Embryonic Stem Cell Differentiation and Morphogenesis

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EMBRYONIC STEM CELL DIFFERENTIATION AND
MORPHOGENESIS

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The primitive endoderm (PrE) appears as an epithelium on the surface of inner cell mass (ICM) at the late blastocyst stage (E4.5). This is the second lineage segregation event in mouse development. The transcription factor *gata6* and the Ras/MAPK signaling pathway are essential for the commitment of PrE. To further study the PrE lineage establishment, we generated a mutant mouse line by replacing *gata6*-coding exons with H2BGFP and monitored *gata6* promoter activity in PrE development. We observed that the *gata6* promoter driven GFP fluorescence signal was uniformly present initially in heterozygous blastocysts (*gata6*\(^{H2BGFP/+}\)) at the 32-cell stage. However, the signal diverged into two populations by the 64-cell stage; the GFP intensity in cells of ICM either intensified or declined upon differentiation into PrE or epiblast (EPI), respectively. The PrE commitment was also accompanied by a reduced cell motility as the cells sorted to assemble into an epithelium. In homozygous mutant blastocysts (*gata6*\(^{H2BGFP/H2BGFP}\)), initially strong GFP fluorescence throughout the ICM subsided and no PrE formed, suggesting the Gata6 protein is required to autoactivate its promoter activity. In embryonic stem cells in vitro, ectopically expressed Gata6 associated with its endogenous promoter and rescued the activity. Mutation of the motif phosphorylated by Erk1/2 at serine residue (S264) of Gata6 abolished its ability to transactivate promoter activity and PrE
differentiation. The results indicate that MAPK phosphorylation of Gata6 protein enhances its association and activation of its own promoter, which then drives a positive feedback regulation and subsequent commitment to PrE lineage.

As the blastocyst implants on the uterus, the pluripotent EPI cells will transform into a cup-shaped epithelium with proamniotic cavity formation in the center of the embryo. A recent study using a new technical approach to culture blastocysts in a matrix support in vitro suggests that the proamniotic lumen forms as a result of epithelial organization and rosette morphogenesis without cell death. Nevertheless, so far no gene mutation is known to impact directly the formation of proamniotic lumen as to provide mechanistic support to either model of embryonic cavitation. Phosphatase and tensin homolog (Pten), a lipid phosphatase originally identified as a tumor-suppressor gene, regulates the phosphoinositol 3 signaling pathway and impacts cell death and proliferation. We analyzed Pten mutant embryos in detail and discovered that the formation of the proamniotic cavity is impaired. Embryoid bodies derived from Pten-null embryonic stem (ES) cells failed to undergo cavitation, reproducing the embryonic phenotype in vitro. Analysis of embryoid bodies and embryos revealed a role of Pten in the initiation of the focal point of epithelial rosette that develops into the proamniotic lumen, and in establishing epithelial polarity to transform the amorphous epiblast cells into a polarized epithelium. We conclude that Pten is required for proamniotic cavity formation by establishing polarity for epiblast cells to form a rosette that expands into the proamnionic lumen, rather than facilitating apoptosis to create the cavity.
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<tbody>
<tr>
<td>Anx2</td>
<td>Annexins 2</td>
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<tr>
<td>aPKC</td>
<td>atypical protein kinase C</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic proteins</td>
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<tr>
<td>CUB</td>
<td>Cubilin</td>
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<tr>
<td>DAH</td>
<td>Differential adhesive affinity hypothesis</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid body</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EPI</td>
<td>Epiblast</td>
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<tr>
<td>ESC</td>
<td>Embryonic Stem Cell</td>
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<tr>
<td>ExE</td>
<td>Extra-embryonic ectoderm</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>Fgf4</td>
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</tr>
<tr>
<td>HSPGs</td>
<td>Heparin sulphate proteoglycans</td>
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<tr>
<td>ICM</td>
<td>Inner cell mass</td>
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<tr>
<td>LRP2</td>
<td>LDL receptor-related protein 2</td>
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<tr>
<td>Oct3/4</td>
<td>Octamer-binding transcription factor 3/4</td>
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<td>ParE</td>
<td>Pariental endoderm</td>
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<td>Term</td>
<td>Description</td>
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<tr>
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<td>-------------------------------------------------</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5 bisphosphate</td>
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<td>PIP3</td>
<td>Phosphoinositol-3,4,5 tri-phosphate</td>
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<tr>
<td>PrE</td>
<td>Primitive endoderm</td>
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<tr>
<td>Pten</td>
<td>Phosphatase and tensin homologue on chromosome 10</td>
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<tr>
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<td>Retinal acid</td>
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<tr>
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<td>Sex determining region Y-box 2</td>
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<td>Trophectoderm</td>
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<tr>
<td>VE</td>
<td>Visceral endoderm</td>
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<td>XEN</td>
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CHAPTER 1: Introduction

1.1 Early cell fate decisions of the mouse preimplantation embryo

1.1.1 Two cell fate decisions during preimplantation stage

A mouse embryo spends nearly 5 days preparing for uterine implantation (Saiz and Plusa, 2013). During this preimplantation phase, a series of important lineage commitment decisions occur. The first decision commences at the eight-cell stage (Dyce et al., 1987; Pedersen et al., 1986). Cells located inside the embryo develop into the inner cell mass (ICM), whereas cells positioned outside the embryo mature into the first extraembryonic tissue, a polarized epithelium termed the trophectoderm (TE) (Tarkowski and Wroblewska, 1967)(Figure 1). The maturation of tight junctions between outer cells enables the generation of a cystic cavity within the embryo known as the blastocoel (Fleming et al., 2000; Stephenson et al., 2012).

The second cell fate decision determines cell fate the two ICM cell types: the pluripotent epiblast (EPI), which generates cells of the future body and the second extraembryonic tissue, primitive endoderm (PrE) (Gardner, 1982; Morris et al., 2010)(Figure 1). It has not yet been established how this second cell fate decision is made. Early work proposed that the cells located on the surface of the ICM in contact with the cavity become the PrE simply by virtue of their position, in a process analogous to that of the TE (Enders et al., 1978; Murray and Edgar, 2001)(Figure 2). However, several researchers have challenged this position-based model. As early as E3.5 stage, it was observed that cells expressing defining PrE and EPI proteins, Gata6 and Nanog, respectively, are distributed randomly with the ICM (Chambers et al., 2003; Chazaud et al., 2006a; Morris et al., 2010; Plusa et al., 2008). As the two
Figure 1. Overview of early mouse embryo development during pre-implantation stage. During this preimplantation phase, two important lineage commitment decisions occur. The first cell fate decision give rise to trophectoderm and inner cell mass. The cell fate decision happens within the inner cell mass which leads to the allocation of primitive endoderm and epiblast. Lineage specific gene expression is labeled below each cell types. E: embryonic day.
nascent lineages emerge, PrE cells are sorted outside to form an epithelium layer covering the EPI cells (Figure 2) and begin to express differentiation-specific genes exclusively such as Gata4, Gata6, Sox7, Sox17 and Dab2 whereas, conversely, EPI cells express pluripotency-associated factors such as Nanog, Sox2, and Oct3/4 (Arceci et al., 1993; Artus et al., 2011; Avilion et al., 2003a; Koutsourakis et al., 1999; Niakan et al., 2010; Plusa et al., 2008; Rossant et al., 2003; Scholer et al., 1990).

At this stage, three distinct germinal lineages are present: the EPI, the PrE, and the TE. Only one of these, the EPI, will give rise to the fetus, yet proper segregation and development of the other two extraembryonic lineages are crucial for the survival and patterning of the embryo. Given the importance of these tissues, understanding the molecular mechanisms establishing these three lineages is key to understanding mouse embryogenesis.

1.12 EPI vs. PrE specification

The PrE forms an epithelium on the surface of the ICM adjacent to the blastocoel cavity at the late blastocyst stage (E4.5). A basal lamina is promptly deposited separating the PrE from the EPI (Poschl et al., 2004). Through cell-lineage tracing studies the potential of PrE cells and EPI cells is now likely irreversibly restricted to their respective lineages (Gardner, 1982).

Transcription factors Gata6 and Nanog are the earliest markers for the PrE and EPI, respectively. Interestingly, around the E3.0 stage, all ICM cells show coexpression of Nanog and Gata6 (Plusa et al., 2008; Stephenson et al., 2012). As embryos develop, however, they synthesize either Gata6 or Nanog exclusively (around E3.5 stage). Cell
Figure 2. Two models for primitive endoderm formation. “Position based Model” proposed that the cells located on the surface of the inner cell mass in contact with cavity become the primitive endoderm by the virtue of their position. “Cell Sorted Model” considered that initial primitive endoderm differentiation occurs randomly, in the interior of the inner cell mass. Subsequently, the differentiated primitive endoderm cells sort to the surface to form a coherent primitive endoderm epithelial structure.
lineage tracing and live cell tracking analyses (Chazaud et al., 2006a; Meilhac et al., 2009; Plusa et al., 2008; Xenopoulos et al., 2015) show that this process occurs in individual ICM cells asynchronously and apparently at random. The stable emergence of the Nanog+ / Gata6− EPI and the Nanog− / Gata6+ PrE is currently the first known sign of the specification process.

Evidences have been provided to demonstrate that the segregation of EPI and PrE lineage is under transcriptional control (Figure 1). Several groups have analyzed Nanog mutant embryos, and found that in the null embryos all ICM cells synthesize Gata6 (Frankenberg et al., 2011b; Messerschmidt and Kemler, 2010b; Mitsui et al., 2003; Silva et al., 2009). In Gata6 mutant embryos all ICM cells uniformly express Nanog without any other PrE marker, indicating the global acquisition of an EPI fate. Overexpression of Gata6 in embryonic stem cells (ESCs) is sufficient to induce PrE differentiation (Fujikura et al., 2002). This suggests a negative regulatory loop exits between Nanog and Gata6, similar to the one that ensures mutually exclusive synthesis of Oct4 and Cdx2 during TE/ICM differentiation. In fact, Nanog and Gata6 might directly repress each other’s transcription, as suggested by their binding sites identified in chromatin immunoprecipitation studies of ESCs (Singh et al., 2007) and of induced extra-embryonic endoderm (iXEN) cells (Wamaitha et al., 2015). A number of other transcription factors have also been identified as involved in this process. For example, during EPI maturation, Oct3/4 and Sox2 function in collaboration with Nanog to enforce EPI lineage commitment (Avilion et al., 2003b; Nichols et al., 1998). Likewise, Sox17 and Sox7 cooperate together with Gata4 and
Gata6 to regulate PrE differentiation. A more detailed discussion of the role of each transcription factor will be introduced later in this chapter.

### 1.13 FGF signaling in EPI vs. PrE specification

A series of studies has revealed that fibroblast growth factor (Fgf) signaling plays an essential role during PrE lineage formation. Mutation of fibroblast growth factor 4 (Fgf4) (Feldman et al., 1995; Goldin and Papaioannou, 2003; Kang et al., 2013a; Krawchuk et al., 2013), fibroblast growth factor receptor 2 (Fgfr2) (Arman et al., 1998) or growth factor receptor-bound protein 2 (Grb2) signal transduction component (Chazaud et al., 2006a; Cheng et al., 1998) results in the absence of Gata6 synthesis and failure to generate the PrE lineage. Blocking Fgf signaling with pharmacological inhibitors against Fgfr2 or downstream Mek1/2 also is sufficient to convert all ICM cells to adopt an EPI fate (Lanner et al., 2010; Nichols et al., 2009; Yamanaka et al., 2010), whereas the addition of Fgf4 will induce the ICM cells to differentiate into PrE cells (Yamanaka et al., 2010).

Detailed transcriptional analysis of single cells indicates an instructive function of Fgf4–Fgfr2 in EPI and PrE lineage diversion. (Guo et al., 2010) The ligand–receptor pair is initially ubiquitously expressed but quickly establishes mutually exclusive patterns following blastocyst formation at the 32-cell stage. (Frankenberg et al., 2011a; Guo et al., 2010; Kurimoto et al., 2006) This antagonistic ligand–receptor pattern occurs prior to the expression of lineage transcription factors Nanog and Gata6, in accordance with an upstream function. Specifically, Fgf4 is originally expressed in the 8- to 16-cell morula and then becomes restricted to the epiblast
precursor cells (Niswander and Martin, 1992; Rappolee et al., 1994). Pluripotency factors Oct3/4 and Sox2 have been shown to regulate the expression of Fgf4 directly (Yuan et al., 1995). Fgf4 expression is also severely downregulated in Nanog mutant EPI cells. (Frankenberg et al., 2011a) The cooperation between Sox2, Oct3/4 and Nanog generates enough Fgf4 for the correct production of mature PrE cells, with regard to both time and number. Fgfr2, on the other hand, appears to be restricted to the TE and PrE lineages (Arman et al., 1998). Fgf4, produced by EPI-biased cells (Guo et al., 2010; Ohnishi et al., 2014), has been proposed to activate the FGF/ERK pathway in neighboring cells, which have Fgfr2 expression. FGF/ERK signal activation will lead to the down-regulation of Nanog and induction of the PrE program. (Hamilton and Brickman, 2014) This differential receptor-ligand expression in prospective EPI/PrE cells presages the exclusive Nanog/Gata6 expression and suggests this could be important for the establishment and maintenance of the lineage restriction.

Interestingly, in Fgf4 mutants, there is still Gata6 expression until early blastocyst stage (Kang et al., 2013a; Krawchuk et al., 2013), which indicates that instead of inducing the initial expression of Gata6, Fgf4 mainly functions to control the proportion of cells that differentiate into PrE lineage. In addition, Fgf4 administration does not induce Sox17 and Gata4 expression in Gata6 mutants (Bessonnard et al., 2014; Schodde et al., 2014) underscoring that both the transduction of FGF signals and functional Gata6 protein are required for the expression of mature PrE markers.
To investigate further how Fgf signaling and Gata6 accelerate PrE lineage commitment, Martinez-Arias lab generated an ESC in vitro model. By quantification study, they suggested that PrE-like differentiation occurs in cells exposed to Gata6 factor levels above a threshold, and the function of Fgf/MAPK signaling is to set this threshold dose (Schroter et al., 2015). However, the mechanism underlying how the Fgf/MAPK stimuli regulate the threshold dose is still unknown.

