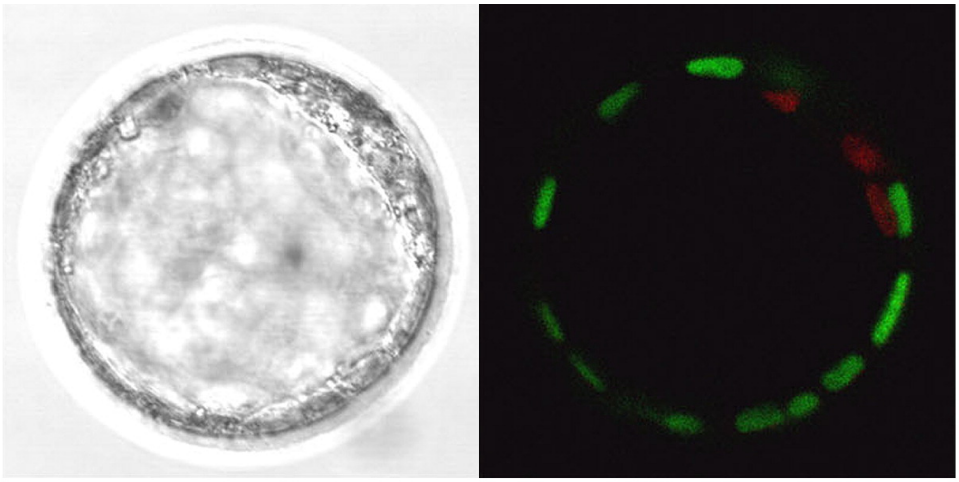
The first cell fate specification event in mouse development



Carine Stapel
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About the cover

The cover shows two images of a mouse blastocyst. On the left a phase contrast image. On the right a merged confocal microscopy image that shows the first two cell types that are observed in the mammalian blastocyst, inner cell mass (ICM) and trophectoderm (TE). ICM is identified by the ICM marker Oct-4 (red). TE is identified by the TE marker Cdx-2 (green). Taken from (Niwa *et al.*, 2005).

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Abstract

The separation of inner cell mass and trophectoderm is the first cell fate specification event in mammals. The inner cell mass will form the embryo proper and contributes to extraembryonic tissues, whereas the trophectoderm will develop into the placenta to support embryo development. Two models have been proposed to describe the mechanisms behind the differentiation of these two cell types. The cell polarity model states that cell polarization followed by asymmetric cell division lies on the basis of this process. According to the inside-outside model, cell position is the ultimate determinant of cell fate. A combination of both models seems to best explain the existing data. Early mouse development displays a high degree of developmental plasticity. A debated issue in the field is whether the differentiation between inner cell mass and trophectoderm additionally contains a component of prepatterning. In this thesis I will describe the support that exists for the different models. To illustrate the mechanisms of cell fate specification I will also describe the role of the trophectoderm specific transcription factor Cdx-2 in this process.

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1. Mouse embryonic development

Mammalian development is unique in that the embryo develops inside the uterus of the mother. In order to obtain the nutrients it needs for development, the embryo will establish a connection with its mother through the placenta. The first differentiation step in mammalian development is geared towards this process. It separates the inner cell mass (ICM), which will form the embryo proper, from the trophectoderm (TE), which is required for placenta formation. In my thesis I will give an overview of the current understandings of this first cell fate specification event in mouse as a model for mammalian development.

The fertilized egg

Mouse embryonic development starts with fertilization of the oocyte. Like for all mammals, mouse oocytes develop in the female ovaries (Figure 1). The oocytes are arrested in prophase of Meiosis I (Wolpert *et al.*). But under the influence of gonadotropins several eggs will proceed through meiosis until the second metaphase of Meiosis II (Wolpert *et al.*). At this stage, the oocyte will leave the ovary during ovulation and starts its journey through the oviduct, where it may be fertilized by a sperm cell (Figure 1).

