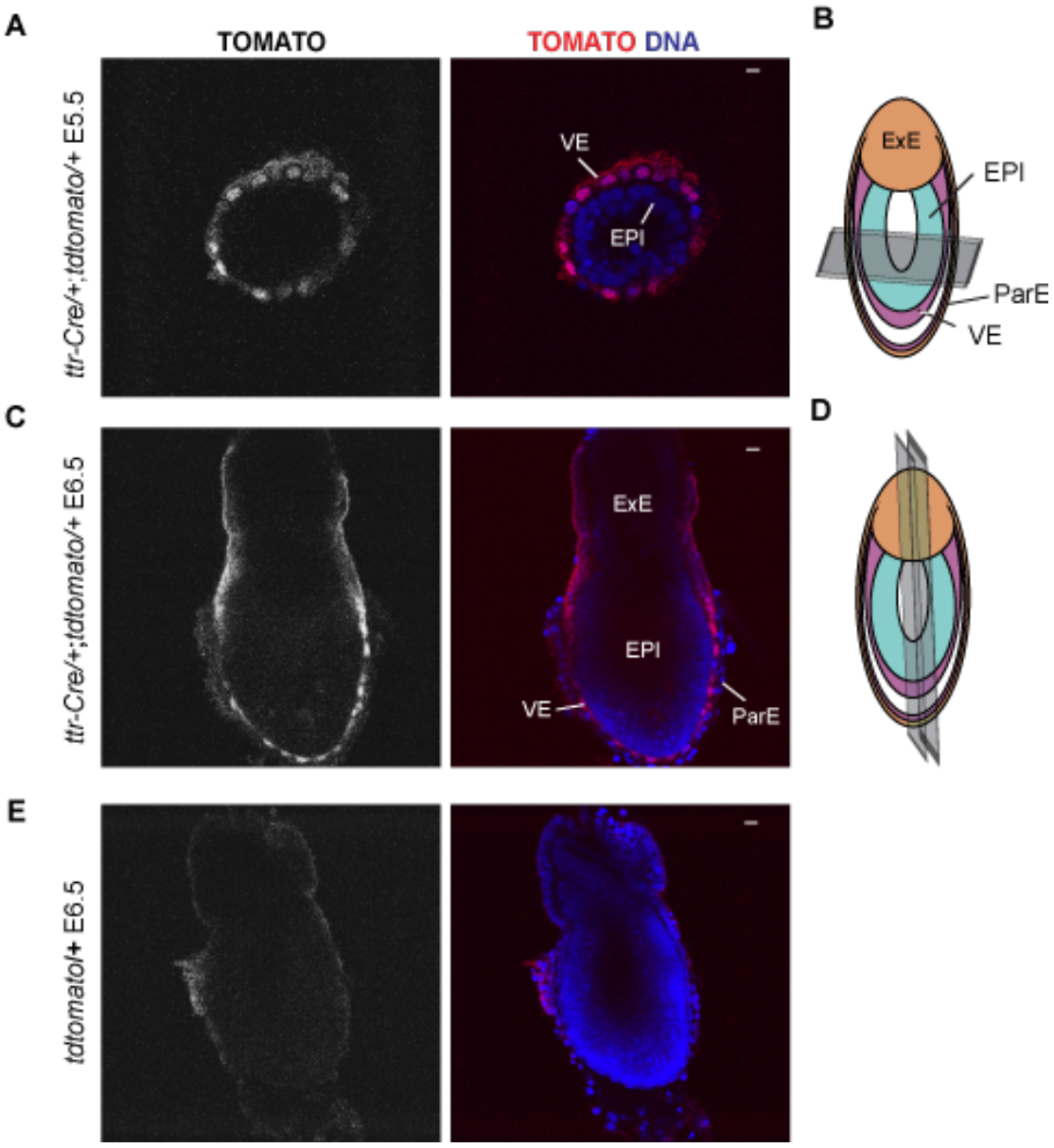
B) Cartoon of E5.5 – E6.5 embryo showing transverse plane shown in (A) of mouse embryo. Orange = TE lineage. Violet = PE lineage. Aqua = EPI lineage. ExE = Extraembryonic Ectoderm. ParE = Parietal Endoderm. VE = Visceral endoderm.

C) TOMATO reporter signal in single sagittal section of ttre-Cre/+; tdtomato/+ mouse embryo at E6.6. EXE, EPI, ParE and VE are labeled. White bar = 20 microns.

D) Cartoon of E5.5 – E6.5 embryo showing sagittal plane shown in (C) and (E).

E) Lack of TOMATO reporter signal in single sagittal section of tdtomato/+ mouse embryo at E6.5.

Chapter 3, Figure 10



CHAPTER 4

Sox2 is regulated by FGF/MAPK and promotes PE development non-cell autonomously by promoting *Fgf4* in EPI cells

4.1 Introduction

To understand how pluripotency is regulated it is essential to understand how the pluripotent transcriptional network is established and maintained. One approach to investigate the role of pluripotency transcription factors is to examine their role in establishing and regulating pluripotency in development. In Chapter 3, I demonstrated that *Oct4* promotes PE development by cell-autonomously promoting the expression of multiple factors required for PE development. The role of *Oct4* in promoting PE cell fate is distinct from the role of the pluripotency factor *Nanog* which non-cell autonomously promotes PE cell fate by promoting *Fgf4* transcription in the EPI (Messerschmidt et al., 2010, Frankenberg et al., 2012). The pluripotency factor SOX2*,* which promotes pluripotency in ES cells together with OCT4 and NANOG is expressed in the preimplantation mouse embryo and has been suggested to be cell-autonomously required in the EPI and TE lineages (Avilion, 2003; Keramari et al., 2010). However, studies on the role of *Sox2* in the TE lineage do not agree on the timing of *Sox2*'s cell-autonomous role in the TE, with one study suggesting *Sox2* is required after implantation in the extraembryonic ectoderm (Avilion, 2003), and the other study suggesting it is required earlier, for TE specification (Keramari et al., 2010). Notably, a role for *Sox2* in TE specification was demonstrated using RNAi to knockdown *Sox2*, which we have shown to be unreliable for inferring gene function from knockdown embryo phenotypes (see chapters 2 and 3). To clarify understand

regulation and functions of the important pluripotency factor *Sox2* in the pre-implantation mouse embryo, I, along with Ralston Lab members Dr. Stephanie Blij and Eryn Wicklow, perform a careful molecular analysis of cell fate in *Sox2* null embryos. In this chapter, I discuss the analysis of cell fate in *Sox2* null embryos and then present my evidence that, similar to *Nanog, Sox2* is regulated by FGF/MAPK signaling and promotes PE cell-fate non-cell autonomously, in part by promoting *Fgf4* transcription in the EPI.