Previous work has already indicated that Fgf signaling can interact with the transcriptional machinery. For example, Erk2 can directly phosphorylate Oct3/4 at multiple sites, and the phosphorylation can regulate Oct3/4 transcription primarily by altering its DNA binding affinity (Brumbaugh et al., 2012). Erk signaling can also enhance Gata6 transcription efficiency by phosphorylating a conserved serine site (Adachi et al., 2008; Kelly et al., 2014). In this study, by demonstrating that MAPK phosphorylation of Gata6 enhances its association and activation of its own promoter, I propose a positive feedback regulation that propels the PrE lineage commitment.

1.14 Molecular regulation of EPI pluripotency

The EPI lineage is segregated from the PrE lineage within the inner cell mass (ICM) of the preimplantation embryo. The EPI proliferates to provide the substrate germlayer specification, undergoes gastrulation to form the embryo proper. Naive early EPI cells can be cultured immortalized in vitro culture in the form of ES cells (Brook and Gardner, 1997; Evans and Kaufman, 1981; Martin, 1981). A select set of core transcription factors in combination governs and thereby defines pluripotency of EPI and ES cells: Oct4 (also known as POU5F1), Sox2 and Nanog. Here, the function of these transcription factors will be discussed individually.
Oct3/4

Octamer-binding transcription factor 4 (Oct3/4) is a POU-domain transcription factor encoded by the Pou5f1 gene. Oct3/4 is a maternally inherited transcript and is strictly regulated during the embryogenesis. The expression of Oct3/4 is maintained at a low level until the four cell stage. (Palmieri et al., 1994) At the four-cell stage, the gene undergoes zygotic activation and all the cells have a high expression of Oct3/4. Later on, during the TE formation, the Oct3/4 is segregated to only the ICM cells and is diminished in the differentiated TE cells (Okamoto et al., 1990; Rosner et al., 1990). Following implantation, Oct3/4 expression is limited in the EPI cells, although it is transiently expressed in PrE cells but vanishes shortly after implantation (Okamoto et al., 1990).

Oct3/4 mutant embryos die at the peri-implantation stage because the ICM degenerates. The mutant embryos strictly adopt a fate of trophoblastic giant cells but no embryonic or extraembryonic endoderm cells survive (Nichols et al., 1998). This phenotype has been further confirmed in ES cells, where Oct3/4 null ES cell adopted the fate of nonproliferating TE (Nichols et al., 1998). These findings show that Oct3/4 represses the trophoblast program during development and is crucial for the maintenance of ES cell self-renewal. Indeed, in the function of regulating pluripotency of ES cells, Oct3/4 acts closely with another two transcription factors Sox2 and Nanog. Collectively, these three proteins form an autoregulatory circuit whereby Oct3/4, Sox2 and Nanog all bind to their own promoters as well as the promoters of the genes coding the two other factors (Boyer et al., 2005; Kuroda et al., 2005; Okumura-Nakanishi et al., 2005). This autoregulation suggests that these three
transcription factors function collaboratively to enhance the stability of their own expression and facilitate the maintenance of the pluripotent state (Alon, 2007; Odom et al., 2006).

However, unexpectedly, overexpression of Oct3/4 in ES cells induces cell differentiation toward PrE lineage. This phenomenon observed in ES cells was also confirmed in an embryo study. Not only is Oct3/4 expressed in both PE and EPI cells (Frum et al., 2013; Palmieri et al., 1994), but Oct4 is also required for ICM cells to respond to exogenous Fgf4 and PrE lineage formation in a cell-autonomous fashion (Frum et al., 2013). Oct3/4 binding sites are highly enriched around several important EPI pluripotency gene and PrE genes including Gata4 and Gata6. Consistent with this, in the absence of Oct3/4, EPI and PrE lineage segregation cannot be initiated in Oct3/4-deleted ICM (Le Bin et al., 2014). Based upon these experiments, a model has been proposed whereby Oct3/4 acts as a three-way regulator to switch between three different cell fates-EPI, PrE and TE- in a dose-dependent manner.

Nanog

The homeobox containing transcription factor Nanog plays an essential role in EPI lineage specification. Nanog is first detected in 8-16 cells stage embryos (Xenopoulos et al., 2015) where it is co-expressed with PrE marker Gata6. During the blastocyst stage, Nanog expression is shut off in TE lineage but maintained in ICM cells specifically the EPI cell lineage. Post implantation, the expression of Nanog in EPI cells is downregulated (Chambers et al., 2003; Chazaud et al., 2006a; Schrode et al., 2013b).
Nanog mutant embryos die due to the loss of both EPI and PrE (Messerschmidt and Kemler, 2010a; Mitsui et al., 2003). The lack of the EPI cell lineage in Nanog null embryos proved that Nanog is required for EPI formation and maintenance of pluripotency in mouse EPI. However, surprisingly, deletion of Nanog from pre-existing ES cells does not abolish their ability to self-renew or contribute to embryos in chimeras, except that the mutant ES cells are more prone to differentiate than wild type (Chambers et al., 2007; Mitsui et al., 2003). In addition, Oct3/4 and Sox2 are still present in the mutant ES cells, which is consistent with the fact that Oct3/4 and Sox2 are sufficient to initiate pluripotency reprogramming (Takaoka et al., 2006). Nevertheless, overexpression of Nanog in mouse ES cells can bypass their requirement for the critical self-renewal Lif/Stat3 cascade and these cells maintain a self-renewing capacity while being differentiation impotent. (Chambers et al., 2003; Mitsui et al., 2003) Thus, the ES cell study demonstrate that Nanog is sufficient, but not necessary for ensuring self-renewal.

It is interesting to note that Nanog is also required for PrE formation but in cell non-autonomous manner (Messerschmidt and Kemler, 2010a). Further analysis of Nanog loss of function in the ICM showed that initial expression of Gata6 occurs in Nanog-negative cells, but downstream expression of PrE markers such as Gata4 and Sox17 requires the presence of wild-type cells (Frankenberg et al., 2011a). This suggests a sequential process in which initiation of Nanog and Gata6 is cell autonomous, but stabilization of the PrE phenotype requires induction of Gata4 and Sox17 by a signal derived from the Nanog-expressing cells, possibly mediated by FGF signaling as is discussed above.
**Sox2**

Sex determining region Y-box 2 (Sox2) is a member of SRY-box containing transcription factor family (Kamachi et al., 2000). Unlike Oct3/4 and Nanog, Sox2 expression is highly dynamic such that it decreases in the embryo until at the 8-cell stage it reaches its lowest levels. At the 16-cell stage, Sox2 expression is limited in the ICM cells but then increases to its highest level (Guo et al., 2010).

Sox2 null embryos die at a slightly later stage than Oct3/4 mutant embryos with defects in EPI. Furthermore, ES cells cannot be derived from the Sox2 null embryos (Avilion et al., 2003a). This indicates that just like Oct3/4, Sox2 is required for ES cell pluripotency. Also, inhibition of Sox2 expression in ES cell lines leads to the formation of TE like cells supporting the concept of Oct3/4 and Sox2 cooperating to repress TE fates in ES cells (Li et al., 2007; Masui et al., 2007).

Sox2 and Oct3/4 expression overlap in the morula, ICM, EPI, and germ cells; thus likely the two transcription factors have parallel roles for the two factors in the maintenance of pluripotency. In fact, Sox2 is an important cofactor of Oct3/4 in maintaining pluripotency. Fgf4 is activated by the cooperation between Oct3/4 and Sox2. Also studies in both human and mouse ES cells suggest that Oct3/4 and Sox2 cooperatively activate their own and the Nanog promoters (Okumura-Nakanishi et al., 2005; Tomioka et al., 2002; Yuan et al., 1995). Subsequently, other targets including Utf-1 (Nishimoto et al., 1999) and Fbx-15 (Tokuzawa et al., 2003) were identified.
1.15 Molecular regulation of PrE differentiation

As mentioned before, Gata6 is the earliest known marker of PrE specification (Morrisey et al., 1996). After specification, PrE cells turn on the other PrE markers including Sox17, Gata4, Dab2 and Pdgfrα (Artus et al., 2010; Niakan et al., 2010; Plusa et al., 2008; Yang et al., 2002). Although Gata6 plays a key role in PrE specification, it appears to act in concert with other signaling factors and pathways during the subsequent stages of PrE maturation. In the following part, details of the key transcription factors that coordinate PrE lineage formation will be discussed respectively.

Gata6

GATA-binding factor 6 (Gata6) is a member of the GATA family of zinc finger transcription factors (Morrisey et al., 1996). Gata6 protein is first detected around the 8-16 cell stage in all blastomeres, where it is coexpressed with Nanog (Chazaud et al., 2006a; Plusa et al., 2008). As the embryo develops to the 64-cell stage, Gata6 and Nanog expression become exclusive. At this stage, Gata6 positive cells have committed to the PrE lineage, which will further develop into the pariental endoderm (ParE) and visceral endoderm (VE) (Morrisey et al., 1996; Schrodé et al., 2013a). After the embryos implant on the uterus, strong Gata6 expression continues in the ParE lineage, but diminishes in the VE lineage (Cai et al., 2008; Kwon et al., 2008).

Gata6 mutant embryos die shortly after implantation due to the defect in PrE lineage (Cai et al., 2008; Koutsourakis et al., 1999; Schrodé et al., 2014). The fact that in the Gata6 mutant blastocyst all cells express the pluripotency-related factor Nanog
provides further proof of the hypothesis that Gata6 and Nanog act in a mutually exclusive manner. The inability of gata6 (−/−) ES cells to undergo extraembryonic endoderm (XEN) differentiation is consistent with the finding that gata6 deletion results in a defects of endoderm development and early embryonic lethality. Overexpression of Gata6 is sufficient to induce the mES cells to properly differentiate into PrE lineage (Shimosato et al., 2007; Wang et al., 2011a). Moreover, a short pulse of Gata6 induction is ample to active both endogenous Gata6 and Gata4 expression, and the auto-regulatory positive feedback between the Gata factors initiate conversion to induced XEN (iXEN) cells independently of exogenous Gata6 (Wamaitha et al., 2015).

In XEN cell lines, Gata6 enriches at the gene-coding region of multiple pluripotency markers including Esrrb, Nanog, and Oct4 whose expression is downregulated in PrE cells. Gata6 is also enriched at a number of rapidly up-regulated XEN-associated genes such as Gata4, Pdgfra and Dab2, further suggesting that Gata6 directly regulates both pluripotency and XEN genes (Wamaitha et al., 2015). Interestingly, the ChIP-seq analysis also shows that Gata6 was enriched at a common locus upstream of the endogenous gata6 promoter (Wamaitha et al., 2015). A previous gata6 promoter study also identified a number of consensus GATA binding elements suggesting that a self-regulatory mechanism may reinforce of Gata6 expression (Molkentin et al., 2000).

Collectively, these findings not only demonstrate that Gata6 is required for activation of the PrE program but also place Gata6 at the top of the hierarchy regulating PrE
specification and functioning as a master transcriptional regulator to induce a XEN program.

**Gata4**

GATA-binding factor 4 (Gata4) is another member of the GATA family of zinc finger transcription factors (Arceci et al., 1993). Gata4 expression starts at around the 64-cell stage, when the cells are committing to the fate of PrE (Guo et al., 2010; Ohnishi et al., 2014). After implantation, Gata4 remains positive on ParE and VE lineage (Cai et al., 2008).

Unlike Gata6, XEN is present in gata4-null embryos (Kuo et al., 1997; Molkentin et al., 1997). And, although gata4-null ES cells fail to undergo spontaneous endoderm differentiation upon aggregation in embryoid bodies, the addition of retinoic acid is sufficient to induce the gata4-null cells to differentiate into endoderm (Bielinska and Wilson, 1997; Soudais et al., 1995). GATA factors, therefore, may serve redundant functions in PrE development.

Although not required for XEN formation, Gata4 appears to have an essential role in regulating cardiac development (Pu et al., 2004; Watt et al., 2004). gata4-null mice display defects in heart morphogenesis and ventral foregut closure, resulting in embryonic lethality by embryonic day E 8.5 to E10.5. (Kuo et al., 1997; Molkentin et al., 1997) Expression of Gata6 is significantly upregulated in the gata4-null embryos, further suggesting that these two transcription factors may belong to a common developmental pathway and that Gata6 may compensate partially for Gata4 in the gata4(-/-) embryos. (Kuo et al., 1997)
Sox7 and Sox17

Sex determining region Y-box 7 (Sox7) and Sex determining region Y-box 17 (Sox17) are members of the SOX (SRY-related high mobility group box) transcription factor family, and specifically belong to the F subfamily (Bowles et al., 2000; Pevny and Lovell-Badge, 1997; Wegner, 1999). Like Gata6, Sox17 is first detected in cells within the 32-cell stage embryo and shows co-expression with Nanog. Around the 64-cell stage, Sox17 expression becomes restricted in PrE precursor cells and Nanog is exclusively expressed in Epi precursor cells. By the 128-cell stage, Sox17 positive cells are sorted outside and form the PrE epithelium. These results demonstrate that, similar to Gata6, Sox17 is initially coexpressed with Nanog, but then gradually becomes partitioned to the primitive endoderm (Niakan et al., 2010). Sox7 expression pattern is more similar with Gata4 and is only detected when the PrE cells form an epithelium covering the EPI cells. However, sox17 and sox7 mutant embryos lack an obvious defect in the establishment of the primitive endoderm (Kanai-Azuma et al., 2002), suggesting a redundant functional role in primitive endoderm development with other transcription factors.

Sox17 targeted gene have been identified in XEN cell lines and include Col4a1, Col4a2, Lama1, Lamb1-1, and Lamc1 suggesting that Sox17 functions in the differentiation of ICM and ES cells to regulate gene expression that encode basement membrane components (Niakan et al., 2010). Sox17 also binds to the regulatory regions of Gata4 and Gata6 indicating that Sox17 might also function to activate and reinforce the transcriptional network governing differentiation (Niakan et al., 2010). In sox17 mutant ES cell; Nanog, Sox2, and Oct3/4 expression are upregulated. Thus
Sox17 may also be required for the proper repression of the ES cell pluripotency transcription program during differentiation (McDonald et al., 2014). This is also confirmed by the fact that Sox17-binding sites have been identified upstream of Sox2 and Nanog (Niakan et al., 2010). Moreover, Sox17 consensus binding sites is similar to the Sox2 binding motif. This further suggests that Sox17 and Sox2 could be acting antagonistically at these shared targets by either directly competing for these target sequences or reciprocally influencing the binding of Nanog or Oct3/4 at these locations (Niakan et al., 2010).