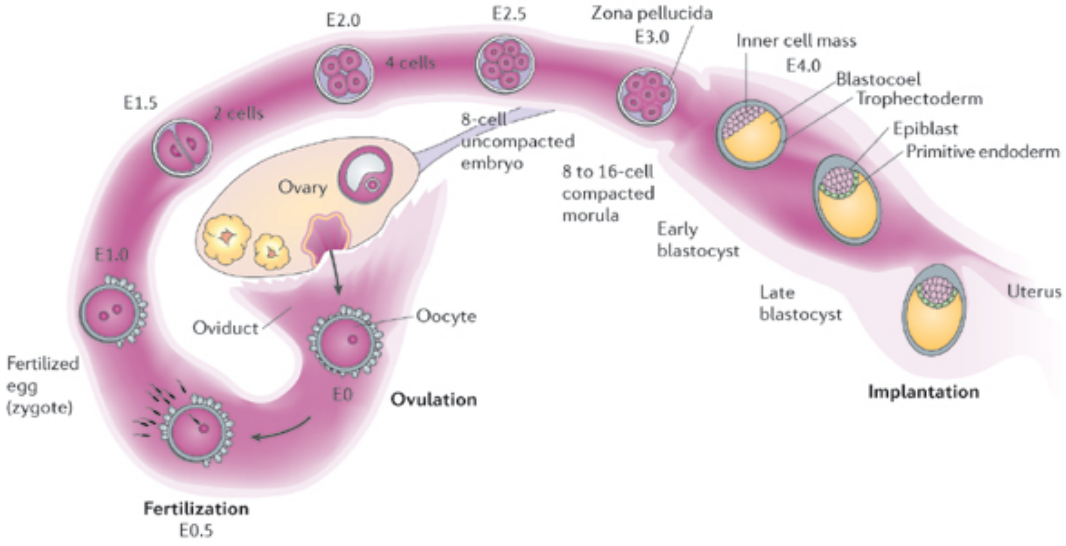

Figure 1. Overview of mouse embryonic development from fertilization to implantation.
After ovulation, the mouse oocyte travels through the oviduct. Embryonic development starts when the oocyte is fertilized by a sperm cell. The male and female genomes merge and the zygote initiates cell division. The E2.5 embryo consists of 8-cells. Soon after interactions between the blastomeres increase and the embryo undergoes compaction giving it a more smooth outside appearance. Fluid is pumped into the embryo to form a blastocyst with blastocyst cavity (blastocoel). In the blastocyst, a clear separation between inner cell mass and trophectoderm is observed. At E4.5 the epiblast and primitive endoderm can be distinguished within the inner cell mass. Soon after, the blastocyst will implant into the uterus where it will continue development. Adapted from (Wang *et al.*, 2006).

At ovulation, the oocyte still has a diploid genome. Upon fertilization, the oocyte nucleus will finish Meiosis II and form the second polar body to extrude its excessive DNA and create the haploid female pronucleus (Wolpert *et al.*). In the mean time, the sperm nuclear envelope is broken down, sperm chromatin decondenses and protamines, which tightly packaged the sperm DNA, are replaced by histone proteins (Li *et al.*, 2010; Wolpert *et al.*). This results in looser packaging of the male DNA. After these events, the male and female pronuclei migrate towards each other while replicating their DNA. When the pronuclei meet, the chromatin condenses and the chromosomes organize in a mitotic spindle (Wolpert *et al.*). The cell will divide and it is only after this, at the two-cell stage, that the male and female DNA truly mix.

Maternal contribution

The oocyte is packed with maternal proteins and RNAs that direct its maturation and the first steps of embryonic development. This is a common phenomenon, observed in many species (Li *et al.*, 2010; Schier, 2007). In the mouse embryo, maternal products regulate processing of the male genome, activation of the zygotic genome and their own degradation (Li *et al.*, 2010).