Regulation of *Sox2* in the blastocyst

In ES cells, *Sox2* regulates of network of pluripotency genes including *Oct4* and *Nanog* (Masui et al., 2007, Li et al., 2007, Okamura-Nakanishi et al., 2005, Tomioka et al., 2002, Kuroda et al., 2005; Chew et al., 2005). ES cells cannot be derived from *Sox2* null embryos (Avilion, 2003), suggesting that similar to ES cells, *Sox2* is essential for pluripotency in the blastocyst. Recent work in the Ralston Lab has demonstrated that *Sox2* is restricted to inside cells at the 16-cell stage, earlier than the pluripotency factors *Nanog* and *Oct4* (Wicklow et al., *in revision*) suggesting that *Sox2* may be regulated by a signaling network upstream of the *Cdx2*-dependent network that restricts *Nanog* and *Oct4* to the ICM (Strumpf et al. 2005, Ralston and Rossant 2008., Ralston et al., 2010). By disrupting the HIPPO signaling pathway components *Lats1/2* and *Tead4* the authors go on to demonstrate that HIPPO signaling pathway members repress *Sox2* in outside cells and that repression of *Sox2* in outside cells is independent of *Cdx2*.

During the resolution of EPI and PE cells fates during the second preimplantation cell fate decision, *Sox2* undergoes a second phase of restriction in

which *Sox2* is repressed in PE cells, leading to expression of *Sox2* in EPI cells only (Fig. 1A,B). Intriguingly, FGF/MAPK-dependent restriction of *Nanog* to the EPI precedes restriction of *Sox2* to the EPI, suggesting the hypothesis that restriction of *Sox2* to the EPI may occur through an FGF/MAPK-independent mechanism. In this chapter, I test this hypothesis and demonstrate that FGF/MAPK signaling is both necessary and sufficient for the restriction of *Sox2* to the EPI and therefore in the second cell fate decision of preimplantation development *Nanog* and *Sox2* are repressed in the FGF/MAPK signaling.

Sox2 promotes PE cell fate

Prior to blastocyst formation, the ICM is a molecularly homogenous mixture of cells containing progenitors for the EPI and PE. Shortly thereafter, homogeneity of the ICM resolves into two distinct molecular profiles that represent EPI and PE cells (Guo et al., 2010). The resolution of EPI and PE cells is driven by FGF/MAPK signaling. First, the pluripotency factor *Nanog* is restricted by FGF/MAPK signaling to EPI cells (Nichols et al., 2009; Yamanaka et al., 2010; Chazaud et al., 2006; Kang et al., 2013; Yamanaka et al., 2013) where it represses *Gata6* (Frankenberg et al., 2011). Second, FGF/MAPK signaling promotes the expression of *Pdgfrα, Sox17,* and *Gata4* in PE by FGF4 secreted from EPI cells (Frankenberg et al., 2011; Kang et al., 2013; Frum et al., 2013).

To investigate the role of *Sox2* in the preimplantation mouse embryo, Wicklow and colleagues analyzes EPI, PE and TE cell fates in *Sox2* MZ null embryos. Analysis of cell fate in *Sox2* MZ null embryos demonstrates that in contrast to *Sox2* knockdown embryos (Keramari et al., 2010), *Sox2* MZ null embryos

form the blastocyst and properly express *Nanog* in a salt-and-pepper pattern, suggesting that specification of EPI and PE cells does not require *Sox2* (Fig. 2B). Somewhat surprisingly, while SOX17 is detected in the appropriate number of cells in E4.25 *Sox2* null embryos, analysis of SOX17 at E3.75 demonstrates that *Sox2* null embryos have a significant delay in initiating expression of *Sox17* (Fig. 2A,C), suggesting that *Sox2* has an important role in promoting PE cell fate.

Based on the role of the other essential pluripotency factors *Nanog* and *Oct4* in promoting PE cell fate, *Sox2* could promote PE cell fate through two mechanisms: *Sox2* could either promote PE cell through a non-cell autonomous mechanism, possibly by regulating *Fgf4* in the EPI*,* similar to the role of *Nanog* in promoting PE cell fate, or, *Sox2* could act similar to *Oct4* and promote cell fate cell-autonomously in the PE. Alternatively, *Sox2* could promote PE development through mechanisms distinct from how *Nanog* and *Oct4* promote PE development.

In this chapter, I clarify our understanding of how the essential pluripotency factor promotes PE cell fate and demonstrate that *Sox2* promotes PE cell fate, non-cell autonomously, in part by promoting *Fgf4* in the EPI.

4.2 Materials and methods

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. 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* (deVries et

al., 2000), *Tead4*^{tm1Hssk} (Nishioka et al., 2009), and *Cdx2*$^{tm1.1Ara}$ (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). Final concentrations of FGFR/MAPK inhibitors were 100 nM PD173074 and 500 nM PD0325901(Stemgent).

Immunofluorescence and confocal microscopy

Embryos were fixed, stained, imaged, and recovered for genotyping as previously described (Ralston and Rossant., 2008) Optical sections 5 µm in thickness were collected using a Leica SP5 confocal microscope and 20X, 0.7 NA objective. Antibody sources were previously described (Chapter 3; Frum et al., 2013; Hirate et al., 2013) and rabbit anti-EOMES (Abcam), rabbit anti-DAB2 (Santa Cruz Biotech) and rabbit anti-LAMA1 (Sigma).

Chimeras

YFP-expressing R1 ES cells (George et al., 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 (Smith et al., 2009).