1.16 The final cell sorting and spatial segregation

The PrE in pre-implantation blastocysts (E4.5) is a polarized epithelium with an apical surface facing the cavity and a basement membrane at the interface with the EPI. The apical surface has microvilli, endocytic receptors such as cubilin (CUB) and LDL receptor-related protein 2 (LRP2), and atypical protein kinase C (aPKC) (Gerbe et al., 2008; Morrisey et al., 2000). Cells within the ICM can migrate in a non-directional, actin-dependent manner (Meilhac et al., 2009). The best-known and comprehensive theory to explain the spontaneous sorting and patterning of the embryonic layers is the differential adhesive affinity hypothesis (DAH) proposed by Malcolm Steinberg (Green, 2008; Steinberg, 1996, 2007). This hypothesis postulates that less adhesive cells sort to the periphery and envelope highly adhesive cells in the center. Such an arrangement achieves lowest free energy. Using E-cadherin null ES cells as the less adhesive component compared to wild type, we previously demonstrated that indeed the sorting pattern is consistent with that predicted by Steinberg’s hypothesis. However, when E-cadherin null ES cells were mixed with
PrE cells differentiated from wild type cells, adhesion affinity no longer dictates sorting pattern (Moore et al., 2009; Rula et al., 2007). Thus, the differential adhesive affinity hypothesis may explain sorting of undifferentiated ES cells, but it cannot explain the patterning of embryonic germinal layers.

Another explanation suggested that endogenous properties of PrE cells, such as their ability to polarize are the driving force of this sorting process (Gerbe et al., 2008; Moore et al., 2009; Rula et al., 2007). In this model, PrE cells reach the surface by random movement, and subsequently the cells generate an apical/basal polarity. Furthermore, the polarized deposition of basement membrane (BM) prevents cells reentry. Basolateral-specific cell-surface receptors, such as integrin and dystroglycan, transduce signals from the BM and promote the adhesion of PrE cells to the substratum (Eastburn and Mostov, 2010). The apical localization of DAB2 and LRP2 is the first sign of cell polarization at the surface of PrE cells (Gerbe et al., 2008). Shortly after, around E4.5, the aPKC protein is enriched apically in the PrE epithelium (Saiz et al., 2013). Furthermore Dab2, an epithelial polarity organizer (Capo-Chichi et al., 2002), plays an important role in PrE morphogenesis formation. Deletion of Dab2 leads to the loss of epithelial polarity and consequently the failure of PrE to position on the surface. The homozygous Dab2-deficient mutant is embryonic lethal (earlier than E6.5) due to defective cell positioning and structure formation of the VE (Yang et al., 2007; Yang et al., 2002). Disruption of aPKC function through RNAi or with a pharmacological inhibitor also impairs the maintenance of the PrE epithelium (Saiz et al., 2013). Study of $\beta 1$ integrin-null blastocysts found that primitive endoderm cells are segregate away from, instead of
forming an epithelial layer covering the inner cell mass, indicating the essential role of BM and integrin-mediated cell adhesion for PrE morphology formation (Moore et al., 2014).

1.2 Epithelialization of the epiblast and the formation of proamniotic cavity

1.21 Epiblast epithelization and proamniotic cavity formation

After the blastocyst hatches out from the zona pellucida, the pre- to post-implantation transition take place and the ensuing morphogenesis is critical for development success (Dey et al., 2004). At the early post-implantation stage, the pluripotent EPI transforms into a polarized epithelium at the distal part of the conceptus and the extra-embryonic ectoderm (ExE) becomes positioned at the proximal part (Tam and Loebel, 2007). Both epithelial layers are enveloped by VE derived from PrE. The VE filters and transports nutrients and waste and plays essential role for the survival and growth of the embryos (Figure3). Also, the interactions take place between the VE and the enveloped EPI and ExE to ensure the normal differentiation and morphogenesis (Bielinska et al., 1999). Shortly after implantation, the proamniotic cavity emerges to establish the tube of egg cylinder (Figure 3). This process is reported to involve the epithelialization of the EPI cells and possibly apoptosis to make space for the cavity (Coucouvanis and Martin, 1995). However, due to the small size and relative inaccessibility of the embryo at this stage of development, the mechanism underlying of this cavitation event still remains a mystery. Functional studies of genes involved in the differentiation of the VE have shown that the failure to assemble a proper VE results in cavitation defects. For example, in Dab2 mutant embryos, there is no cavity
Figure 3. A model of proamniotic cavitation in egg cylinder stage. (A) In the first two days post-implantation, trophectoderm gives rise to polyploid trophoblast giant cells, ectoplacental cone and the extra-embryonic ectoderm. Primitive endoderm develops into parietal endoderm that covers inner surface of mural trophectoderm and visceral endoderm that covers egg cylinder and epiblast. (B) Immunofluorescence staining of an implanted embryo. ZO-1 staining illustrates the cavity formation area. Red: ZO-1. Green: E-cadherin. Blue: DAPI.
formation in the EPI (Yang et al., 2002). Additionally, defective interactions with the basal lamina, caused by loss-of-function mutation of r1-laminin, β-integrin, or Integrin linked kinase, prevent EPI cells from becoming polarized and forming an epithelium (Li et al., 2002a; Moore et al., 2014; Murray and Edgar, 2000; Sakai et al., 2003; Smyth et al., 1999).

1.22 The apoptosis model of cavity formation

The formation of the proamniotic cavity in early mouse embryos has been a classic model to study programmed cell death, as apoptotic cells are observable in the formation of the cavity in early mouse embryos and more obvious in embryoid body models (Coucouvanis and Martin, 1995, 1999). It had been postulated the VE is the source of the signal for programmed cell death in the EPI, and a second signal for survival is provided by the surrounding basal membrane to the cells only in direct contact (Coucouvanis and Martin, 1995). Other studies in embryoid bodeis have shown that bone morphogenetic protein (BMP) signaling is capable of reproducing the activity of the death signal. Blocking BMP activity prevents cavitation and addition of the BMP protein to cultures promotes cavitation. However, the identity of the survival signal in the early embryo is still unknown. Also, we are not aware of any previous report of a gene knockout in mice that shows a deficiency in cell death and the formation of proamniotic cavity. Particularly, no knockouts of components of the apoptotic pathways, such as family members of p53, Bcl, apaf-1, etc, demonstrate any defective phenotype of embryonic cavitation. Also, knockouts of caspase family members individually or in combination do not affect embryonic cavitation (Sadowski-Debbing et al., 2002; Zheng and Flavell, 2000; Zheng et al., 1999).
Although autophagy was suggested to participate in proamniotic cavity development since atg5 and beclin 1 knockout embryoid bodies fail to cavitate, the in vitro observation is not true in beclin 1-deficient embryos (Qu et al., 2007). Thus, programmed cell death may not be important in the formation of proamniotic cavity, or alternatively redundant genes prevent the identification of cavitation genes.

1.23 The rosette model of cavity formation

Recently, another model has been provided to explain the cavitation. In this model, rather than cell death, a previously unknown morphogenetic event transforms the amorphous EPI into a rosette of polarized cells. The luminal space then appears in the center of the embryo through hollowing of the apical membranes of EPI cells (Bedzhov and Zernicka-Goetz 2014). Specifically, in this model, by generating an in vitro embryos culture system, the researchers were able to reveal a sequence of events that take place during the implantation. Around E4.5 stage, the polar TE and PE secrete extracellular matrix (ECM) proteins that establish a BM that wraps around the EPI. Within the next 24 hours, ECM proteins provide polarization cues that signal through 1-integrin receptors to establish a basal-apical axis of the EPI cells. The apically localized adherin junctions and constriction of the actomyosin network reshape the initially round cells and form a rosette-like structure. The small lumenal space then appears in the center of the rosette as a result of charge repulsion of apical membranes coated by anti-adhesive glycoproteins such as podocalyxin. Fluid filling mechanisms are likely to contribute to further enlarging the lumen. Similar morphogenic changes occur in the ExE. Nevertheless, so far no gene mutation is
known to impact directly the formation of the proamniotic lumen as to provide mechanistic support to this model of embryonic cavitation.

### 1.24 Pten and polarity establishment

Pten (phosphatase and tensin homologue on chromosome 10) is the enzyme that converts phosphoinositol(3,4,5)-triphosphate or PtdIns(3,4,5)P$_3$ (PIP3) into phosphatidylinositol 4,5 bisphosphate or PtdIns(4,5)P$_2$ (PIP2) (Gerisch et al., 2012; Maehama and Dixon, 1998; Sha et al., 2010; Vazquez and Devreotes, 2006). Pten deletion in mice results in early embryonic failure prior to gastrulation, indicating the essential role of Pten for embryo development (Di Cristofano et al., 1998; Knobbe et al., 2008; Podsypanina et al., 1999; Stambolic et al., 1998; Suzuki et al., 1998). The most well studied function of Pten is as a classic tumor suppressor (Di Cristofano et al., 1998; Li et al., 1997; Steck et al., 1997). This tumor suppressing mechanism of the PTEN gene likely involves several candidate pathways, including the FAK pathway (Cheng et al., 2009), the MAPK pathway (Bouali et al., 2009; She et al., 2005), and the PI3K/AKT pathway (Ding et al., 2009; Gao et al., 2009). Currently, the PI3K/AKT pathway is regarded as the key pathway by which PTEN exerts its antioncogenic effects. PTEN encodes a protein with lipid phosphatase activity, which can dephosphorylate PIP3 to form PIP2. By dephosphorylating PIP3, Pten inhibits growth factor signal transduction pathways regulated by PI3K/AKT which play key roles in regulating critical cellular functions, including proliferation, apoptosis, glucose homeostasis, cell size, nutrient response and DNA damage.
As a result, PTEN activity in tumor cells results in cell cycle arrest at the G1 phase and induction of apoptosis.

Phosphoinositides have been also described as key regulators of apical-basal polarity (Martin-Belmonte et al., 2007; Shewan et al., 2011). Reduction of PTEN activity caused defects in the segregation of PIP3 and PIP2 and disruption of lumen formation, indicating an essential role of Pten in polarity and lumen establishment. PAR-3 is the first protein of the PAR/aPKC complex to show asymmetric localization to the apical cortex during epithelial polarity establishment (Harris and Peifer, 2005). PAR-3 scaffolding protein can directly bind to PTEN through its PDZ domains (von Stein et al., 2005), which are conserved in mammals and necessary for the establishment of epithelial polarity (Feng et al., 2008). The interaction between PAR-3 and PTEN can recruit PTEN to the membrane, consequently preventing apical accumulation of the PIP3 and localized production of PIP2, and resulting in the establishment of apical membrane identity. Following establishment of PIP2 asymmetry, the actin cytoskeleton in particular becomes highly enriched in the subapical region of epithelial cells during lumen formation and is responsible for numerous cellular processes such as vesicle trafficking and junction formation/stability. Annexins (Anx) can also provide an important connection between PIP2 and cytoskeletal reorganization. Anx2 binds PIP2 and is recruited to sites of actin assembly (Hayes et al., 2004; Rescher et al., 2004). Disruption of Anx2 function compromises lumen formation in a similar manner to loss of PTEN during MDCK cyst formation (Martin-Belmonte et al., 2007). In addition, Anx2 also binds the Rho-family small GTPase Cdc42, another key modulator of actin. Similarly, reducing Cdc42 level with siRNA
disrupts the apical actin cytoskeleton and causes similar lumen formation defects to reduction of Anx2 and PTEN (Martin-Belmonte et al., 2007). Collectively, these results define a molecular pathway involving PTEN/Anx2/Cdc42 that links the production of PIP2 to actin reorganization during apical membrane biogenesis and lumen formation.
CHAPTER 2: Gata6 Positive Feedback Propels Primitive Endoderm Commitment

2.1 Introduction and background

The development of PrE, one of the earliest cell lineages in mammalian embryos, has been fervently investigated to understand the basis of embryonic differentiation (Chazaud and Yamanaka, 2016; Hermitte and Chazaud, 2014). The analyses of mouse embryos with mutation in Fgf4 (Feldman et al., 1995; Kang et al., 2013b), the receptor tyrosine kinase (RTK) Fgfr2 (Arman et al., 1998), or the RTK adaptor protein Grb2 (Chazaud et al., 2006b; Cheng et al., 1998; Wang et al., 2011b) established that the Ras/MAPK pathway is essential for PrE differentiation (Frankenberg et al., 2011a), though the substrate(s) for Erk1/2 or the mechanism is not understood. Another essential gene for PrE differentiation is gata6, which is thought to be induced by Ras/MAPK signaling and is placed downstream of the pathway (Frankenberg et al., 2011a; Hermitte and Chazaud, 2014).

However, the mechanisms underlying how FGF/ERK signal pathway regulates Gata6 expression and further primitive endoderm formation has not been addressed. To further understand PrE lineage establishment, we generated a GFP reporter of Gata6 (Gata6: H2B-GFP). From the Gata6-GFP knock in mice, we collected the gata6H2BGFP/+ (gata6+/−) and gata6H2BGFP/H2BGFP (gata6−/−) embryos. In the heterogeneous embryo, we observed the fluctuation of GATA6 expression during the formation of PrE and demonstrated that the early spatial pattern of differentiation of PrE is stochastic and the spatial order emerges at a late stage, consistent with the GATA6 expression pattern observed in wild type embryos. With the knock out embryo, using the GFP as readout, we demonstrated that Gata6 expression is
maintained via an auto-regulatory mechanism. Furthermore, we showed that activated Erk signals can enhance Gata6 promoter binding efficiency by phosphorylating Gata6 on a conserved serine residue. Another member of the GATA family of zinc-finger transcription factor, Gata4, which is downstream of Gata6 expression in PrE lineage (Cai et al., 2008; Capo-Chichi et al., 2005), can also bind to the promoter region of gata6 and reinforce Gata6 expression. We also confirmed that gata6 null mutant embryos lack a PrE entirely, and exhibit pan-ICM expression of the pluripotency-associated factor NANOG (Schrode et al., 2014).