Maternal to zygotic transition

A very important role of maternal products is to regulate zygotic genome activation. In mouse, the first zygotic transcripts can already be observed at the 1-cell stage. These transcripts result from leaky transcription of the male nucleus (Li *et al.*, 2010; Schultz, 2002). This process is called minor zygotic genome activation (ZGA). Two features that can explain minor ZGA are protamine to histone exchange and the presence of specific chromatin modifications (Li *et al.*, 2010; Schultz, 2002). During the replacement of protamines with histone octamers, the DNA is more loosely packed and accessible to transcription factors. Once the histone octamers are in place, the chromatin of the male nucleus is hyperacetylated and hypomethylated, two features that allow for higher accessibility to transcription factors and transcriptional activation (Hamatani *et al.*, 2004; Li *et al.*, 2010; Schultz, 2002). Interestingly, it appears that these early mRNAs are not translated until full ZGA (Hamatani *et al.*, 2004; Schultz, 2002). Thus, minor ZGA seems to be a side effect of chromatin remodeling rather than an essential regulatory step in mouse development.

At the late 2-cell stage, the zygotic genome will be fully activated during major ZGA. Little is known about what initiates ZGA (Li *et al.*, 2010; Schier, 2007), but the process is associated with extensive chromatin remodeling (Schultz, 2002). In fact, several chromatin-modifying enzymes are maternally provided (Li *et al.*, 2010). The importance of chromatin remodeling for ZGA is further supported by the fact that hyperacetylation of the paternal genome coincides with early transcription and that experimentally induced hyperacetylation of the female genome at the 1-cell stage has the same effect (Schultz, 2002). The first wave of transcription at the 2-cell stage produces transcripts that are mainly involved in basic cellular processes (Hamatani *et al.*, 2004; Wang *et al.*, 2004). A second, smaller wave of transcription is observed at the 4-cell stage and

provides transcripts to support specific developmental processes from the 8-cell stage onwards (Hamatani *et al.*, 2004; Wang *et al.*, 2004).

Degradation of maternal products

Maternal products in mice only need to function very transiently and are degraded early on during development (Li *et al.*, 2010). By the 2-cell stage, over 90% of maternal RNAs has been degraded (Hamatani *et al.*, 2004; Schultz, 2002). At this stage the zygotic genome is still largely inactive. Maternal products must therefore play a major role in this process. Downregulation of maternal RNAs is regulated by both poly-A tail deadenylation (Bachvarova *et al.*, 1985; Bachvarova, 1992; Schier, 2007) and miRNA mediated degradation (Giraldez *et al.*, 2006; Lykke-Andersen *et al.*, 2008). Maternally provided proteins will also be degraded (Li *et al.*, 2010).

Compaction and blastocyst formation

In most organisms, the first embryonic cell divisions are synchronized. Mammals are unique in that early cleavages often occur asynchronous (O'Farrell *et al.*, 2004; Wolpert *et al.*). The embryo will keep dividing without obvious morphological changes until the 8-cell stage. At the 8-cell stage the outer appearance of the embryo changes in a process called compaction (Levy *et al.*, 1986). During compaction, cell-cell contacts are enforced and contact surfaces are maximized, creating a ball of cells with a smooth appearance (Figure 1).

The key protein in compaction regulation is E-cadherin, encoded by the cadherin 1 (*Cdh1*) gene (Larue *et al.*, 1994). E-cadherin is maternally provided and is initially equally distributed over the cytoplasm. At the 8-cell stage its localization becomes confined to the basolateral cell membrane and the protein is activated by posttranslational phosphorylation, inducing adherens junction establishment (Eckert *et al.*, 2008; Larue *et al.*, 1994). Interestingly, compaction still occurs if translation of the zygotic genome is blocked after the 2-cell stage (Levy *et al.*, 1986). This underscores the importance of posttranslational modifications in this process.

The formation of adherens junctions is essential for compaction and also for subsequent tight junction formation (Eckert *et al.*, 2008; Kan *et al.*, 2007; Larue *et al.*, 1994). Tight junction formation is initiated after compaction and is finished by the 32-cell stage (Eckert *et al.*, 2008). At this point a waterproof seal is formed between the outer cells of the embryo. This is essential for the next step of development, blastocyst formation (Figure 1).