4.3 Results

Sox2 is restricted to EPI cells by an FGF/MAPK-dependent mechanism

In the preimplantation mouse embryo, *Nanog* is repressed by FGF/MAPK signaling in PE cells shortly after blastocyst formation (Plusa et al., 2008; Kang et al., 2013, Krawchuk et al., 2013). Accordingly, treatment of preimplantation mouse embryos with FGF4/Heparin or FGFR/MAPK inhibitors leads to repression or expression of *Nanog* throughout the entire ICM (Nichols et al., 2009; Yamanaka et al., 2010). Analysis of SOX2 in blastocyst stage embryos demonstrates that while initially expressed in all ICM cells at blastocyst formation, SOX2 is repressed from the PE by E4.0 (Fig. 1C, D). I hypothesize like *Nanog, Sox2 is* repressed in the PE by FGF/MAPK signaling. To test if *Sox2* is repressed in the PE by FGF/MAPK signaling I cultured wild-type embryos in small molecule inhibitors of FGFR and MAPK from E2.75 to E4.5 and labeled SOX2 and SOX17 by immunofluorescence. In wild-type embryos cultured in control media, SOX2 was detected only in EPI cells and SOX17 was detected only in PE cells. In wild-type embryos treated with FGFR/MAPK inhibitors SOX17 was undetectable, confirming the efficacy of our inhibitor treatment (Yamanaka et al., 2010, Frum et al., 2013), and SOX2 was

detected in all ICM cells (Fig. 1G). Therefore, unlike in the TE where *Sox2* is repressed by the HIPPO signaling component TEAD4 (Wicklow et al. *in revision*), *Sox2* is repressed by FGF/MAPK signaling in the PE lineage.

Intriguingly, comparison of NANOG and SOX2 in E3.75 blastocysts clearly demonstrates that repression of NANOG precedes repression of *Sox2* in presumptive PE cells (Fig. 1A, B). It is unclear whether the difference in timing of *Nanog* and *Sox2* repression in the EPI is due to differences in when *Nanog* or *Sox2* become sensitive to FGF/MAPK signaling, differences in the level of FGF/MAPK signaling required for repression, or difference in NANOG and SOX2 turnover.

Sox2* promotes PE development through regulating *Fgf4

I next sought to investigate the mechanism by which *Sox2* promotes PE development. *Sox2* null embryos exhibit a delay in PE development, as demonstrated by the absence of SOX17 in E3.75 *Sox2* null embryos (Wicklow et al., *in revision;* Fig. 2A, C). *Fgf4* is required for PE development (Kang et al., 2013; Yamanaka et al., 2013) and is co-regulated in ES cells by OCT4 and SOX2 (Yuan et al., 1995; Ambrosetti et al., 1997). Intriguingly, *Fgf4* levels are reduced in *Sox2* null embryos compared to non-mutant littermates (Wicklow et al., *in revision*). This led me to hypothesize that *Sox2* promotes PE development through regulating transcription of *Fgf4* in the EPI.

I predicted that PE development is delayed in *Sox2* null embryos because *Sox2* is required for expression of *Fgf4* and therefore, treatment of *Sox2* null embryos with FGF4/Heparin will rescue the timing of PE development. Therefore, I cultured E2.75 *Sox2* null embryos and non-mutant littermates in either control

medium or medium containing FGF4/Heparin to E3.75 and examined NANOG and SOX17 by immunofluorescence (Fig. 3A).

To confirm that any rescue of the PE observed in FGF4/Heparin treated *Sox2* null embryos is due to a FGF4/Heparin treatment and not due to a difference in developmental timing, I first sought to confirm that the timing of development and the *Sox2* PE phenotype was not altered by *in vitro* culture or FGF/Heparin treatment. In non-mutant embryos cultured in control medium SOX17 was detected in approximately half of the ICM, similar to E3.75 non-mutant embryos developing *in utero* (Fig. 3C, D). In *Sox2* null embryos cultured in control medium SOX17 was significantly reduced compared to non-mutant embryos, phenocopying E3.75 *Sox2* null embryos *in utero* (Fig. 3C, D). Additionally, non-mutant and *Sox2* null embryos cultured in either control medium or medium containing FGF4/Heparin had similar numbers of total cells as E3.75 non-mutant and *Sox2* null embryos *in utero* (Fig. 3B). Thus, *Sox2* null embryos cultured in control medium from E2.75 for 30 hours are equivalent to E3.75 *Sox2* null embryos *in utero* in terms of total cell number, and have the same PE phenotype at E3.75 *Sox2* null embryos *in utero*.

I next examined SOX17 in non-mutant and *Sox2* null embryos cultured in FGF/Heparin. In non-mutant embryos cultured in medium containing FGF4/Heparin from E2.75 to E3.75 the number of ICM cells expressing SOX17 was significantly increased compared to untreated embryos (Fig. 2C,D), as expected (Yamanaka et al., 2010). In *Sox2* null embryos treated with FGF4/Heparin SOX17 expression was significantly increased compared to *Sox2* null embryos cultured in control medium (Fig. 3C,D). The robust increase of SOX17 in FGF4/Hep treated *Sox2* null embryos

suggests that the delay in PE development observed in *Sox2* null embryos is either partially, or fully the consequence of reduced *Fgf4* in *Sox2* null embryos.

Intriguingly, the proportion of SOX17-positive cells in FGF4/Heparin treated *Sox2* null embryos was slightly but significantly reduced compared to non-mutant embryos treated with FGF4/Heparin. It is unclear why FGF4/Heparin treatment induces a slightly smaller proportion of SOX17-positive cells in *Sox2* null embryos than in non-mutant embryos but one possible explanation is that in addition to promoting *Fgf4*, *Sox2* regulates additional PE promoting pathways, either non-cell or cell-autonomously.

Continued treatment of *Sox2* null embryos with FGF4/Heparin until E4.5 led to equivalent proportions of SOX17-positive cells in non-mutant and *Sox2* null embryos (Fig. 3E,F), suggesting that if *Sox2* does indeed promote PE cell fate through mechanisms other than promoting *Fgf4* transcription, these mechanisms are not required for PE cell fate at E4.5. I conclude that a major contribution of *Sox2* in promoting PE development is through *Sox2* promoting *Fgf4* in the EPI.

Sox2 regulates PE development non-cell autonomously

Because we observed that treatment of *Sox2* null embryos with FGF4/Heparin had a slightly but significantly reduced proportion of SOX17-positive cells compared to non-mutant embryos treated with FGF4/Heparin, this leaves open the possibility that *Sox2* has FGF4-independent roles in PE development. To distinguish whether *Sox2* regulates PE development non-cell autonomously or cell-autonomously we generated chimeric embryos consisting of wild-type EPI and a *Sox2* null PE by aggregating YFP-labeled ES cells with 8-cell pre-compaction stage

Sox2 null embryos and cultured the resulting embryos until E3.75 (Fig. 4A). We first confirmed that our aggregation procedure did not alter the timing of development by comparing the total host cell number of aggregated embryos with the total cell number of E3.75 flushed embryos (Fig. 4B). In non-mutant chimeras, aggregated ES cells occupied the EPI compartment of the ICM and all host ICM cells were SOX17-positive. *Sox2* null chimeras exhibited maximal response to the aggregated ES cells, containing SOX17 expression indistinguishable from that of non-mutant littermates (Fig. 4C,D). Our results indicate that *Sox2* is dispensable for PE development in the presence of a wild-type EPI and therefore *Sox2* is required non-cell autonomously for PE development.