2.2 Development of a GATA6-H2BGFP reporter line

We generated Gata6-H2BGFP knock-in ES cells in which the Gata6 promoter controls GFP expression. Briefly, the enhanced green fluorescent protein (EGFP) gene was fused in-frame to the ATG at the Gata6 translation start site in the 5’ homologous arm. (Fig. 4A) Targeted ES cell clones were obtained by homologous recombination in ES cells. The targeted Gata6H2BGFP knock-in ES cells were then injected into blastocysts of C57BL/6 mice to obtain chimeric founders. The obtained Gata6-H2BGFPneo/+ heterozygotes were backcrossed with Sox2 cre/+ mice to delete the neomycin resistance gene. The heterozygous gata6$^{H2BGFP+/+}$ (gata6+/-) mice were then obtained which express Gata6 from one allele and GFP from the other. Genotype analyses were performed by PCR to confirm the mice genotype.

To validate transgene activity, we examined GFP expression in transgenic gata6$^{H2BGFP+/+}$ (gata6+/-) ES cells under different culture conditions. First, we cultured the ES cells in the presence of leukemia inhibitory factor (LIF), which maintained the ES cells in undifferentiated state. ES cells in this condition showed no expression of
GFP (Fig. 4B), as well as the absence of the other differentiation markers including Gata6, Gata4 and Dab2 (Fig. 4B). The positive staining of pluripotency marker Oct3/4 revealed that the ES cells remained in undifferentiated state. Then we treated the \textit{gata6H2BGFP/+(gata6+/-)} ES cells with retinoid acid (RA) for 3 days to induce the ES cell differentiation to PrE cell lineage. As shown in Fig. 4C, ES cells showed GFP expression. We also observed correlation of reporter activity with Gata6 protein expression. Furthermore, the Gata6 positive cells also showed positive staining of other primitive endoderm markers including Gata4 and Dab2. The signal of pluripotency marker Oct3/4 also reduced in this RA treated ES cells. \textit{gata6H2BGFP/+(gata6+/-)} ES cells were also cultured in suspension to promote the formation of embryoid bodies, which recapitulate several developmental events in embryo development including the formation of an outer layer of cells that resembles PrE. By immunofluorescence, we observed that in 7 days embryoid bodies the GFP positive cells covered the outer layer of embryoid bodies. The GFP positive cells also showed positive Gata6 staining, indicating the formation of the primitive endoderm layer. Gata4 and Dab2 positive expression also confirmed the establishment of PrE (Fig. 4D). To assess H2BGFP expression in mouse embryos, \textit{gata6H2BGFP/+(gata6+/-)} blastocysts were collected at E4.5 and imaged by laser scanning confocal microscopy. At this stage, the PrE is fully established. We first confirmed that Gata6 protein is expressed in the PrE cells localized within ICM cells adjacent to the blastocyst cavity, and investigated whether the Gata6-H2BGFP line could serve as a reporter of PrE. In E4.5 \textit{gata6H2BGFP/+(gata6+/-)} embryos, all detectable GFP signal co-localized with the endogenously expressed Gata6, which was found mainly in nuclei (Fig. 4E).
We also demonstrated the co-localization of GFP with markers of the PrE, including Gata4 and Dab2 (Fig. 4E). TE marker Cdx2 and ICM marker Oct3/4 expression were also examined in gata6<sup>H2BGFP/+(gata6+/-)</sup> embryos (Fig. 5A and B). Oct3/4 is expressed in both GFP positive and negative cells within ICM, and we confirmed the presence of Oct3/4 in both PrE and EPI cell lineages at the early blastocyst stage. Cdx2 expression was observed in the TE cell lineage. The co-expression of the H2BGFP reporter and endogenous Gata6 protein confirmed that the Gata6-H2BGFP line could serve as a fully functional reporter for noninvasive monitoring of Gata6 expression by detecting GFP fluorescence.

### 2.3 Dynamic expression of Gata6 in gata6<sup>H2BGFP/+(gata6+/-)</sup> embryos

Crossing the gata6<sup>H2BGFP/+(gata6+/-)</sup> male and female mice, we generated the gata6<sup>H2BGFP/+(gata6+/-)</sup> and gata6<sup>H2BGFP/H2BGFP(gata6-/-)</sup> embryos. As shown in Fig. 6, at E3.5 stage, gata6<sup>H2BGFP/H2BGFP(gata6-/-)</sup> homozygous blastocysts did not express Gata6 and had higher GFP expression level compared with the gata6<sup>H2BGFP/+(gata6+/-)</sup> heterozygous embryos, possibly due to the biallelic expression of GFP reporter in homozygous embryos.

To study the expression pattern of GFP in blastocysts, we analyzed the pattern of reporter expression in gata6<sup>H2BGFP/+(gata6+/-)</sup> embryos that had been fixed and stained for lineage-specific markers such as Gata6 for PrE and Nanog for EPI. In morula (16-32 cells), GFP was observed throughout the blastocyst (Fig. 7). The GFP-positive cells also showed positive Gata6 and NANOG expression indicating a Gata6+NANOG+ state (Fig. 8).
Figure 4. Gata6 H2BGFP knock-in strategy and reporter expression during endoderm differentiation of ES cells and blastocysts. (A) The wild-type Gata6 and targeted Gata6/H2BGFP alleles. (B) gata6<sup>H2BGFP</sup>/+ (gata6+/-) ES cells do not express GFP while maintained in the pluripotent state in the LIF containing medium. Differentiation markers including Gata6, Gata4 and Dab2 were examined by immunofluorescence as well as pluripotency marker Oct3/4. (C) Retionic acid was added into ES cell medium for 3 days to induce ES cell differentiation. A directed gata6<sup>H2BGFP</sup>/+ (gata6+/-) ES cells differentiation into endoderm lineage resulted in GFP expression. The GFP positive ES cells also showed positive Gata6 and the other differentiation marker Gata4 and Dab2 expression. Reduced expression of Oct3/4 was also observed. (D) Embryoid body are formed from gata6<sup>H2BGFP</sup>/+ (gata6+/-) ES cells. Retionic Acid was added into culture medium for 7 days to induce cell differentiation. Expression of GFP occurred on the out layer of the embryoid bodies. The GFP positive cells also showed positive Gata6 and the other differentiation marker Gata4 and Dab2 expression. (E) Colocalization of GFP with endogenous Gata6 expression in E4.5 Gata6<sup>H2BGFP</sup>+/-(Gata6+/-) heterozygous blastocysts. Immunofluorescence for Dab2 and Gata4 and their relative coexpression with GFP are also shown.
Figure 5. Trophoderm maker Cdx2 and ICM marker Oct3/4 expression in 
gata6^{H2BGFP+} (gata6+/−) heterozygous blastocysts. (B) Expression of Cdx2 and GFP in E4.5 
gata6^{H2BGFP+} (gata6+/−) heterozygous blastocysts. Blue in Merge: DAPI.
In early blastocysts (32-64 cells) stage, as cells within the ICM expressed certain EPI-specific or PrE-specific transcription factors, the different levels of GFP expression were also evident (Fig. 7). Specifically, the GFP signal co-localized with Gata6 expression (Fig. 7). The subset of GFP-positive cells almost invariably showed negative Nanog expression (Fig. 8). In mid- to late- blastocysts (64 cells to 128 cells), a time point when the embryos gradually establish mature lineage of Gata6+ PrE and NANOG+ EPI, the sorting of EPI and PrE populations to adjacent tissue layers was observed. The GFP positive cells showing positive Gata6 staining formed a layer of PrE covering the EPI cells with positive Nanog staining (Fig. 7 and Fig. 8). Our observation of Gata6-H2BGFP reporter expression revealed distinct phases during PrE formation that correlated with developmental stage as defined by total cell number.

2.4 Cell mobility and stabilization during the formation of primitive endoderm

Even though it is well accepted that exclusive Gata6 expression is established as the primitive endoderm lineage matures, we still do not know whether fluctuating expression of Gata6 expression occurs during the PrE specification. To address this question, we investigated the GFP signaling in \textit{gata6}^{H2BGFP/+} (\textit{gata6}+/-) embryos using time-lapse imaging. Using confocal microscopy, we observed that in early blastocysts, cells displayed highly dynamic reporter activity (Fig. 9A). At late blastula stage, after ICM lineage specification, the sorting of PrE and EPI population is completed; we observed that the GFP positive cells formed an epithelialized layer positioned adjacent to the EPI at the interface with the blastocyst cavity (Fig. 9A). We also qualified the GFP intensity of individual cells at different time points (Fig. 9B...
and Fig. 9C). The result further proved our previous observation that the initial ICM cells showed homogenous GFP expression and during later stage, the homogenous cell population diverse into two populations: (1) GFP-negative cells lying deep within the ICM were presumed as EPI; (2) GFP-positive ICM cells positioned in proximity to the blastocyst cavity were presumed as PrE lineage (Fig. 9B). The GFP intensity of TE cell lineage is also measured as control. We observe that during embryo develops, the GFP signal of TE cell lineage is relatively constant (Fig. 9C).

By single cell tracing, we observed that at early blastula stage both the intensity of the GFP signal and the position of GFP positive cells in the ICM fluctuate dynamically (Fig. 10 and Fig. 11). A few instances exhibited an interchange between GFP-low/EPI progenitor cells and GFP-hi/PrE progenitor state as cells migrate in the ICM, indicating that at this stage both the cell fate and position is not fully determined (Fig. 10). At late blastula stage, GFP positive cells remained consistently high intensity of GFP signal (Fig. 11), demonstrating that the GFP positive cells had less developmental plasticity than before. Furthermore, during this stage, the majority of GFP positive cells sorted out to the epithelialized layer and retained their relative positions (Fig. 11). Based on these data, we conclude that at early stage, the PrE and EPI cells remain plastic (or pluripotent) with interchangeable cell lineage commitment and dynamic movement of PrE precursor cells within ICM. At late blastocyst stage, PrE and EPI progenitor cells are committed to their fates and the position of the PrE and EPI remained relatively stable.
Figure 6. E3.5 wild type, gata6<sup>H2BGFP/+</sup> (gata6+/-) embryos and gata6<sup>H2BGFP/H2BGFP</sup> (gata6-/-) embryo. E3.5 wild type, gata6<sup>H2BGFP/+</sup> (gata6+/-), and gata6<sup>H2BGFP/H2BGFP</sup> (gata6-/-) embryos from mating of Gata6<sup>H2BGFP/+</sup> parents were collected and identified by immunofluorescence. Positive GFP signals were observed in gata6<sup>H2BGFP/+</sup> (gata6+/-) and gata6<sup>H2BGFP/H2BGFP</sup> (gata6-/-) embryos, while absence in wild type embryo. Positive Gata6 stainings were observed in wild type and gata6<sup>H2BGFP/+</sup> (gata6+/-) embryos, while absence in gata6<sup>H2BGFP/H2BGFP</sup> (gata6-/-) embryos. Blue in Merge: DAPI.
Figure 7. GFP and Nanog expression pattern in *gata6^{H2B:GFP+} (gata6+/-)* embryos. Localization and distribution of Nanog and GFP reporter in *gata6^{H2B:GFP+} (gata6+/-)* embryos at the 16-32 cells, 32-64 cells and 64–128 cell stage, respectively. Blue in merge: DAPI.
Figure 8. GFP and Gata6 expression pattern in gata6^{H2B:GFP/+} (gata6^{+/-}) embryos. Localization and distribution of pluripotency marker Gata6 and GFP reporter in gata6^{H2B:GFP/+} (gata6^{+/-}) embryos at the 16-32 cells, 32-64 cells and 64–128 cell stage, respectively. Blue in merge: DAPI.
Figure 9. Time-lapse video analysis of gata6^{H2BGFP/+} (gata6^+/+) embryos. (A) An example shows a series of snapshots from a time-lapse movie of a Gata6^{H2BGFP/+} (Gata6^+/+) blastocyst from E3.75 (around 70 cells) to E4.5 (around 120 cells) stages (B) GFP intensity of individual ICM cells in the gata6^{H2BGFP/+} blastocyst were analyzed along the time course and plotted. The cells gained a more intense GFP signal at the later time course (green) became PrE. (C) GFP intensity of the trophectoderm (TE) cells in the gata6^{H2BGFP/+} blastocysts were monitored along the time course. Each dot represents one single cell.
Figure 10. Dynamic cell sorting and GFP expression in *gata6*^{H2BGFP/+} (*gata6* +/-) blastocyst at E3.75 stage. (A) An example shows a sequence of snapshots from a time-lapse movie of a *gata6*^{H2BGFP/+} (*gata6* +/-) blastocyst from E3.75 (around 70 cells) stage. The movement of selected individual ICM cells was monitored, analyzed with Volocity software to quantitate the GFP intensity and cell movement, and plotted. (B) Quantification of movement of single nuclei in the *gata6*^{H2BGFP/+} is shown. Individual tracked cells are highlighted by different colors, shown in the upper panel. The area of the ICM is indicated by the dotted line. (C) Quantification of GFP intensity of single nuclei in the *gata6*^{H2BGFP/+} is shown at around 70 cells stage.
Figure 11. Dynamic cell sorting and GFP expression in $gata6^{H2BGFP^{+/+}}$ ($gata6^{+/-}$) blastocyst at E4.5 stage. (A) An example shows snapshots from a time-lapse movie of a $gata6^{H2BGFP^{+/+}}$ ($gata6^{+/-}$) blastocyst at round the 120-cell stage. Four distinctive PrE cells were traced, noted by the colors. (B) The movement of the PrE cells is plotted. The dotted lines indicate the starting (lighter line) and ending (darker line) of the PrE layer. (C) Quantification of GFP intensity of single nuclei in the $gata6^{H2BGFP^{+/+}}$ is shown at around 120 cells stage.
2.5 gata6 promoter activity in gata6<sup>H2BGFP/H2BGFP</sup> (gata6<sup>-/-</sup>) embryo

We also analyzed the pattern of reporter expression in gata6<sup>H2BGFP/H2BGFP</sup> (gata6<sup>-/-</sup>) embryos that had been fixed and stained for Gata6 in morula and blastula stage. In morula (16-32 cells), GFP was observed throughout the blastocyst (Fig. 12). The possible explanation is that, at morula stage, Gata6 expression is regulated by transcriptional machinery that can be activated at the absence of Gata6 protein. However, at blastula stage, we observed that within the ICM, all cells showed diminished GFP signals (Fig. 12). The decreased GFP signals indicated that Gata6 promoter activity is reduced at time of primitive endoderm commitment in gata6<sup>H2BGFP/H2BGFP</sup> (gata6<sup>-/-</sup>) embryo. gata6<sup>H2BGFP/H2BGFP</sup> (gata6<sup>-/-</sup>) embryos expressed NANOG starting at the 8-cell stage in all cells, and thereafter throughout the ICM (Fig. 13). We hypothesized that during the blastula stage, the consistent of GFP expression in the gata6<sup>H2BGFP/H2BGFP</sup> (gata6<sup>-/-</sup>) blastocyst requires Gata6 protein, indicating the existence of a positive self-regulation loop required for dynamic expression of Gata6 during primitive endoderm cell lineage establishment and maintenance. We also investigated the GFP expression in gata6<sup>H2BGFP/H2BGFP</sup> (gata6<sup>-/-</sup>) embryos using time-lapse imaging (Fig. 14A). In morula (16-32 cells), GFP was observed throughout the embryo. However, as the embryo develops into mid to late blastocysts, the GFP positive cells within the ICM gradually lose the GFP signals, indicating the deceased activity of gata6 promoter (Fig. 14A). This trend was also confirmed by quantitatively analyzing the intensity of GFP signal of individual cells as time goes. As shown in Fig. 14B, in the gata6<sup>H2BGFP/H2BGFP</sup> (gata6<sup>-/-</sup>) embryo, the ICM cells GFP intensity decreases along with time. While the TE cell GFP signals
remain relatively the same (Fig. 14C). Apoptosis within ICM also occurred as blastocyst development with an increase of condensed GFP signals (Fig. 15A). When the mutant embryo implanted on the uterus, the ICM cells diminished with only TE cells left (Fig. 15C). Based on this observation, we propose a model that the phase of stochastic gene expression starts the initiation of the specification of EPI and PrE lineage independent of Gata6 expression. As embryo develops, the following signal reinforcement of Gata6 expression by Gata6 itself and/or other downstream factors is required to drive the lineage segregation and the establishment of the PrE lineage.