Blastocyst formation starts at the 32-cell stage when a fluid-filled cavity, the blastocyst cavity, is formed inside the embryo. Cavity formation is a result of water transport into the embryo driven by an increased osmotic pressure of sodium ions (Kawagishi *et al.*, 2004; Madan *et al.*, 2007). It involves transport of sodium ions to the inside of the embryo. Transport is driven by a Na+/K+-ATPase that becomes localized basally just before the 32-cell stage (Madan *et al.*, 2007). The Na+ pool in the cell is replenished by an apical Na+/H+ exchanger (Kawagishi *et al.*, 2004).

Blastocyst cavity formation is a gradual process and starts with the formation of multiple small cavities (Marikawa *et al.*, 2009; Motosugi *et al.*, 2005). The cavities will

grow and fuse together to form the mature blastocyst cavity. With blastocyst formation, the first axis of the embryo, the embryonic-abembryonic (Em-Ab) axis, will also become apparent. Due to space constraints imposed by the zona pellucida (ZP), a glycoprotein shell that surrounds the embryo, the blastocyst cavity will end up on one side of the embryo, pushing the inside cells to the other side (Motosugi *et al.*, 2005). This cluster of inside cells constitutes the inner cell mass (ICM). The single-cell layer surrounding blastocyst cavity and ICM is called the trophectoderm (TE).

Allocation of ICM and TE

ICM and TE represent the first cell lineages that are established during mouse embryonic development. The ICM consists of a cluster of apolar cells that will develop into the embryo proper and contribute to extraembryonic tissues. The TE consists of epithelium-like polar cells that will solely form extraembryonic tissues. However, TE is essential for the development of the embryo. It is required for implantation and placenta formation and contributes to patterning of the ICM through secretion of Fgf2 (Arnold *et al.*, 2009; Wu *et al.*, 2011). Here I will describe the mechanisms behind the differentiation between ICM and TE.

Two models exist to explain how ICM and TE become separated, the cell polarity model (Johnson *et al.*, 1981) and the inside-outside model (Tarkowski *et al.*, 1967) (reviewed in Yamanaka *et al.*, 2006). The cell polarity model states that asymmetric cell division at the 8-cell stage is responsible for the generation of one polarized and one apolar cell, each with different developmental capacities (Figure 2). The inside-outside model states that cells adopt different fates according to their position due to positional signaling and differences in their environment (Figure 2). A combination of both models can probably best explain the currently existing data.

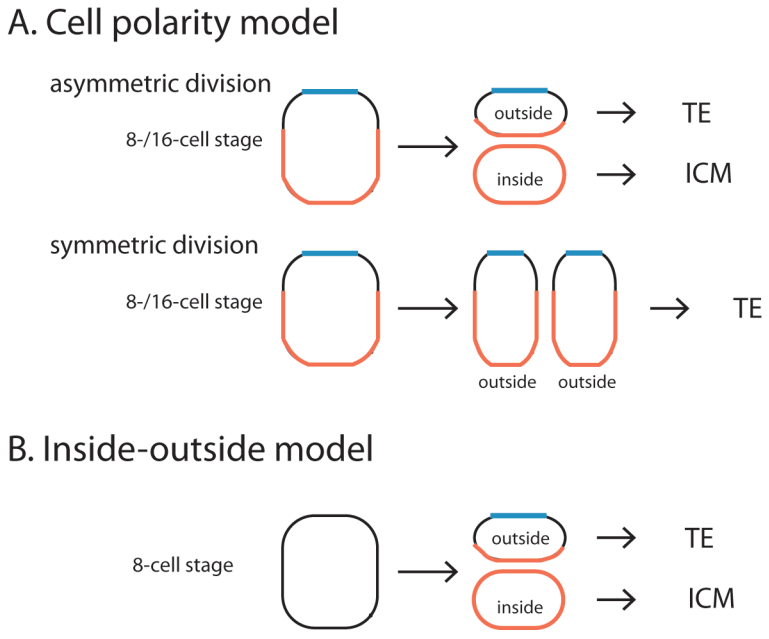

Figure 2. Models for the specification of ICM and TE. The polarity model states that asymmetric distribution of specific factors (blue and orange), followed by asymmetric cell division is responsible for the separation of TE and ICM precursors. Cells that inherit the polarized domain will adopt a TE fate. According to the inside-outside model, cell position is ultimately responsible for cell fate specification. Inside cells will acquire an ICM fate whereas outside cells will form the TE.