4.4 Discussion

In this chapter, I have contributed to our understanding of the regulation of cell fates in the embryo by investigating how *Sox2* is regulated during development and *Sox2*'s role in resolving ICM cell fates. I have demonstrated that *Sox2* is repressed in the EPI blast through an FGF/MAPK dependent mechanism during the second lineage decision in preimplantation mouse development and that *Sox2* regulates PE development non-cell autonomously, in part through promoting *Fgf4* in the EPI. These findings significantly clarify our understanding of the role of the pluripotency factor SOX2 during development and help to elucidate the signaling and transcriptional networks that regulate the establishment of pluripotency *in vivo* (Fig. 5).

4.5 Figures and Legends

Figure 1: *Sox2* expression is regulated by FGF/MAPK signaling

A) SOX2 and NANOG immunostaining in wild-type embryos at E3.75. White arrowhead = SOX2-positive, NANOG-negative ICM cell. White bar = 20 μm. N = number of embryos.

B) Average proportion of SOX2-positive and NANOG-positive ICM cells in wild-type embryos at E3.75. Error bars = standard deviation.

C) SOX2 and SOX17 immunostaining in wild-type embryos at E3.75 in which SOX2 is undergoing restriction to the PE. Note presence of SOX17/SOX2-double positive cells and SOX17-positive, SOX2-negative cells. White bar = 20 μm. N = number of embryos.

D) SOX2 and SOX17 immunostaining in wild-type embryos at E4.25. Note SOX2 is restricted to the EPI. White bar = 20 μm. N = number of embryos.

E) SOX2 and SOX17 immunostaining in wild-type embryos cultured in control medium or medium containing FGFR and MAPK inhibitors from E2.75 to E4.5.

F) Average proportion of SOX2-positive ICM cells in wild-type embryos cultured in control medium or medium containing FGFR and MAPK inhibitors. Error bars = standard deviation.

G) SOX2 and SOX17 immunostaining in wild-type embryos cultured in control medium or medium containing FGF4/Heparin from E2.75 to E4.5.

H) Average proportion of SOX2-positive ICM cells in wild-type embryos cultured in control medium or medium containing FGF4/Heparin. Error bars = standard deviation.

Chapter 4, Figure 1

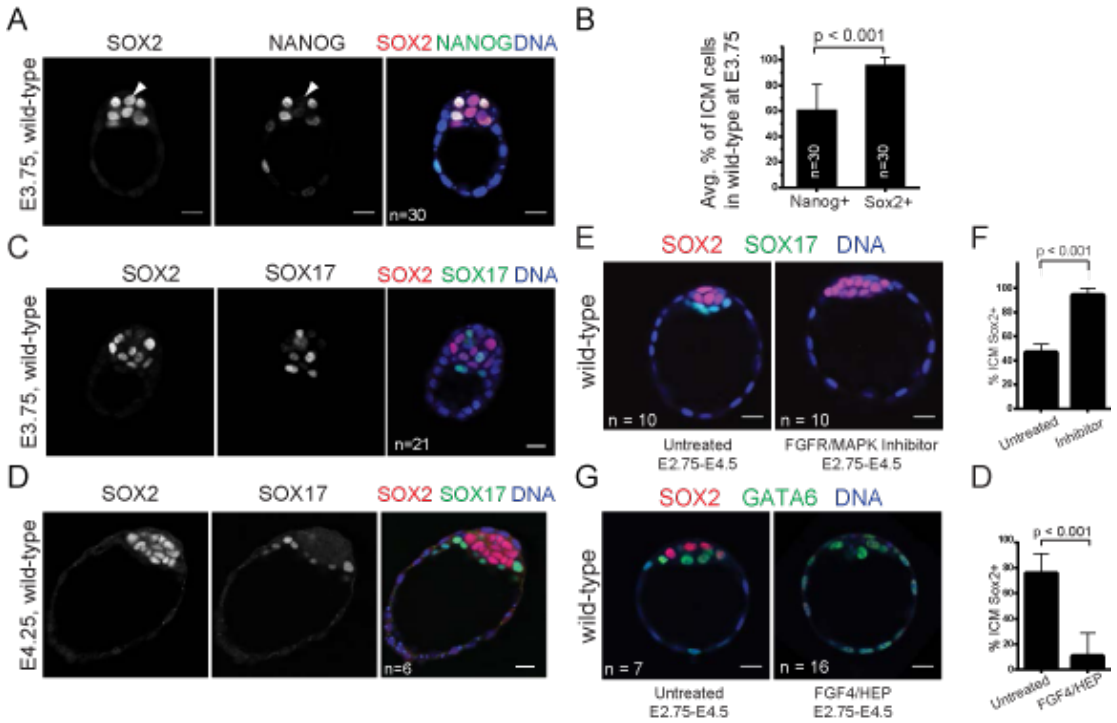


Figure 2: PE development is delayed in *Sox2* null embryos

A) SOX17 and NANOG immunostaining in *Sox2* null embryo at E3.75. White bar = 20 μm. N = number of embryos.

B) Average proportion of NANOG-positive ICM cells in non-mutant, *Sox2* maternal null, *Sox2* null, and *Sox2* maternal/zygotic null embryos at E3.75. Error bars = standard deviation.

C) Average proportion of SOX17-positive ICM cells in non-mutant, *Sox2* maternal null, *Sox2* null, and *Sox2* maternal/zygotic null embryos at E3.75. Error bars = standard deviation.