2.6 Exogenous expression of Gata4 or Gata6 rescued Gata6 promoter activity in gata6 null ES cells

To further test our hypothesis, we used exogenous expression of Gata6 and Gata4 to rescue gata6 promoter activity in gata6 null ES cells. We generated ES cells from the gata6H2BGFP/+(gata6+/-) blastocysts and gata6H2BGFP/H2BGFP(gata6-/-) blastocysts. gata6H2BGFP/+(gata6+/-) ES cells were used as positive control for the rescue experiment. gata6H2BGFP/(gata6+/-) ES cells underwent spontaneous differentiation. ES cells showed GFP expression. We observed that GFP positive cells showed Gata6 protein expression and negative staining of pluripotency marker Oct3/4 (Fig. 16A). Furthermore, the GFP positive cell also showed positive staining of other primitive endoderm markers including Gata4 and Dab2 (Fig. 16A). When cultured in the presence of LIF, gata6H2BGFP/H2BGFP(gata6-/-) ES cells in this condition showed no expression of GFP. Immunostaining of ES cells showed the positive staining of pluripotency markers Oct3/4, with no expression of differentiation markers including Gata6, Gata4 and Dab2 (Fig. 16A).
Figure 12. GFP and Gata6 expression pattern in \textit{gata6}^{+/+} \textit{H2BGFP}/\textit{H2BGFP} (\textit{gata6}^{+/-}) embryos. (A) Localization and distribution of Gata6 and GFP reporter in \textit{gata6}^{+/+} \textit{H2BGFP}/\textit{H2BGFP} (\textit{gata6}^{+/-}) embryos at the 16-32 cells, 32-64 cells and 64–128 cell stage, respectively. Blue in merge: DAPI.
Figure 13. GFP and Nanog expression pattern in $gata6^{H2BGFP/H2BGFP}$ ($gata6^{-/-}$) embryos. (B) Localization and distribution of pluripotency marker NANOG and GFP reporter in $gata6^{H2BGFP/H2BGFP}$ ($gata6^{-/-}$) embryos at the 16-32 cells, 32-64 cells and 64–128 cell stage, respectively. Blue in merge: DAPI.
Figure 14. Time-lapse video analysis of gata6\textsuperscript{H2BGFP/H2BGFP} (gata6\textsuperscript{-/-}) embryos. (A) An example shows a series of snapshots from a time-lapse movie of a gata6\textsuperscript{H2BGFP/H2BGFP} (gata6\textsuperscript{-/-}) blastocyst from E3.75 (around 70 cells) to E4.0 (around 120 cells) stages. (B) GFP intensity of ICM cells from the gata6\textsuperscript{H2BGFP/H2BGFP} blastocyst was analyzed along a time course. The tracing of GFP levels on a panel of ICM cells were plotted. (C) GFP intensity of the trophectoderm (TE) cells in the gata6\textsuperscript{H2BGFP/H2BGFP} (gata6\textsuperscript{-/-}) blastocyst was analyzed.
Figure 15. Apoptosis in \textit{gata6}^{H2BGFP\text{H2BGFP}} (\textit{gata6}^{-/-}) blastocyst and implanted embryos. (A) \textit{gata6}^{H2BGFP\text{H2BGFP}} (\textit{gata6}^{-/-}) blastocyst collected at E4.5 stage, the white arrow points to the apoptotic cells with condensed nuclei. (B) Embryos from a mating between \textit{gata6}^{H2BGFP\text{H2BGFP}} (\textit{gata6}^{+/-}) heterozygous mice were collected at E5.5 stage and analyzed following cryo-sectioning. The embryos on slides were analyzed for GFP fluorescence, and were genotyped by immunostaining with Gata6 as well as PCR of cells collected from the slides. Representative \textit{gata6}^{H2BGFP\text{H2BGFP}} (\textit{gata6}^{+/-}) heterozygous and \textit{gata6}^{H2BGFP\text{H2BGFP}} (\textit{gata6}^{-/-}) homozygous mutant embryos were shown for GFP fluorescence, and counterstained with DAPI.
To further prove that Gata6 can bind to its own promoter and induce the Gata6 expression, we tried to rescue the phenotype with gata6 plasmid transfection. We observed that only those $gata6^{H2BGFP/H2BGFP}(gata6{-/-})$ cells successfully transfected with gata6 plasmid were able to express GFP. Also the GFP positive cell showed positive Gata4 and Dab2 expression with decreased expression of pluripotency marker Oct3/4 expression, indicating the exogenous gata6 induced the differentiation of the ES cells into the primitive endoderm lineage (Fig. 16A).

Gata4 is another transcription factor of the GATA family, which is also expressed in the PrE. Gata6 is one of the first lineage-specific transcription factors to be observed, being expressed at around the 16- to 32-cell stage. Gata4 is induced by GATA6 (Cai et al., 2008), at around the 64-cell stage, when the “salt-and-pepper” distribution of transcription factors is evident and thus, when cells are more likely fated to form PrE.

To test if expression of Gata4 also can bind to the $gata6$ promoter to reinforce Gata6 expression and thus the commitment of the primitive endoderm lineage, we also transfected with $gata6^{H2BGFP/H2BGFP}(gata6{-/-})$ ES cells with Gata4 plasmid. By microscope, we also observed positive GFP signals. Interestingly, the GFP positive cells also showed positive Gata4 expression, indicating the successful transfection of exogenous Gata4 was able to bind to the $gata6$ promoter and induce the GFP expression. But the Gata4 alone cannot induce the Gata6 expression, which confirmed that Gata4 expression is downstream of GATA6 expression. Meanwhile, Gata4 also failed to rescue the Dab2 expression (Fig. 16A). We then transfected the $gata6^{H2BGFP/H2BGFP}(gata6{-/-})$ ES cells with both Gata6 and Gata4 plasmid.
The co-transfection rescued the GFP expression successfully. And the transfected ES cells differentiated into primitive cell lineage (Fig. 16A).

2.7 Gata4 and Gata6 occupy gata6 promoter in primitive endoderm cells

We further performed CHIP assay to confirm that Gata4 and Gata6 can bind to gata6 promoter and compared the binding efficiency between gata4 and gata6. From the CHIP assay, we treated wild type ES cells with RA for 4 days to induce the ES cells into PrE lineage. gata6H2BGFP/H2BGFP (gata6-/-) ES treated with RA and undifferentiated wild type ES cells were used as negative control. The results confirmed that both Gata6 and Gata4 bind to the promoter of gata6 and induce Gata6 expression (Fig. 16B). Gata4 and Gata6 showed similar capacity in binding to the gata6 promoter. These results showed that both Gata6 and Gata4 could directly regulate gata6 promoter activity and reinforce Gata6 expression, allowing the cells to fully commit to the PrE lineage.

2.8 Activated Erk signals can enhance Gata6 promoter binding efficiency by phosphorylating Gata6 on a conserved serine residue

Phosphorylation of Gata-1,-2,-3 and -4 is known to modulate their DNA-binding and protein–protein interactions resulting in gene-specific transcriptions. (Cai et al., 2008)(Cai et al., 2008)(Cai et al., 2008) Likewise, Gata-6 contains consensus ERK phosphorylation sites (PYS\textsuperscript{264}P). We therefore investigated whether Gata-6 is phosphorylated by ERK in response to upstream Ras activation signals in differentiated embryonic stem cells. Anti-Gata6 antibody immunoprecipitated from wild type ES cells undergoing spontaneous differentiation (remove LIF from culture medium) and induced differentiation by RA, gata6\textsuperscript{H2BGFP/H2BGFP} (gata6-/-) ES cells
used as negative control. As expected, in differentiated wild type ES cells, MAPK substrates phospho-serine signals were detected. Our results indicated that during ES cells differentiation serine residues of Gata-6 are selectively phosphorylated in response to Ras/ERK signaling (Fig. 17A). To determine serine phosphorylation is responsible for Gata-6 binding efficiency to its promoter, we generated two mutant Gata6 plasmids: constitutively phosphorylated form of Gata6 (S264D) or a nonphosphorylatable form of Gata6 (S264A) (Fig. 17B). Gata6 mutants were transfected into gata6H2BGFP/H2BGFP(gata6-/-) ES cells. We found that both the mutants and wild type Gata6 plasmids can rescue the GFP signals.

However, compared to wild type and constitutively phosphorylated form of Gata6, the GFP signals induced the nonphosphorylatable form of Gata6 (S264A) are much lower, indicating a lower binding efficiency of Gata6 (S264A) to Gata6 promoter (Fig. 17C). We also treated the gata6H2BGFP/H2BGFP(gata6-/-) ES cells with Mek inhibitor which can impair the Erk activation and then transfected with the constitutively phosphorylated Gata6 (S164D) and wild type Gata6 plasmid. We found that the wild type plasmid cannot rescue the GFP signals under the Erk activity inhibition but the constitutively actively Gata6 (S-D) plasmid can still rescue the GFP signal. Together, the results demonstrate that ERK-dependent phosphorylation of GATA-6 on serine 264 is essential for the trans-activation of the gata6 promoter by GATA-6 in response to Ras/ERK signaling.
Figure 16. Gata6 binding to and activation of its promoter in primitive endoderm differentiation. 

a. *gata6*^{H2BGFP/+} (gata6 +/−) and *gata6*^{H2BGFP/H2BGFP} (gata6 −/−) ES cells were either treated with retinoic acid to induce PrE differentiation, or transfected with Gata6 and/or Gata4 expression plasmids. Two days following treatment, the cells were analyzed for GFP fluorescence, and were analyzed for PrE markers including Gata6, Gata4, and Dab2 by immunofluorescence microscopy. 

b. Schematic illustration shows the *gata6* gene promoter site targeted for ChIP assay. The sequence contains multiple GATA binding sites. 

c. Association of Gata6 with *gata6* promoter was analyzed by ChIP-qPCR assays. Briefly, nuclear extracts from wildtype or *gata6* (−/−) ES cells with or without differentiation by RA treatment were used for immunoprecipitations using antibodies to Gata4 or Gata6, and the immunoprecipitations were analyzed by PCR amplification of an about 200 base pair fragment to detect the enrichment of *gata6* promoter sequences. Immunoprecipitation was performed in triplicate and the results are presented as mean fold enrichment with standard deviation.
Figure 17. Phosphorylation of Gata6 and its regulation of its own promoter activity. (A) Wildtype ES cells of undifferentiated, spontaneously differentiated upon removal of LIF (- LIF), and RA-differentiated were lysed, and the whole cell lysates were used for immunoprecipitation using anti-Gata6. Both the lysate inputs and immunoprecipitations were analyzed by Western blot with anti-PYS*P and anti-Gata6 antibodies. (B) Expression vectors of wildtype and mutant plasmids: Gata6, Gata6 (S264A), and Gata6 (S264D) were constructed and sequencing was performed to verify the targeted mutation. (C) Undifferentiated gata6H2BGFP/H2BGFP (gata6-/-) ES cells were transfected with expression vectors of wildtype and mutant plasmids: Gata6, Gata6 (S264A), and Gata6 (S264D). At 48 hours after transfection, the cells were monitored for GFP signals, and were also analyzed by immunofluorescence microscopy for Gata6 and Gata4. Scale bar: 20um. (D) About 20 GFP expressing cells in each group were quantitated for GFP levels. GFP signals stimulated by Gata6 (S264A) were statistically lower than those stimulated by wildtype or S264D Gata6 (*** p < 0.001). (E) gata6H2BGFP/H2BGFP (gata6-/-) ES cells were transfected with wildtype and Gata6(S264D) plasmid and treated with Mek inhibitor in the meantime. After transfection for 48 hours, the cells were monitored for GFP signals. (F) About 20 cells in each group were quantitated for GFP levels.
2.9 Gata6 is essential for primitive endoderm lineage commitment