Cell polarity model

Until the 8-cell stage, cells of the embryo show no differences on the morphological level. By the late 8-cell stage the embryo undergoes compaction. During this process blastomeres start to polarize and obtain an epithelial-like appearance (Ducibella *et al.*, 1977; Johnson *et al.*, 1986a; Vinot *et al.*, 2005). Cells flatten and microvilli that used to be equally distributed over the cell membrane of blastomeres become localized to the apical cell membrane. Inside the cell, cytoplasmic organelles and microtubules adopt an asymmetric localization (Ducibella *et al.*, 1977; Johnson *et al.*, 1986a). Additionally, asymmetric localization is observed at the protein level. For example, PAR proteins, which are implicated in asymmetric cell division in many organisms, have also been shown to become asymmetrically localized in mouse embryos at the 8-cell stage (Plusa *et al.*, 2005; Vinot *et al.*, 2005).

A common mechanism to create two different daughter cells is by asymmetric cell division. Indeed some of the 8-cell blastomeres undergo asymmetric division (Figure 2). Johnson and Ziomek first described this phenomenon in 1981 (Johnson *et al.*, 1981). They observed that single (polarized) blastomeres, isolated from the 8-cell stage and 16-cell stage embryo, often give rise to one polarized and one apolar daughter cell due to differential inheritance of the apical domain. They suggested that this process lies at the basis of ICM and TE specification, with apolar cells giving rise to ICM, while polar cells continue to form TE. Indeed apolar cells usually end up on the inside of the embryo, which will form the ICM. Their stable inside localization seems to be a result of their preferential interaction with other cells, due to the lack of an apical domain (Dard *et al.*, 2009). According to the same mechanism, polar cells preferentially localize with their polar domain away from other cells on the outside of the embryo (Dard *et al.*, 2009). Interestingly, cell polarization also occurs in single blastomeres that are isolated before compaction (Houliston *et al.*, 1989; Johnson *et al.*, 1986b). In this case orientation of the polarization domain is independent of cell-cell contact. These blastomeres give rise to one polar and one apolar cell (Takashi Hiiragi, personal communication). This shows that polarization is hard-wired in the blastomeres.

The question remains whether fate of these cells is established once asymmetric division has occurred or whether their fate depends on their position. The second option is further explored in the inside-outside model.

Inside-outside model

According to the inside-outside model, the position of a blastomere determines its fate (Figure 2). Strong support for the inside-outside model comes from the high developmental plasticity that is observed in the early mouse embryo. Transplantation of outside cells from morula (16-cell) stage embryos, to an 8-cell embryo frequently results in their contribution to both ICM and TE instead of TE alone (Rossant *et al.*, 1980). This result does not come completely unexpected, since blastomeres of the unmanipulated morula will also contribute to the ICM through asymmetric divisions. However, the reverse experiment yields a similar result. Aggregation of early blastocyst stage ICMs with morulas results in chimeras that show contribution of the ICM donor-cells to the trophectoderm (Rossant *et al.*, 1979).

In both cases the change in cell fate of transplanted cells does not depend on the less-differentiated blastomeres that they were combined with. Pure aggregates of multiple ICMs or outside cells resulted in the formation of embryonic structures consisting of both ICM and TE derived tissues (Rossant *et al.*, 1979; Rossant *et al.*, 1980; Spindle, 1978). Aggregates of outside cells reconstitute blastocysts with well-defined ICMs within 24 hours (Rossant *et al.*, 1980). Likewise, the outside cells of ICMs that were isolated before the late blastocyst stage start to show features of polarization shortly after isolation and have the potential to form blastocyst structures within 24 hours, both in case of *in vitro* (Spindle, 1978) and *in utero* development of the aggregates (Rossant *et al.*, 1979). The fact that cell divisions are not required for these processes to occur confirms that TE and ICM reconstruction are not the result of incomplete elimination of the respective cell types (Rossant *et al.*, 1979; Spindle, 1978).