Chapter 4, Figure 2

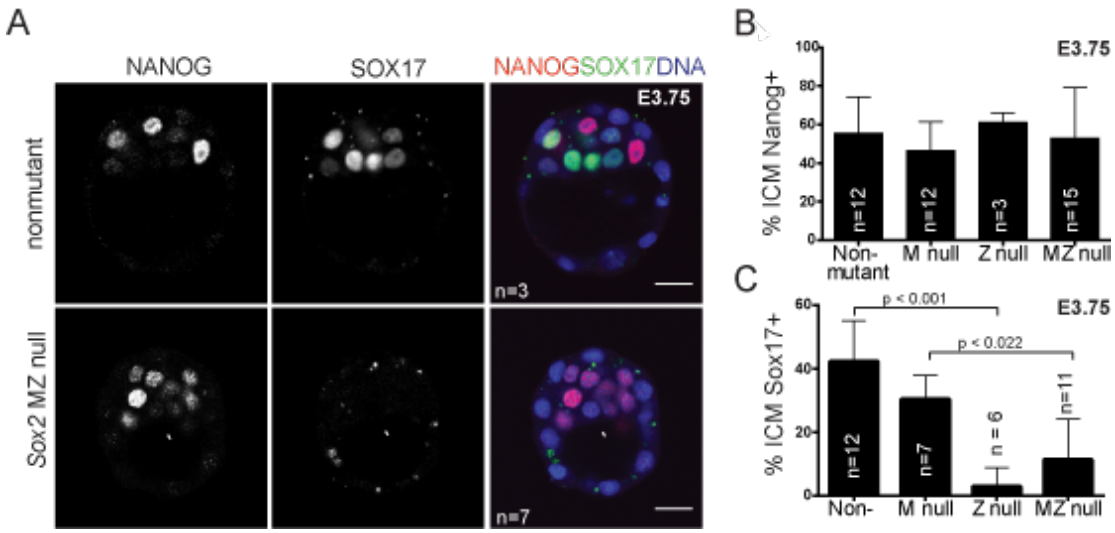


Figure 3: FGF4 treatment rescues the timing of PE development in *Sox2* null embryos

A) Schematic for experiments to test if FGF4 treatment rescues SOX17 expression defect in part A and C.

B) Comparison of number of cells in embryos cultured in control medium and medium containing FGF4 and Heparin from E2.75 +30 hrs to embryos developing *in utero* until E3.75.

C) Proportion of SOX17-positive ICM cells in *Sox2* null and non-mutant embryos cultured in control medium or medium containing FGF4 and Heparin from E2.75 + 30 hrs. Error bars = standard deviation.

D) SOX17 and NANOG immunostaining in *Sox2* null and non-mutant embryos cultured in control medium or medium containing FGF4 and Heparin from E2.75 + 30 hrs. White bars = 20 μm. N = number of embryos.

E) SOX17 and NANOG immunostaining in *Sox2* null and non-mutant embryos cultured in control medium or medium containing FGF4 and Heparin from E2.75 to E4.5 hrs. White bars = 20 μm. N = number of embryos.

F) Proportion of SOX17-positive ICM cells in *Sox2* null and non-mutant embryos cultured in control medium or medium containing FGF4 and Heparin from E2.75 to E4.5. Error bars = standard deviation.

Chapter 4, Figure 3

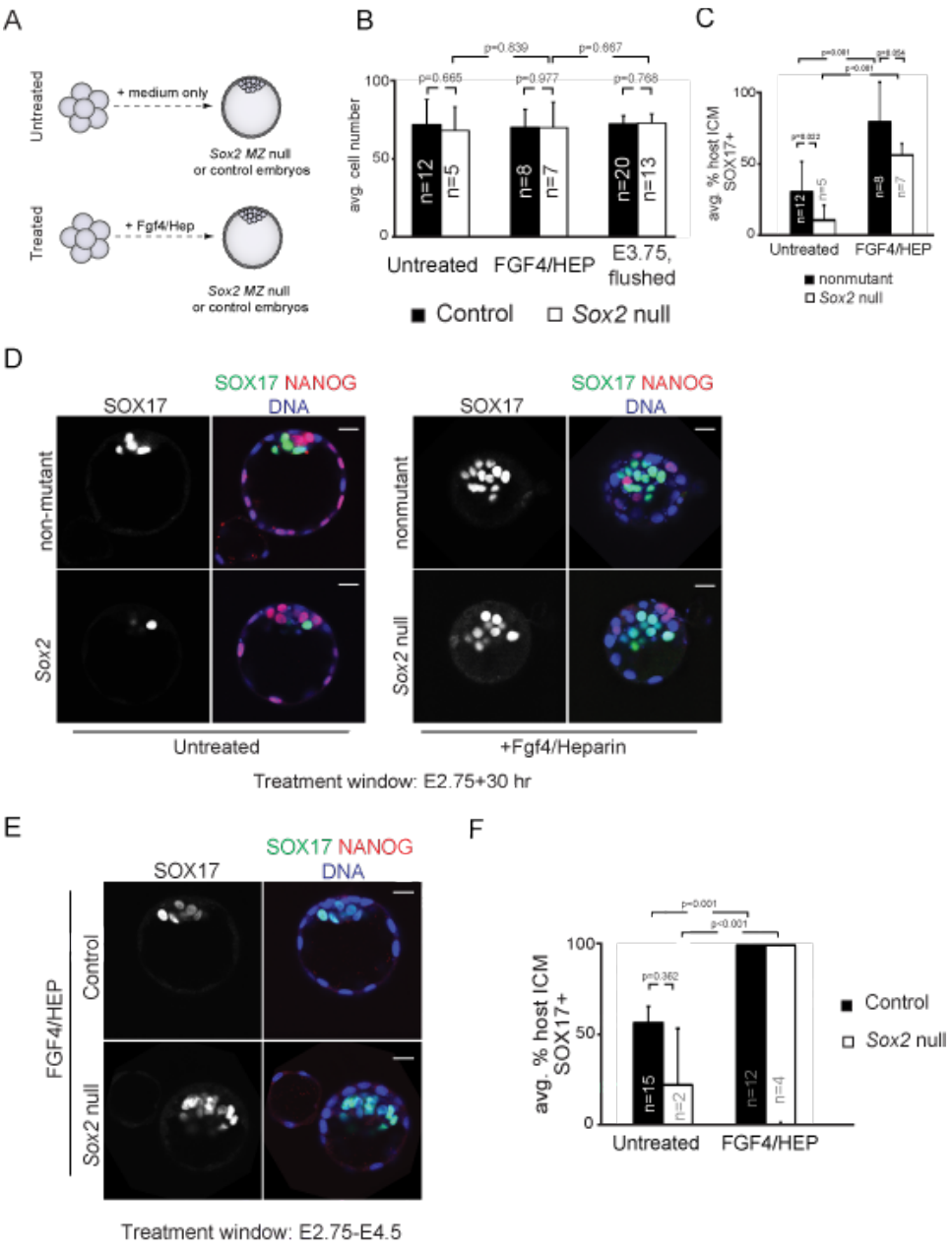


Figure 4: *Sox2* acts non-cell autonomously in PE development

A) Schematic of experiments to test if Sox2 acts non-cell autonomously or cell-autonomously in PE development.