Gata6 expression is first detected at around the 8-cell stage in every cell of blastomeres, and around 64 cells stage it is restricted to the PrE progenitor cells. Thus Gata6 is considered as the earliest PrE marker. Furthermore, ectopic expression of Gata6 in mouse ES cells is sufficient to induce the differentiation of ES cells to PrE-like stage. In order to investigate the role of Gata6 in PrE cell fate specification, we collect the heterogeneous \( gata6^{H2BGFP/+}(gata6+/-) \) embryo and homogeneous \( gata6^{H2BGFP/H2BGFP}(gata6-/-) \) embryos at E4.5 stage. In with heterogeneous blastocyst, we confirmed that PrE formation is marked by exclusive expression of differentiation-specific genes such as Gata4 and Dab2 (Fig. 18A and Fig. 18B). However, in the \( gata6 \) mutants, embryos in the absence of Gata6 failed to active the Gata4 and Dab2 expression, indicating the PrE program is disabled in the absence of Gata6 (Fig. 18A and Fig. 18B). We further confirmed the conclusion in vitro system. First, we induce the \( gata6^{H2BGFP/+}(gata6+/-) \) and \( gata6^{H2BGFP/H2BGFP}(gata6-/-) \) ES cells with RA which can arbitrary induce the ES cell differentiate. After treated with RA for 3 days, we observed that \( gata6^{H2BGFP/+}(gata6+/-) \) ES cell underwent differentiation marked by the expression of Gata6, Gata4 and DAB2. However, in the \( gata6^{H2BGFP/H2BGFP}(gata6-/-) \) ES cells, the cells failed to express Gata4 and Dab2. Under the same exposure time, the GFP signal is much less than the \( gata6^{H2BGFP/+}(gata6+/-) \) ES cells (Data not shown).
Figure 18. PrE lineage is absent in the Gata6 mutant blastocysts. (A). Localization and expression of PrE markers Gata4 in gata6H2BGFP+/+(gata6+/+) embryos and gata6H2BGFP/H2BGFP (gata6−/−) embryos at E4.5 stage, respectively. Blue in merge: DAPI. (B). Localization and expression of epithelial markers DAB2 in gata6H2BGFP+/+(gata6+/+) embryos and gata6H2BGFP/H2BGFP (gata6−/−) embryos at E4.5 stage, respectively. Blue in merge: DAPI.
Figure 19. PrE lineage cannot be specified in gata6H2BGFP/H2BGFP (gata6−/−) EBs. gata6H2BGFP+/+(gata6+/-) and gata6H2BGFP/H2BGFP (gata6−/−) ES cells were culture in suspension to produce embryoid bodies for 7 days. GFP signals, pluripotency markers Oct3/4 and Nanog were examined as well as the PrE markers Gata6, Gata4 and Dab2. Blue in merge: DAPI.
Figure 20. PrE lineage cannot be specified in gata6$^{H2BGFP/H2BGFP}$ (gata6$^{-/-}$) ES cells. (A) Wildtype (WT), gata6$^{H2BGFP/+}$ (gata6$^{+/-}$) (HET) and gata6$^{H2BGFP/H2BGFP}$ (gata6$^{-/-}$) (KO) ES cells, with or without differentiation by retinoic acid, were analyzed by Western blot for GFP, Gata6, Gata4, Oct3/4 and Dab2 protein levels. (B) Wildtype (WT), gata6$^{H2BGFP/+}$ (gata6$^{+/-}$) (HET) and gata6$^{H2BGFP/H2BGFP}$ (gata6$^{-/-}$) (KO) ES cells, with or without differentiation by retinoic acid, were analyzed by RT-PCR for the mRNA levels of GFP, GATA6, GATA4, Oct3/4, Nanog and Dab2 levels.
*gata6*\(^{H2B GFP/+}(gata6+/-)\) and *gata6*\(^{H2B GFP/H2B GFP}(gata6-/-)\) ES cells were also cultured in suspension to promote the formation of embryoid bodies (EBs), which are aggregates of ES cells capable of differentiating into a primitive endoderm outermost layer, yet retaining the pluripotent stem cells in the interior of the sphere. By immunofluorescence, we observed that in *gata6*\(^{H2B GFP/+}(gata6+/-)\) 7days EBs the GFP positive cells formed the outside layer on the EBs. The GFP positive cells also showed positive Gata6 staining, indicating the formation of PrE layer. Gata4 and Dab2 positive expression also confirmed the establishment of PrE. However, in the *gata6*\(^{H2B GFP/H2B GFP}(gata6-/-)\) EBs, GFP expression was negative and differentiation markers were absent (Fig. 19A). When the *gata6*\(^{H2B GFP/+}(gata6+/-)\) and *gata6*\(^{H2B GFP/H2B GFP}(gata6-/-)\) EBs were treated with RA, the *gata6*\(^{H2B GFP/+}(gata6+/-)\) EBs showed thicker primitive endoderm layer with more cell with positive GFP and Gata6. In the *gata6*\(^{H2B GFP/H2B GFP}(gata6-/-)\) EBs, RA could not bypass Gata6 to induce the expression of Gata4 and Dab2 (Data not shown). Collectively, we concluded that Gata6 is required for PrE cell fate specification and the establishment of the PrE lineage. A western blot and rtPCR also confirmed the observation in immunofluorescence in ES cells (Fig. 20). A gene dosage effect was observed between the differentiated wild type ES cells and the heterozygous ES cells.

### 2.10 Summary and Significance

The current study achieved three significant findings, that increased and diminished Gata6 expression lead to the lineage commitment of ICM to PrE and Epi, respectively; that Gata6 binds to its own promoter to achieve a positive feedback in PrE differentiation; and that Erk phosphorylation of Gata6 at serine 264 enhances it
activity in transcriptional activation of its own promoter. The findings suggest a plausible model for the lineage differentiation of PrE and Epi (Figure 21). We postulate that all cells of the early (32-cell stage) blastula exhibit a moderate level of Gata6 expression. FGF4 activation in a subset of the ICM cells leads to phosphorylation of Gata6 by Erk1/2, and the phosphorylated Gata6 gains an increased affinity to its promoter to initiate a positive feedback loop that augments Gata6 expression. These cells ultimately commit to the PrE lineage. In the remaining cells not receiving the FGF signal and possessing low Erk1/2 activity, the initial Gata6 expression subsides, possibly caused by suppression mediated by Nanog and Oct3/4, and subsequently the cells proceed into the Epi lineage. Therefore, our findings reveal a Gata6-positive feedback regulatory loop and provide a mechanistic explanation for the long sought impact of Ras/MAPK pathway in the development of PrE.
Figure 21. Model for Gata6 phosphorylation and self promotion of gata6 promoter in the commitment of primitive endoderm lineage.

The current study produced several significant findings to support a model for PrE lineage commitment: 1. Gata6 binds to its own promoter to achieve a positive feedback in Gata6 expression. 2. Erk phosphorylation of Gata6 at serine 264 enhances its activity in transcriptional activation of its own promoter.

We postulate that all cells of the early (32-cell stage) blastula exhibit a moderate level of Gata6 expression. FGF4 activation in a subset of the ICM cells leads to phosphorylation of Gata6 by Erk1/2, and the phosphorylated Gata6 gains an increased affinity to its promoter to initiate a positive feedback loop that augments Gata6 expression. These cells ultimately commit to the PrE lineage. In the remaining cells not receiving the FGF signal and possessing low Erk1/2 activity, the initial Gata6 expression subsides, possibly caused by suppression mediated by Nanog and Oct3/4, and subsequently the cells obligate into the Epi lineage.
CHAPTER 3: Pten Facilitates Epiblast Epithelialization and Proamniotic Cavity Formation

3.1 Introduction and background

Pten (phosphatase and tensin homologue on chromosome 10) possesses lipid phosphatase enzymatic activity that converts phosphoinositol-3,4,5 tri-phosphate (PIP3) into phosphatidylinositol 4,5 bisphosphate (PIP2) (Maehama and Dixon, 1998; Morimoto et al., 2000). It is a classic tumor suppressor (Gerisch et al., 2012; Sha et al., 2010; Steck et al., 1997; Vazquez and Devreotes, 2006) that negatively regulates the PI3K-Akt-mTor-S6K pathway (Steck et al., 1997). The Pten knockout mice die at an early stage during embryonic development (Di Cristofano et al., 1998).

In the current study, we investigated Pten mutant embryos and embryoid bodies, to understand epiblast organization and the formation of the proamniotic lumen. Shortly following implantation of the blastocysts, the pluripotent Epi covered by the PrE transforms into a polarized columnar epithelium, the ectoderm, while the PrE differentiate into the visceral endoderm (VE) and the parietal endoderm (PE) (Cantley and Neel, 1999). The organization of the ectoderm coincides with the emergence of a proamniotic cavity (Knobbe et al., 2008; Manning and Cantley, 2007). A now classic study suggested that cavitation is the result of factor(s) derived from the enveloping visceral endoderm stimulating programmed cell death in the Epi and rescuing of an epithelial layer by a survival signal provided by direct contact to the visceral endoderm-derived basement membrane (Coucouvanis and Martin, 1995; 1999). The apoptotic cells are rapidly removed by efferocytosis (Vandivier et al., 2006) and are difficult to be observed in the early embryos (Coucouvanis and Martin, 1995).
However, a more recent study using a new technical approach to culture blastocysts in a matrix support in vitro suggests that the proamniotic lumen forms as a result of epithelial organization and rosette morphogenesis without the need for cell death (Bedzhov and Zernicka-Goetz, 2014). In this model, the basement membrane dividing the PrE and Epi imparts polarization cues through beta1-integrin-mediated adhesion to establish a basal-apical axis of the Epi cells and hence the opening of lumen at the apical side.

In this chapter, we have used the Pten mutant line derived from the floxed allele in C57BL6/J background (Tam and Loebel, 2007) for the analysis of the Pten null embryonic phenotypes. We conclude that the key role of Pten in embryonic cavitation is not to promote apoptotic cell death; rather, Pten facilitates epithelial polarization and the formation of a rosette that expands into the future proamniotic lumen. The results favor the non-cell death model of embryonic cavitation (Stern and Downs, 2012) and provide new understanding for the mechanism of proamniotic cavitation.

### 3.2 Pten is essential for proamniotic cavity formation

Pten is essential for early embryogenesis in mice, but the precise mechanism for the developmental failure of the knockout embryos has not been settled. We analyzed the phenotypes of Pten knockout embryos immediately after implantation for the morphology of extraembryonic endoderm and ectoderm. A section of each embryo within uteri from timed mating between Pten heterozygous parents was used for immunostaining to distinguish Pten-positive (wildtype or heterozygous) from Pten-deficient/knockout embryos (Fig. 22A). Eight out of the 38 E5.5 embryos were determined to be Pten negative by immunostaining and considered to be pten (-/-),
Figure 22. Formation of visceral endoderm in both Pten-positive and -deficient embryos. Mouse embryos harvested from timed matings between pten (+/-) mice were sectioned and analyzed by immunofluorescence microscopy. Cross sections of the embryos are shown. The relative proximo-distal position of the sections is indicated as the dashed line. Immunostaining of Pten was used to distinguish Pten-negative (considered pten (-/-)) embryos from Pten-positive (pten (+/-) or (+/+)) embryos. (A) Representative examples are shown of an E5.5 wildtype (upper panel) and Pten-null (indicated as “(-)”) embryo (lower panel) analyzed for Pten and the PrE marker Dab2. DAPI (blue) was used for nuclear counterstaining. (B) Representative examples of a Pten-positive and a Pten-null E6.5 embryo are shown. Sequential sections were analyzed by immunofluorescence microscopy for Dab2 and E-cadherin and counterstained with DAPI.
which approximated the expected Mendelian ratio. In all these Pten-null E5.5 embryos, extraembryonic visceral endoderm appeared morphologically normal as indicated by positive Dab2 staining (Fig. 22A, B). At around the E5.5 stage, the most obvious morphological difference of the Pten-null embryos was the lack of a proamniotic cavity (Fig. 22A, B). The central cavity was apparent in wildtype embryos, shown by E-cadherin staining that depicted the outline of a lumen (Fig. 19B). No cavity was visible in all consecutive sections through the E5.5 Pten null embryos, shown in a representative example (Fig. 22B). In all the 8 Pten-deficient E5.5 embryos identified, no evidence of cavitation could be recognized. Even at E6.5 stage, when all Pten-positive embryos had a well-developed epithelialized embryonic ectoderm and prominent proamniotic cavity, all Pten null (6 identified among 34 total) embryos exhibited an enlarged cell mass enveloped within a visceral endoderm layer and lacked a cavity. No organized ectoderm was recognizable in E6.5 Pten null embryos (Fig. 23A). Even at E7.5, while Pten-positive embryos had advanced morphogenesis, the Pten deficient embryos lacked an ectoderm layer and exhibited abnormal cavitation, as shown in an example (Fig. 23B). In 8 mutant embryos identified at around E7.5 stages, 5 embryos showed no cavity, and 3 other mutant embryos exhibited an interior hollow space but also contained excessive cells without a mature ectoderm (Fig. 23C). Thus, Pten is not required for the development of extraembryonic endoderm, but is essential for the formation of the proamniotic cavity and the patterning of embryonic ectoderm.
Figure 23. Histology of Pten positive and deficient embryos. (A) Representative examples of histology of E5.5 and E6.5 wildtype and Pten null (indicated as “(-)”) embryos. The wildtype and pten (+/-) embryos from the same litter were distinguished from the Pten-negative embryos by immunostaining for Pten. (B) Representative examples of histology of E7.5 wildtype (WT) and Pten-null embryos. The E7.5 wildtype and Pten heterozygous embryos from the same litter were distinguished from Pten-null embryos by immunostaining of Pten. Adjacent sections were stained for Dab2 and Gata4. (C) Examples of histology of E7.5 Pten-null embryos. Three E7.5 Pten-null embryos were identified by immunostaining for Pten. Adjacent sections were stained for Gata4 and Dab2. Scale Bar: 100 um.
3.3 Pten is required for marking of a dominant rosette prior to proamniotic cavity formation