In conclusion, although polarity is important to make cells different from each other and constitutes the first identifiable difference between inside and outside cells, the transplantation experiments described above show that development remains flexible after polarization. In the end the position of the blastomere will determine its fate.

Post-implantation development

Complete separation between ICM and TE is only established in the blastocyst after the 32-cell stage. At this stage, transplanted cells will solely contribute to the tissue from which they originated (Grabarek *et al.*, 2012; Rossant *et al.*, 1979; Rossant *et al.*, 1980). While the blastocyst matures, two cell types are specified in the ICM, the epiblast (EPI) and the primitive endoderm (PE) (Chazaud *et al.*, 2006; Morris *et al.*, 2010; Plusa *et al.*, 2008). A clear separation between these cell types is observed in the late blastocyst at E4.5 (Figure 3). However, EPI and PE precursors can already be identified before this stage on the basis of specific markers like Nanog (EPI) and GATA6 (PE) (Chazaud *et al.*, 2006; Plusa *et al.*, 2008). Sorting of these two precursors to their final position is established through directional movement during cell division and cavitation, position dependent regulation of gene expression, and apoptosis (Chazaud *et al.*, 2006; Plusa *et al.*, 2008). In the late blastocyst, PE is located adjacent to the blastocyst cavity, while EPI is enclosed by TE and PE (Arnold *et al.*, 2009). PE forms the extraembryonic endoderm and will as such contribute to support embryonic development. EPI is on its own responsible for the formation of the embryo proper and will generate all three germ layers (Arnold *et al.*, 2009).

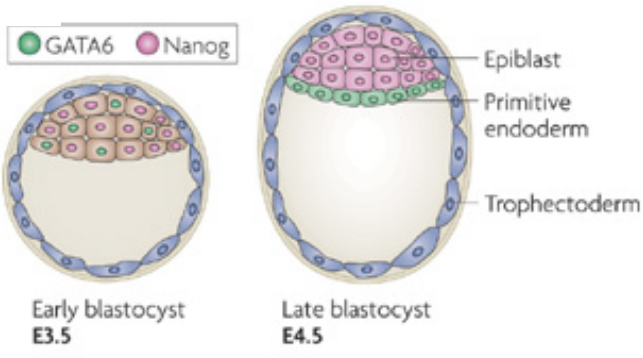

Figure 3. Specification of epiblast and primitive endoderm. From the early blastocyst stage onwards, epiblast precursors can be identified by the expression of Nanog, whereas primitive endoderm precursors uniquely express GATA6. Only at the late blastocyst stage do the two cell types adopt their final positions within the ICM through cell sorting and apoptosis. Adapted from (Arnold *et al.*, 2009).

At E4.5 the blastocyst has reached the uterus and will implant into the uterine wall to ensure its access to nutrients for future development (Figure 1) (Wang *et al.*, 2006). At around E6 the mouse embryo will start gastrulation to form the three germ layers, ectoderm, mesoderm and embryonic endoderm (Arnold *et al.*, 2009). During this process the embryo adopts a cup-like shape and is appropriately named the embryonic cup (Arnold *et al.*, 2009). The germ layers will continue their differentiation process to form all cells present in the mature mouse. After 18-22 days post fertilization, the mouse embryo is born and continues its growth outside of the uterus (Wolpert *et al.*).