B) Comparison of total average number of cells in *Sox2* null and non-mutant chimeras to embryos developed *in utero* to E3.75.

C) Average proportion of SOX17-positive ICM cells in *Sox2* null and non-mutant chimeras at equivalent total cell number to E3.75

D) SOX17 immunostaining and YFP signal in *Sox2* null and non-mutant chimeras at equivalent total cell number to E3.75.

Chapter 4, Figure 4

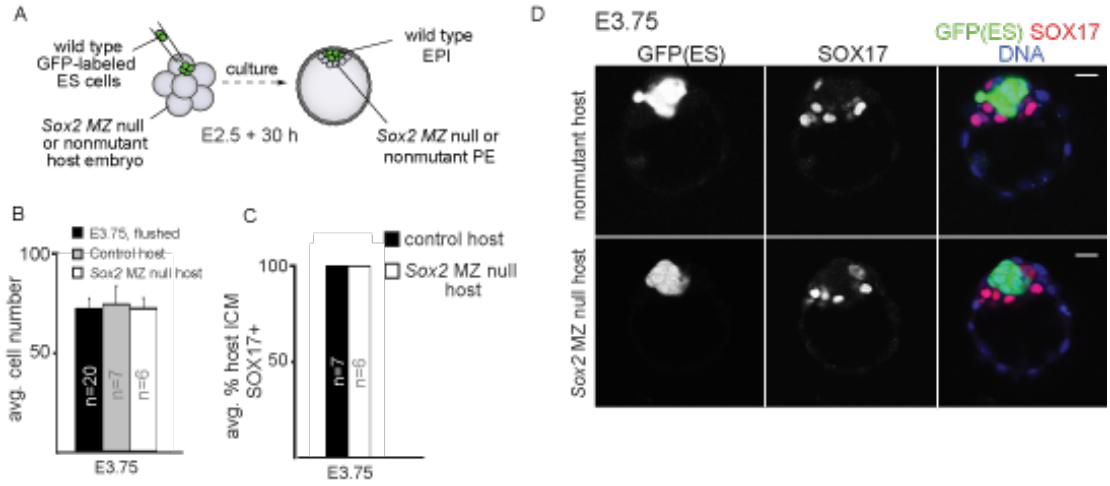
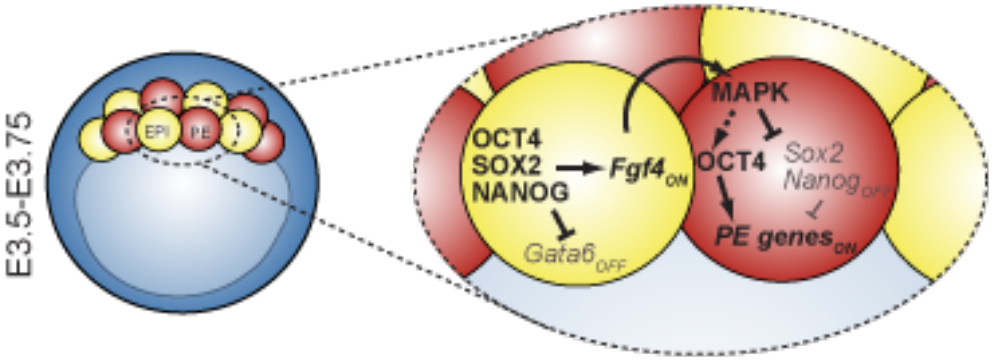


Figure 5: Model of the regulation and roles of *Sox2* in resolving EPI and PE fate in the mouse blastocyst

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 (Frum et al., 2013; Chia Le Bin et al., 2014) and represses *Sox2* and *Nanog*.

Chapter 4, Figure 5



CHAPTER 5

Final thoughts and future direction

5.1 Introduction

In this thesis, I have greatly increased our understanding of how pluripotent and other cell fates are established and regulated during preimplantation mouse development. In chapter 2 and 3, I used a genetic strategy to demonstrate that maternally provided *Cdx2* and *Oct4* are not maternal effect genes, supporting a model where cell fate in the preimplantation mouse embryo is plastic rather then pre-determined, implying that pluripotency and other cell states are acquired during preimplantation mouse development rather than being established in the oocyte. In chapter 3, I clarified the requirement for *Oct4* in the EPI, TE and PE lineages in preimplantation mouse development and uncovered a novel role for *Oct4* in cell-autonomously promoting development of a non-pluripotent cell type, the PE. This changes our view of *Oct4* from being a factor associated only with pluripotency, to viewing *Oct4* as a factor that can control different essential roles in different cellular and environmental contexts. Finally, in chapter 4, I demonstrated that *Sox2* has a similar role in the preimplantation mouse embryo in promoting PE cell fate as the pluripotency factor *Nanog,* but also reported subtle differences between the *Sox2* and *Nanog* null embryo phenotypes that suggest *Nanog* and *Sox2* have overlapping and independent roles in preimplantation mouse development.

The studies presented in chapter 2, 3 and 4 raise some key unanswered questions that provide exciting avenues for investigation that would greatly advance understanding of preimplantation development and stem cell biology.

Distinct roles for essential pluripotency factors in the preimplantation mouse embryo.

In ES cells, pluripotency is maintained through an autoregulatory loop of transcription factors, including NANOG, OCT4 and SOX2 that promote the expression of genes associated with pluripotency and repress the expression of genes associated with differentiation. This model of pluripotency in ES cells has led to the assumption that in mouse development, *Nanog*, *Oct*4 and *Sox2* are only required in the EPI lineage. However, my analysis of *Oct4* null and *Sox2* null embryos reveals differences between *Oct4* null, *Nanog* null and *Sox2* null embryo phenotypes that highlights distinct roles for each of these pluripotency factors in the preimplantation mouse embryo. For example, while *Nanog* null and *Sox2* null embryos both exhibit similar PE phenotypes at E3.75, in contrast to *Nanog* null embryos in which PE development remains distrupted at E4.25 (Messerschmidt et al., 2010) the PE of *Sox2* null embryos eventually recovers (Wicklow et al., *in revision*), highlighting distinct requirements for *Nanog* and *Sox2* in non-cell autonomously promote PE cell fate. An unbiased, global view of gene expression in *Oct4* null, *Sox2* null and *Nanog* null embryos would be invaluable in teasing apart the molecular differences responsible for the phenotypic differences between *Oct4* null, *Sox2* null and *Nanog null* embryos.