A recent finding indicates that proamniotic cavitation is initiated from a rosette following apical constriction and enlargement of the opening (Bielinska et al., 1999). In embryos around the E5.0 stage prior to observable cavitation, a concentrated presence of Pten expression was observed at the center of the epiblast was observed (Fig. 24). Although several rosettes marked by ZO1 staining were present, the location with high Pten expression likely indicates the center of a forming dominant rosette of the developing cavity. Coincident with focal Pten expression, an emerging foci marked by the presence of the tight junction ZO1 protein could be observed (Fig. 25). In contrast, in Pten null embryos, ZO1 staining was scattered and marked a few potential rosettes, but no dominant foci was present (Fig. 26), suggesting Pten is asymmetrically expressed in the epiblast and required for the initiation of a rosette in the formation of cavity. The increased Pten expression was apparent at the site of a rosette marked by E-cadherin (Fig. 27A) and b-actin (Fig. 27B) staining in Pten-positive embryos. By E6.5 stage, a cavity was apparent in Pten-positive embryos as indicated by the polarized staining with ZO1 (Fig. 28A), αPKC (Fig. 28B), and b-actin (Fig. 28C). Focal ZO1 expression marked the tight junctions lining the ectoderm around the embryonic cavity (Fig. 28A). The apical lining of the cavity also showed stronger αPKC expression (Fig. 28B), and the presence of F-actin was also polarized at the apical tip of the ectoderm cells (Fig. 28C). In Pten-null E6.5 embryos, no asymmetric distribution of ZO1, αPKC, or beta-actin was seen, and no cavitation occurred (Fig. 28).
Figure 24. Pten is required for marking of rosette prior to cavitation of E5.5 embryos. Precavitation E5.5 mouse embryos harvested from timed matings between pten (+/-) mice were analyzed by immunofluorescence microscopy. (A) Representative examples are shown of E5.5 wildtype embryos as a longitudinal or cross sections stained for Pten and Dab2. An image at a higher magnification of the same slide is shown in the lower panel.
Figure 25. Establishment of adherent junction and tight junction in E5.5 wild type embryos. Pre-cavitation E5.5 mouse embryos harvested from timed matings between pten (+/-) mice were analyzed by immunofluorescence microscopy. Representative examples are shown of E5.5 wildtype embryos as a longitudinal or cross section stained for E-cadherin and ZO1. An image of the same slide at a higher magnification is shown in the lower panel.
Figure 26. Disruption of adherent junction and tight junction polarity in E5.5 pten(-) embryos. Pre-cavitation E5.5 mouse embryos harvested from timed matings between pten (+/-) mice were analyzed by immunofluorescence microscopy. Representative examples are shown of E5.5 Pten-null embryos as a longitudinal or cross sections stained for E-cadherin and ZO1.
Figure 27. Pten expression is apparent at the site of a rosette in E5.5 embryos. Precavitation E5.5 mouse embryos harvested from timed matings between pten (+/-) mice were analyzed by immunofluorescence microscopy. (A) A representative example is shown of E5.5 wildtype embryos stained for Pten and E-cadherin. (B) A representative example is shown of E5.5 wildtype embryos stained for Pten and β-actin.
Figure 28. Lack of a proamniotic lumen in Pten-null E6.5 embryos. E6.5 mouse embryos harvested from timed matings between pten (+/-) mice were analyzed by immunofluorescence microscopy comparing Pten-positive and null embryos of the same litter. (A) Representative examples of a pair of Pten-positive and null embryos stained with E-cadherin and ZO1. (B) Representative examples of a pair of Pten-positive and null embryos stained with Dab2 and aPKC. (C) Representative examples of a pair of Pten-positive and null embryos stained with Dab2 and β-actin.
In the absence of Pten, αPKC and F-actin appeared evenly distributed in all epiblast cells at the cell-cell junctions, and no polarized or concentrated distribution of the markers was established. Thus, an increased and concentrated Pten expression appears to mark the site of a rosette that initiates the development of the proamniotic cavity.

3.4 Pten-null embryoid bodies are incapable of cavitation

Embryoid body models are able to recapitulate extraembryonic endoderm morphogenesis and proamniotic cavitation in culture (Bedzhov and Zernicka-Goetz, 2014; Lesche et al., 2002). We analyzed embryoid bodies derived from the Pten-null ES cells, and were able to reproduce the defective cavitation phenotype in vitro (Fig. 29). In 5 wildtype embryoid bodies selected to show different stages of cavitation, E-cadherin and ZO-1 stainings of epithelial tight junctions illustrated the marking of a rosette to its expansion into a well-formed lumen (Fig. 29). Similar to those of wildtype, embryoid bodies derived from pten (-/-) ES cells harbored a surface layer of Dab2-positive extraembryonic endoderm (Fig. 30A). While a central cavity is present in most wildtype embryoid bodies by day 5, all pten (-/-) embryoid bodies lacked a central cavity (Fig. 30A). In about 50 aggregates/embryoid bodies from 3 fields, 80% of the wildtype embryoid bodies, though none of the pten null EBs, possessed a lumen by day 5. Although Oct3/4 is present in the internal ectoderm cells, the pten (-/-) cells, unlike wildtype ES cells, did not organize into an epiblast epithelial layer (Fig. 30B). Similar to that observed in embryos, in embryoid bodies tight junction markers ZO1, αPKC, and b-actin were polarized around the developing cavity, but no polarized distribution of these proteins was seen in pten (-/-) embryoid
Figure 29. Development of a rosette and progression to a lumen in embryoid bodies. Representative 5-day-old wildtype embryoid bodies were analyzed by immunofluorescence microscopy for E-cadherin and ZO1. Five selected examples of images show different stages of rosette marking and lumen formation.
Figure 30. Failure of cavity formation in pten (-/-) embryoid bodies. Representative images from 5-day-old wild type and Pten-null embryoid bodies were analyzed by immunofluorescence microscopy. (A) Examples of a pair of wildtype and pten (-/-) embryoid bodies stained for Dab2 and E-cadherin. (B) Examples from a pair of wildtype and pten (-/-) embryoid bodies stained for laminin, megalin, and Oct3/4 are shown. (C) Examples from a pair of wildtype and pten (-/-) embryoid bodies stained for E-cadherin and ZO1, and images at a higher magnification (lower panel) are shown. (D) Examples from a pair of wildtype and pten (-/-) embryoid bodies stained with Dab2 and αPKC are shown. (E) Examples from a pair of wildtype and pten (-/-) embryoid bodies stained with Dab2 and α-actin are shown.
bodies (Fig. 30 C, D, E). From these results, we conclude that in Pten-null embryoid bodies, the mutant ES cells can undergo normal primitive endoderm and extraembryonic tissue differentiation in vitro but are unable to establish a cavity in embryoid bodies.

3.5 E-cadherin regulation of focal Pten expression and cavity formation

Previously we observed that embryoid bodies derived from E-cadherin null ES cells are able to form an extraembryonic surface layer but fail to form cavity (Bedzhov and Zernicka-Goetz, 2014). Thus, we compared Pten null and E-cadherin null embryoid bodies (Fig. 31). In wild type embryoid bodies, the Pten protein was enriched at the apical surface and the cell-cell contact adherent junctions (Fig. 31A). Similar to those of Pten null, E-cadherin knockout embryoid bodies failed to form a cavity, and also lacked focal expression of Pten, which was detected in embryoid bodies from wild type ES cells (Fig. 31A). Western blot analysis indicated that Pten expression was reduced in E-cadherin (-/-) embryoid bodies (Fig. 31B), and was quantitated to be 50 - 60% of wild type (Fig. 31C). These results suggest that E-cadherin modulates the levels and subcellular localization of Pten at the onset of cavitation, which is critical for setting up the focal location of cell tight junctions to establish a dominant rosette. The mechanism for the role of E-cadherin in inducing Pten expression has been suggested in epithelial cells (Coucouvanis and Martin, 1995; Rula et al., 2007). Thus, failure of cavitation in E-cadherin null embryoid bodies is due to the inability for cell-cell adhesion to induce Pten expression to create an initiating rosette.
Figure 31. E-cadherin regulates Pten protein levels in embryoid bodies. (A) Immunofluorescence staining of E-cadherin and Pten in representative wildtype (WT), pten (-/-), and E-cadherin (-/-) embryoid bodies. (B) Western blot analysis of Pten and E-cadherin in 3-day and 6-day wildtype, pten (-/-), and E-cadherin (-/-) embryoid bodies. (C) The Pten protein levels in the Western blot were quantified using NIH Image J program. The difference in Pten protein levels between wildtype and E-cadherin (-/-) embryoid bodies was analyzed and determined to be statistically significant (p<0.05). Experiments were repeated 3 times and consistent conclusions were reached.
3.6 Occurrence of apoptosis in embryonic cavity formation

Apoptosis is generally thought to play an important role in morphogenesis of lumens in embryogenesis, and classic studies suggested a model that cell contact survival and programmed cell death induced by a visceral endoderm-derived factor facilitate proamniotic cavity formation in mouse embryos (Fournier et al., 2009; Lau et al., 2011). Since loss of Pten is known to enhance cell survival by allowing unsuppressed activation of the AKT/mTOR signal pathway, we investigated if suppression of apoptosis contributes to the failure of cavitation in Pten null embryos and embryoid bodies.

In embryoid bodies derived from wildtype, pten (-/-), or E-cadherin (-/-) ES cells, dead cells were revealed by their uptake of fluorescent propidium iodide (PI) (Fig. 32A). In all three genotypes, a slight amount of cell death was observed at day 3 in embryoid bodies and was abundant by day 6. The cell death monitored by PI staining was concentrated at the centrally in wildtype, peripherally in pten (-/-), and randomly in E-cadherin (-/-) embryoid bodies (Fig. 32A). Surprisingly, pten (-/-) and E-cadherin (-/-) embryoid bodies had higher levels of apoptosis as judged by Western blot analysis of cleaved/activated caspase 3 and proteolytic cleavage of polyADP ribose polymerase (PARP) (Fig. 32B). The increased apoptosis correlated with a reduction of phospho-Erk1/2 (p-Erk1/2) levels (Fig. 32B). Activation of PI3K/mTOR signaling pathway was also examined, but no significant difference was observed between wildtype and pten (-/-) or E-cadherin (-/-) embryoid bodies (Fig. 32C).

Using immunofluorescence microscopy to examine activated/cleaved caspase 3 as an indicator of programmed cell death, apoptotic cells were largely located at the center
of wildtype embryoid bodies, but were randomly distributed throughout those of \textit{pten} (-/-) or E-cadherin (-/-) (Fig. 32D). Regarding embryos, the presence of activated caspase 3-positive apoptotic cells was observed in nearly every wildtype E5.5 embryo, but was not observed in four Pten null embryos analyzed for the activation of caspase 3 (Fig. 32E).

These results indicate that the absence of Pten does not significantly affect the activation of caspase 3 and apoptosis, and Pten is important in initiation and development of a rosette into the embryonic lumen. Although apoptosis may contribute to embryonic cavitation, and apoptotic cells are especially more obvious during the cavitation of embryoid bodies that often have more excessive cells, cell death appears not to be essential in the development of the embryonic lumen. Although the absence of Pten may reduce the degree of cell elimination, the more critical role of Pten in epithelial polarization and the establishment of the initial rosette as the focal point of future cavity likely underlies the mechanism of developmental failure of the Pten mutant embryos.
Figure 32. Apoptotic cells in the cavitation of embryoid bodies and embryos. (A) Live embryoid bodies derived from wild type, pten (-/-), and E-cadherin (-/-) ES cells were stained with propidium iodide (PI) to detect the presence of dead cells (with permeated plasma membranes). (B) Protein extracts from the monolayer ES cells and 3- and 6-day embryoid bodies were analyzed by Western blot for p-ERK1/2, PARP, and cleaved-caspase 3. Actin levels are shown as loading controls. (C) Western blot analysis of phospho-mTOR and phospho-P13K in monolayer ES cells (day 0), and embryoid bodies on day 3 and day 6, of wildtype, pten (-/-), and E-cadherin (-/-) genotypes. (D) Representative embryoid bodies were analyzed by immunofluorescence microscopy for E-cadherin and cleaved/activated caspase 3. (E) Representative Pten-positive and -null E5.5 embryos were analyzed by immunofluorescence microscopy for Dab2 and cleaved/activated caspase 3.
3.7 Summary and Significance

The Pten knockout mice die at an early stage during embryonic development (Coucouvanis and Martin, 1995, 1999). Two independently targeted Pten null mutations in the BL6 background fail at an embryonic stage prior to E7.5 (Knobbe et al., 2008), whereas embryos homozygous for a third Pten null allele in a CD1 outbred background allow longer survival to E9.5, with defects in chorio-allantoic fusion, formation of the cranial neural folds and aberrant mesoderm migration (Di Cristofano, Pesce et al. 1998, Knobbe, Lapin et al. 2008). Subsequent studies indicate that the defect in axis specification in CD1 background mutant embryos is caused by abnormal behavior of anterior visceral endoderm cells, whereby Pten mutant AVE cells initiate movement but fail to migrate to the same extent as that in wild type embryos (Di Cristofano et al., 1998; Knobbe et al., 2008). In another study using the embryoid body model, the authors conclude that Pten is required for apoptosis-mediated cavitation by regulating the expression of HIF-2α and Bnip3, but not by suppression of PI3K or Akt (Podsypanina et al., 1999), though the intriguing mechanisms were not tested or confirmed in embryos.

Here, we have used the Pten null line derived from the floxed allele in C57BL/6J background for the analysis of the Pten null embryonic phenotypes. We determined that the absence of Pten leads to failure of proamniotic cavity development, due to a defect in creating a polarized rosette. The results were consistent to demonstrate a role of Pten in the organization of ectoderm epithelium and the creation of proamniotic lumen. However, we conclude that the key role of Pten in embryonic cavitation is not promoting apoptotic cell death; rather, Pten mediates epithelial
polarization and the formation of a rosette that expands into the future proamniotic lumen. The results favor the non-cell death model of cavitation. Based on the results, we propose a model for the formation of pro-amniotic cavity (Fig. 33). As established previously, during embryo development, differentiated PrE cells produce laminin and other components of the basement membrane. As established previously, during embryo development, differentiated PrE cells produce laminin and other components of the basement membrane (Bloomekatz et al., 2012; Lesche et al., 2002; Qi et al., 2015). The basement membrane wraps up the embryonic lineage and provides organization cues that passed through β1-integrin receptors to determine orientation of the axis of polarity in the Epi cells during their epithelialization and also determines the apical surface (Bedzhov and Zernicka-Goetz, 2014; Tonary and Carnegie, 1996). On the other hand, the formation of adherent junctions between adjacent Epi cells may also orchestrate the expression and localization of Pten to the apical domain. The apical localized Pten allows the enrichment of PIP2 to the apical plasma membrane and PIP3 to the basal plasma membrane (Moore et al., 2014; Murray and Edgar, 2000; Nadijcka and Hillman, 1974). PIP2 acts as a key determinant of the apical surface development. Apical PIP2 recruits Anx2, which subsequently recruits Cdc42. Cdc2 binds and localizes the Par/aPKC complex to the apical domain and promotes the establishment of epithelial polarity and the formation of an initiating rosette (Bryant and Mostov, 2008; Li et al., 2002b; Sakai et al., 2003). The epithelial tight junctions maintain cell polarity by preventing the lateral diffusion of integral membrane proteins between the apical and lateral/basal surfaces (Comer and Parent, 2007; Elias et al., 2015; Pinal et al., 2006), and contraction of tight
junctions around the rosette leads to expansion of the opening and formation of an epithelial lumen (Bryant and Mostov, 2008). On the other hand, apoptosis occurs to remove excessive cells and may contribute to the establishment of the cavity, but this seems not to be the essential or critical.
Figure 33. Working model for the formation of proamniotic lumen. At E5.5 stage, E-cadherin mediated cell-cell adhesion intensifies in the epiblast and stimulates an increased Pten focal expression that initiates the formation of a dominant rosette. The epithelial tight junction and apical surface are matured at the rosette, which expands into the proamniotic lumen composed of polarized epithelial cells. Excessive cells located within the lumen may be removed by apoptosis. When Pten is absent, cell adherent junctions form at random cell-cell, and no concentrated localization of tight junctions are initiated to establish a dominant rosette. Thus, Pten is required for proamniotic cavitation of mouse embryos prior to gastrulation, by mechanism of epiblast cell polarization and rosette formation rather than apoptosis.
Chapter IV: Materials and Method

Generation of the Gata6 H2BGFP mice and Construction of the targeted allele

A targeting vector was constructed such that a 1.36kb fusion of histone 2B (H2B) and eGFP replaced exon 2 of gata6, specifically with the endogenous ‘ATG’ substituted by that of histone 2B. A gene conferring neomycin resistance (neo) followed the H2BGFP fusion enabling the selection of transfected R1 ES cells. PCR screening was performed to identify correctly targeted ES cells that had undergone homologous recombination. Chosen ES were then microinjected into C57BL/6 host blastocysts and chimeras selected. The neo gene, which was flanked by loxP sequences, was irreversibly excised by breeding chimeras with the Sox2Cre line. The resulting F1 mosaics were bred with C57BL/6 to establish the Gata6:H2BGFP strain lacking the neo gene and Sox2Cre. When synthesized, the fluorescent reporter protein was constitutively targeted to the chromatin of interphase nuclei or mitotic figures of dividing cells.