Evidence for additional signaling pathways promoting EPI and PE cell fate

The widely accepted model for how EPI and PE cells are specified is that the level of FGF/MAPK signaling experienced by an individual ICM cell determines cell fate, such that cells that experience a low level of FGF/MAPK signaling become

pluripotent EPI, while ICM cells that experience a high level of FGF/MAPK signaling differentiate into PE. My studies in which I treated *Oct4* null and *Sox2* null embryos with FGF4/Heparin or ES cells reveals that ES cells are a more potent inducer of PE cell fate in both these mutant embryo contexts, suggesting that ES cells provide factors that promote PE development in addition to FGF4. This is especially apparent in *Oct4* null embryos, in which both ES cells and FGF4/Heparin induce miniscule amounts of SOX17, but ES cells induce significantly more than FGF4/Heparin treatment alone. ES cells are known to produce the BMP signaling ligand BMP4 and the NODAL signaling ligands GDF3 and CRIPTO. Intriguingly, our RNA-seq analysis of *Oct4* null embryos reveals that *Bmp4, Gdf3* and *Tdgf1* (CRIPTO) are all significantly reduced in *Oct4* null embryos. Therefore, BMP and NODAL signaling are attractive candidates for additional signaling pathways that regulate EPI and PE cell fate.

To test the hypothesis that BMP signaling regulates EPI and PE cell fate I cultured wild-type embryos in medium supplemented with BMP4 from E2.75 to E4.5 and evaluated EPI and PE cell fate by the expression of NANOG and SOX17. We detected no difference in the expression of NANOG or SOX17 in BMP4 treated wild-type embryos compared to control embryos cultured in control medium (Fig. 1A, B). Surprisingly, when BMP4 was added to wild-type embryos in combination with FGF4/Heparin, we detected a reduction in the number of SOX17-positive cells, and an increase in NANOG-positive cells in BMP4 + FGF4/Heparin treated embryos compared to embryos treated with FGF4/Heparin alone, which only express SOX17 and do not express NANOG (Fig 1C, D, E, F). The attenuation of the effect of FGF4/Heparin by including BMP4 suggests that BMP signaling opposes FGF/MAPK

signaling and promotes EPI cell fate. We are currently generating embryos that lack maternal and zygotic *Bmp4* in order to define the role of *Bmp4* in preimplantation mouse development.

The HIPPO signaling pathway components YAP and TEAD4 are also intriguing as candidate regulators of EPI and PE cell fate. At the first preimplantation lineage decision, which establishes the TE and ICM lineages, SOX2 is repressed by TEAD4 in the TE lineage (Wicklow et al., *in revision*). TEAD4 activity is controlled by the presence of YAP in the nucleus and YAP nuclear localization is regulated by cell-cell contact. Asymmetries in cell-cell contact exist in the ICM during the second preimplantion lineage decision, in which EPI and PE lineages are established. In the ICM, cells in contact with the blastoceal cavity experience less cell-cell contact that cells deeper in the ICM. Intriguingly, we have detected that YAP is localized in the nucleus of E4.25 ICM cells in contact with the blastoceal cavity (Fig. 2C). Because PE cells migrate to the blastoceal cavity prior to implantation, YAP and TEAD4 could work together to integrate positional information during the resolution of EPI and PE cell fates.

5.2 Figures and Legends

Figure 1: BMP4 inhibits the effect of ectopic FGF4

A) Proportion of SOX17-positive ICM cells in wild-type embryos cultured in control media and wild-type embryos cultured in the presence of either 100 ng/ml or 500 ng/ml of BMP4 from E2.75 to E4.5. Error bars = standard deviation.

B) Proportion of NANOG-positive ICM cells in wild-type embryos cultured in control media and wild-type embryos cultured in the presence of either 100 ng/ml or 500 ng/ml of BMP4 from E2.75 to E4.5. Error bars = standard deviation.

C) SOX17 and NANOG immunostaining in wild-type embryos cultured in the presence of FGF4/Heparin alone or FGF4/Heparin and 100 ng/ml or 500 ng/ml BMP4 from E2.75 to E4.5.

D) Proportion of SOX17-positive ICM cells in wild-type embryos cultured in the presence of FGF4/Heparin alone or FGF4/Heparin and 100 ng/ml or 500 ng/ml BMP4 from E2.75 to E4.5. Error bars = standard deviation.

E) Proportion of NANOG-positive ICM cells in wild-type embryos cultured in the presence of FGF4/Heparin alone or FGF4/Heparin and 100 ng/ml or 500 ng/ml BMP4 from E2.75 to E4.5. Error bars = standard deviation.

F) Proportion of unlabeled (SOX17-negative and NANOG-negative) ICM cells in wild-type embryos cultured in the presence of FGF4/Heparin alone or FGF4/Heparin and 100 ng/ml or 500 ng/ml BMP4 from E2.75 to E4.5. Error bars = standard deviation.

Chapter 5, Figure 1

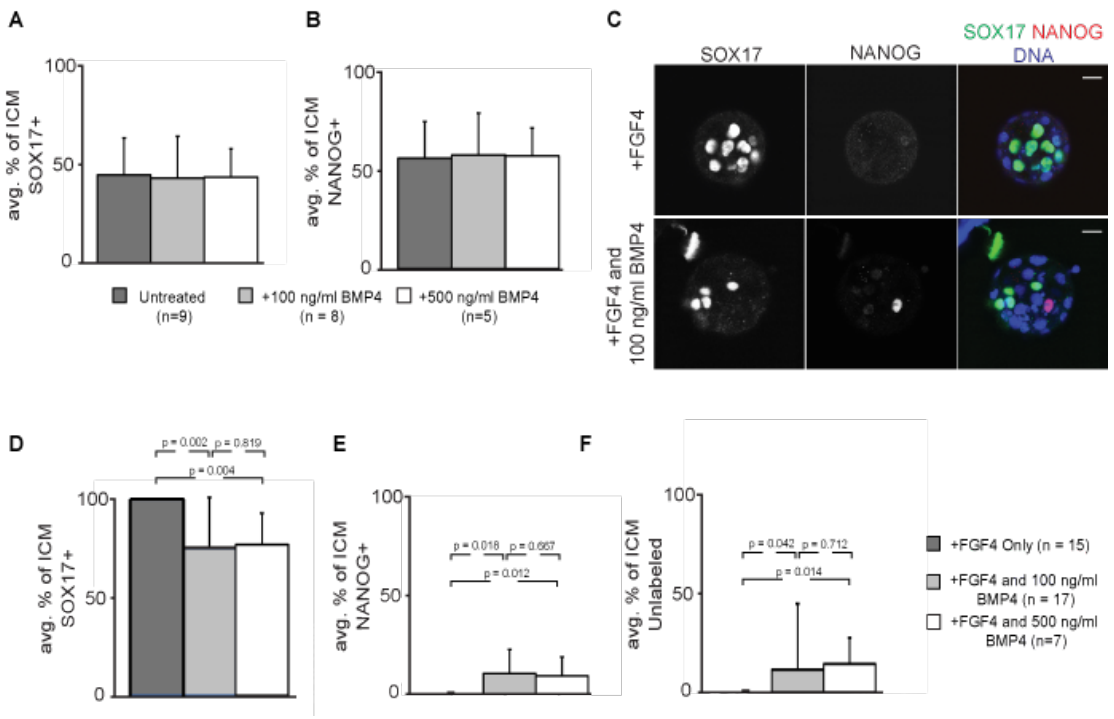


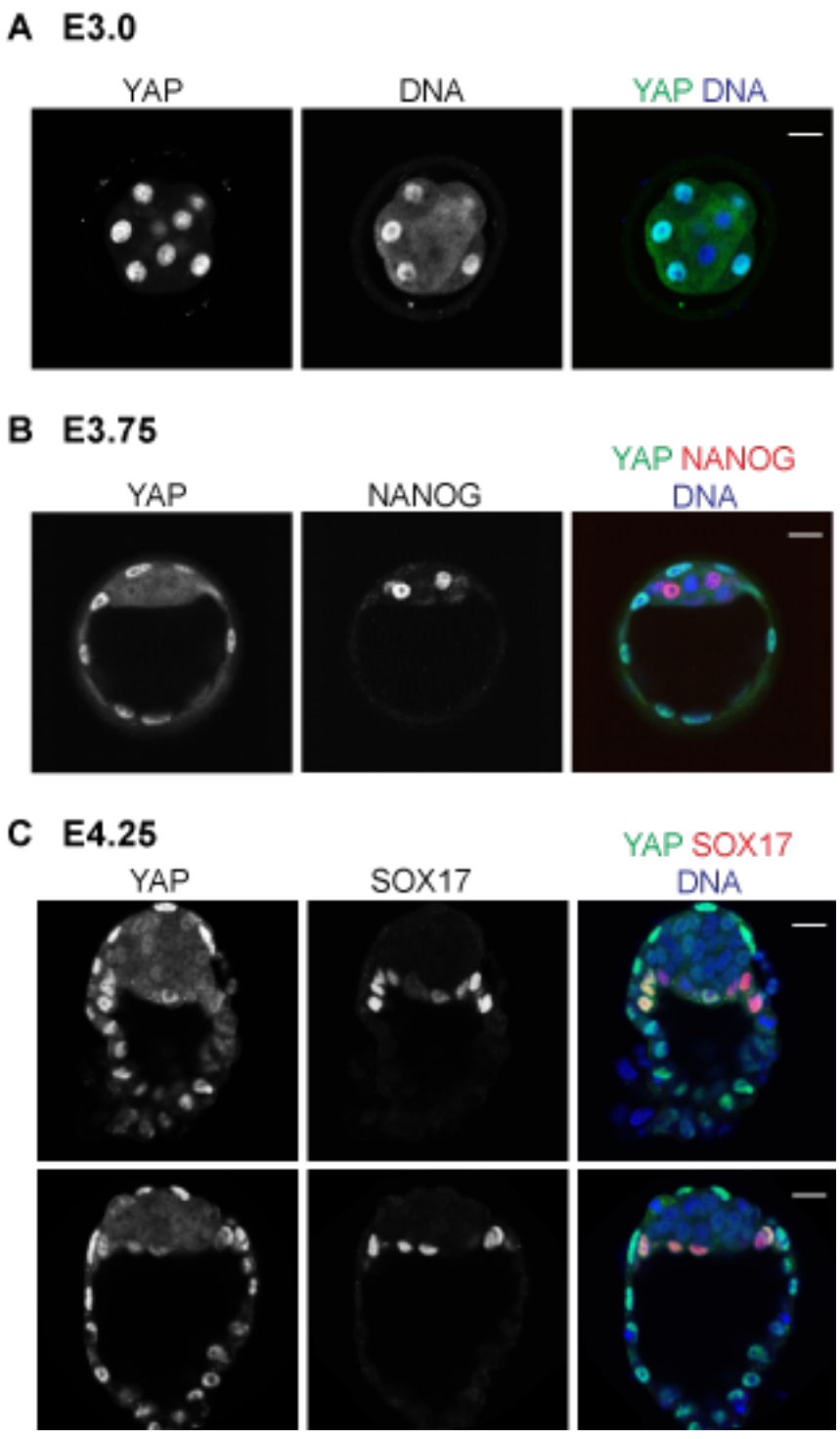
Figure 2: YAP is nuclear localized in outside/TE cells from E3.0 to E4.25 and nuclear localized in PE cells at E4.25

A) YAP immunostaining in E3.0 wild-type embryo. YAP is in all cells and is enriched in the nuclei of cells on the outside of the embryo. White bar = 20 microns.

B) YAP and NANOG immunostaining in E3.75 wild-type embryo. YAP is in all cells and is enriched in the nuclei of cells on the outside of the embryo. White bar = 20 microns.

C) YAP and SOX17 immunostaining in E4.25 wild-type embryos. YAP signal is in all cells and enriched in the nuclei of cells on the outside of the embryo and enriched in the nuclei of cells in contact with the blastocoel cavity. ICM cells with enriched nuclear YAP costain with the PE marker SOX17. White bar = 20 microns.

Chapter 5, Figure 2



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