Generation of ES cells from mouse embryos

Preimplantation mouse embryos were flushed from uteri at 3.5 days post coitus. They were cultured individually in wells of a 24 well plate, containing gamma-irradiated fibroblasts, in ES cell media. Post attachment and growth the embryonic outgrowths were trypsinized and triturated then replated into a new tissue culture well. ES cell clones visible after 7-10 days were serially propagated, PCR genotyped and cryopreserved.
Mouse and ES cell PCR genotyping

Mice and ES cells were PCR genotyped using the following oligonucleotides: 5’ CCG GTG TGG AAC AGC TAT TTA 3’, 5’ ACC TCC CTA TCC CAC TTC GT 3’ and 5’ CTG ATC AAT TCC GTC TTC GC 3’. The wild type allele amplified at 195bp and Gata6:H2BGFP at 127bp.

Other ES cell lines

E-cadherin-null (9j) and RW4 (wildtype) mouse ES cell lines have been described previously (Moore et al., 2009). Pten-null ES cells were provided by Dr. Ramon Parsons (Puc et al., 2005).

ES cell Culture

All ES cell lines were maintained in a pluripotent state by culturing in ES cell medium supplemented with 1,000 U/ml of recombinant LIF (ESGRO, Chemicon International). The ES cell culture medium was DMEM with 15% (v/v) fetal bovine serum, 2 mM L-glutamine, 1x non-essential amino acids, 50 IU/ml penicillin, 50mg/ml streptomycin, and 0.1 mM β-mercaptoethanol. The ES cells were routinely cultured on gelatin-coated tissue culture grade plastic in a humidified incubator with 5% CO2. For ERK inhibition experiments, 1 uM ERK1/2 inhibitor PD0325901 (Sigma) or 50uM AZD6244 (Selleckchem) was added to the culture.
Generation and collection of embryoid bodies

ES cells were cultured in suspension in non-adhesive plates (coated with polyhama) to form cell aggregates (embryoid bodies). Typically, $6 \times 10^6$ cells in suspension following dissociation with trypsin and EDTA in 10 ml of ES cell medium lacking LIF were used to initiate the embryoid body cultures. The medium was replenished after 2 days by collecting the spheroids with a brief centrifugation. For histochemical analysis, cell aggregates were fixed in formalin, washed twice with PBS, mixed with 3% low melting point agarose, and processed for embedding in paraffin. The samples were subsequently subjected to sectioning, staining, and analysis.

Immunohistochemistry and immunofluorescence

For histochemical analysis, embryoid bodies and mouse uterus were fixed in formalin, washed twice with PBS, mixed with 3% low melting point agarose, and embedded in paraffin. The samples were subsequently subjected to sectioning, staining, and analysis. Embryos were fixed directly with formalin, paraffin embedded and sectioned at 5 μm thickness. Slides were deparaffinized in graded xylene series, dehydrated in ethanol, washed in water, and boiled in antigen retrieval solution (DakoCytomation). Primary antibodies used include: anti-E-cadherin (BD Transduction Lab, Inc., #610181), mouse anti-Dab2 (BD Transduction Labs, Inc., #610465), rabbit anti-Dab2 (in house rabbit polyclonal), anti-Pten (Cell Signaling, Inc., #9188), anti-Oct3/4 (Santa Cruz Biotechnology Inc., sc-1705279), anti-Nanog (Calbiochem, Inc., SC1000), anti-megalin (Santa Cruz Biotechnology,
Inc., sc-16478), anti-aPKC (PKCζ isoform) (Santa Cruz Biotechnology, In., sc-216), anti-ZO1 (Invitrogen, Inc., #61-7300), anti-Gata4 (Santa Cruz Biotechnology, Inc., sc-9053), and anti-activated/cleaved caspase 3 (Cell Signaling, Inc., #9661). Following incubation with primary antibodies, corresponding species-specific secondary antibodies were applied. Multiple secondary antibodies conjugated with the appropriate Alexa fluorochrome were used for simultaneous imaging of multiple antigens. DAPI (4’-6-diamidino-2- phenylindole) solution was used as nuclear counterstain and applied after the secondary antibody.

**ES cells Immunofluorescence**

Live ES cells were fixed with 4% PFA at room temperature for 20 minutes, following by incubation in 0.1% Triton CX-100 for 5 min. Standard immunostaining procedure was applied and the slides were mounted in Prolong Gold.

**Mouse Blastocyst in vitro culture and immunofluorescence staining**

E3.5 day blastocysts were flushed from uterine horns of female mice, collected into one 60-mm culture dish and incubated in 300ul of KMSO medium (EMD-Millipore) in a 37°C, 5% CO2 incubator. For immunofluorescence, the blastocysts were fixed in 4% paraformaldehyde plus 5% sucrose and 1% Triton X-100 for 1 h at room temperature, washed twice for 5 min in 100 µl PBS by transferring to fresh wells. Then the blastocysts were blocked overnight in 5% BSA at 4°C. The following day, blastocysts were incubated with the appropriate primary antibodies for 1 h at room temperature, washed, and blocked overnight in 2% BSA at 4°C. Following incubation with secondary antibodies for 1 h at room temperature, the blastocysts were washed twice, incubated for 30 min with DAPI, washed again, and finally transferred to and
stored at 4°C in PBS in a glass bottom culture dish (MatTek, Ashland, MA) until imaged. The primary antibodies and concentration were as follows: 1:300, rabbit anti-Nanog (Abcam, ab80892, Cambridge, MA) 1:300, rabbit polyclonal anti-Gata4 (Santa Cruz, sc9053); 1:1000, rabbit polyclonal anti-Gata6 (Capo-chichi et al., 2006); 1:300, mouse monoclonal anti-Oct3/4 (Santa Cruz, sc-5279); 1:1000, mouse monoclonal anti-Dab2 (BD, 610464). The secondary antibodies were Alexa fluor-conjugated (Alexa488, Alexa555, Alexa647) secondary antibodies (Molecular Probes, Thermo Fisher Scientific, Waltham, MA)

**Live Embryo Imaging and analysis**

For live imaging, embryos were cultured in glass-bottomed dishes (MatTek). KMSO medium were used for in vitro culture of the embryo and HEPE solution was added into the culture medium. Live image data were acquired using Zeiss Imager M2 LSM 700. Images were acquired 20 times objective. 20–30 xy planes separated by 17um were acquired per z stack, every 20 min. Movies of 3D time-lapse sequences were compiled and annotated using Volocity® 3D Image Analysis Software from Perkin Elmer (Thermo Fisher Scientific). We also used the Volocity software to quantitate GFP intensity and cell movement of the individual nuclei.

**CHIP-qPCR**

Chip assay was performed with the SimpleChIP® Enzymatic Chromatin IP Kit (Agarose Beads)(Cell signaling #9002). Chromatin was immunoprecipitated by incubating with GATA6 antibody (R&D systems AF1700) or GATA4 antibody (Santa Cruz Biotechnology, sc-9053), overnight at 4C. Mouse IgG antibody was used
as a negative control. For quantitative CHIP-qPCR, total input served as internal control. Primer sequence for gata6 gene promoter was purchased from QIAGEN (#GPM1033446(+01A)).

**Plasmid constructions**

The wild type mouse GATA6 plasmid was purchased from addgene (plasmid #51929). GATA6 sites-mutated plasmids were prepared by using Q5® Site-Directed Mutagenesis Kit (New England BioLab #E0554S). Site-directed mutants of mouse Gata6 plasmid, Gata6 (S264A) and Gata6 (S264D) were generated by overlap extension method. Primer sequences are listed as follows: Gata6 (S264A) Forward: 5’ CTTTCCTACGCAGGCAGCCGC 3’, Reverse: 5’ CGTGCAGGCGTCGTGGCC 3’.

Gata6 (S264D) Forward: 5’ CTTTCCTACGATCAGCCAGCCGC 3’, Reverse: 5’ CGTGCAGGCGTCGTGGCC 3’.

**ES cells Transfections**

ES cells were cultured in 6-well plates at 5 x 10^5 cells/well (or normal seeding density) and incubate overnight using ES cell media. The next day, transfection was performed with Lipofectamine 2000(LF2000) reagent. 4 μg/well DNA and 8-12 μl/well LF-2000 were each diluted in 400 μl/well Opti-MEM. Combined diluted DNA/LF-2000 were incubated for 20 min at room temperature and added into ES cells. After 4 hours, EB was added to feed the cells. After 48 hours, the cells were collected for further analysis. The GFP intensity of the ES cells were measured by ImageJ software.
Western blotting and antibodies

Primary mouse monoclonal antibodies used include: anti-Dab2 (BD Transduction Labs #610465), anti-Oct3/4 (Santa Cruz Biotechnology, sc-170 5279), anti-GATA4 (Santa Cruz Biotechnology, sc-9053), anti-GFP (abcam, ab32146), rabbit polyclonal anti-Gata6, anti-cleaved caspase 3 (Cell Signaling, Inc., #9661), anti-Pten (Cell Signaling, Inc., #9188), anti-b-actin (Sigma, Inc., #A5441), anti-PARP (Cell Signaling Inc., #9542), anti-phospho-Erk1/2 (Cell Signaling, Inc., #4370), anti-Phopho-mTOR (Cell Signaling, Inc., #2971), and anti-Phospho-PI3K (Cell Signaling, Inc., #4228). The above antibodies were also used for immunofluorescence microscopy and immunohistochemistry. The secondary antibodies include: horseradish peroxidase (HRP) conjugated goat or mouse anti-rabbit or anti-mouse (BioRad; Jackson Immunolab; Zymed). SuperSignal West Extended Duration SuperSignal West Extended Duration Substrate (PIERCE) was used for chemoluminescence detection of protein bands.

rtPCR Assay

mRNA was isolated from ES cells using RNA extraction kit (QIAGEN,#80204), following the manufacturer’s instruction. cDNA was synthesized using iScript™ cDNA Synthesis Kit (Bio-RAD 1708891). Power SYBR Green Supermix (Bio-Rad, 1725264) was utilized to perform qRT-PCR using the Bio-RAD CFX96 Real-Time system (Bio-RAD, Hercules, CA, USA). GAPDH gene transcript was measured as a normalizer to determine the other gene relative transcripts (DDCt). Primer sequences were listed in Table 1.
Table 1

Sequences of primers used for qRT-PCR.

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<th>Gene Name</th>
<th>Forward</th>
<th>Reverse</th>
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<td>OCT3/4</td>
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</table>

**Immunoprecipitation.**

ES cells were lysed in lysis buffer (20mM Tris, 250mM NaCl, 3mM EDTA, 2mM DTT, 0.5% CA-360) with protease inhibitor cocktail. IP was performed with Protein G Agarose Beads (Cell Signaling #9007) bound with anti-Gata6 antibody (R&D #AF1700) at 4°C with rotation overnight. The precipitates were dissolved in SDS buffer and boiled for 10 minutes and detected by immunoblotting with anti PYSP antibody (Cell Signaling) and rabbit polyclonal anti-Gata6.

**Pten mutant mice and genotyping**

In this manuscript, “Pten” refers to mouse or human gene; “pten” is for mouse gene; and “Pten (-)” indicates Pten-null determined by immunostaining rather than PCR genotyping. Founder pairs of the conditional ‘’floxed’’ Pten strain (Lesche et al.,
were purchased from Jackson Laboratory, Inc. The constitutive germline deletion lacking exon 5 (delta flox) was achieved by crossing with the Sox2-Cre strain (Hayashi et al., 2002). The mice maintained in the C57BL6/J background were housed and bred inside the barrier area of the mouse facility of the University of Miami Miller School of Medicine following NIH guideline, and the use of mice and experimental protocols for the current study was reviewed and approved by institutional IACUC committee. Genotyping by PCR followed the published protocol (Lesche et al., 2002). Briefly, the three primers used are: 5’ TCC CAG AGT TCA TAC CAG GA 3’; 5’ GCA ATG GCC AGT ACT AGT GAA C 3’; and 5’ AAT CTG TGC ATG AAG GGA AC 3’. The PCR products were 500 bp, 650 bp, and 300 bp for pten wildtype, flox, and delta flox respectively. Genomic DNA templates used for PCR were acquired from tail biopsy, pre-implanted blastocysts or outgrowths, later stage embryos (E6.5 or later), or fixed cells scraped from slides.
Reference


Ding, J., Ning, B., Gong, W., Wen, W., Wu, K., Liang, J., He, G., Huang, S., Sun, W., Han, T., et al. (2009). Cyclin D1 induction by benzo[a]pyrene-7,8-diol-9,10-epoxide via the phosphatidylinositol 3-kinase/Akt/MAPK- and p70s6k-dependent pathway promotes cell transformation and tumorigenesis. The Journal of biological chemistry 284, 33311-33319.