2. Patterning in the early mouse embryo

In many organisms, the first cell fate specification events and establishment of the embryonic axes is prepatterned and depends on maternal factors. In *C. elegans* for example asymmetric domains of PAR proteins are established upon fertilization (Munro *et al.*, 2004). From this moment on, all cell division patterns and cell fate specification events are set. In *Drosophila*, prepatterning occurs even earlier, in the oocyte. Maternal nurse cells produce proteins and RNAs that are transported to the oocyte and are distributed asymmetrically (Bastock *et al.*, 2008). Again, PAR proteins play an important role in the establishment of asymmetry. Another well-known example of prepatterned development is dorsalization in for example *Xenopus* and zebrafish development. In these species, maternal factors specify the dorsal area of the embryo and thus the dorso-ventral axis (Schier *et al.*, 2005; White *et al.*, 2008; Wolpert *et al.*). In all these organisms, disruption of cell division axes or knock down or reallocation of maternally provided patterning factors severely affects the scheme of development. The mouse embryo is different in that respect. Its high degree of developmental plasticity is demonstrated by the transplantation experiments described in Chapter 1 (Rossant *et al.*, 1979; Rossant *et al.*, 1980; Spindle, 1978). This does however not exclude the possibility that the scheme of development may be biased by asymmetries that exist in the zygote. These factors could for example guide asymmetric versus symmetric division of blastomeres from the 8-cell stage onwards so as to bias a cell's contribution to the ICM. In this Chapter, I will explore the respective importance of prepatterning and regulative development in mouse embryonic development.

Prepatterning

A potential role for prepatterning in the mouse embryo would have to fulfill three requirements (Johnson *et al.*, 1986a): i. reproducible asymmetry of proteins and/or RNAs in the mouse zygote, ii. differential distribution of asymmetrically localized components over the daughter cells by the means of regulation of division planes iii. asymmetrically localized components affect cell fate. In this chapter I will describe the arguments in favor and against the fulfillment of the first two of these criteria in mouse development. The third criterion will be discussed in Chapter III from the perspective of the TE specific transcription factor Cdx-2.

Orientation of the first cell division

In order for maternally provided factors to be asymmetrically distributed over the blastomeres, the first cleavage divisions need to occur in a regulated manner. Whether or not this is the case is a strongly debated issue in the field (Gardner, 2001; Hiiragi *et al.*, 2004; Hiiragi *et al.*, 2006a; Hiiragi *et al.*, 2006a; Hiiragi *et al.*, 2006b; Plusa *et al.*, 2002; Vogel, 2005).

The timing of ICM and TE specification is closely related to the occurrence of the first embryonic axis, the embryonic – abembryonic (Em-Ab) axis. The Em-Ab axis is defined by the location of the ICM on the embryonic side and the blastocyst cavity on the abembryonic side (Figure 4B). In 2001 Richard Gardner described a correlation between the first cleavage plane and the Em-Ab axis (Gardner, 2001). In his experiments he marked the cleavage plane with small oil drops in the zona pellucida (ZP) at the 2-cell stage. Assuming that the embryo does not rotate within the ZP during its development to the blastocyst stage, he observed a correlation between the first cleavage plane and the Em-Ab axis. In the majority of embryos, the first cleavage was oriented orthogonally in relation to the Em-Ab axis (Figure 4). The same result was observed when 2-cell embryos were embedded in an alginate gel to restrict their movements.

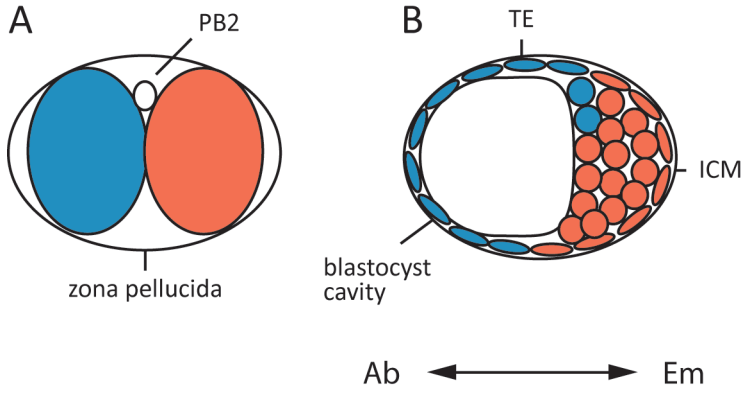

Figure 4. Correlation between the first cleavage division and the embryonic-abembryonic axis.
A. If the zona pellucida has an oval shape, the 2-cell embryo aligns along the long axis. **B.** In the absence of embryo rotation, an orthogonal relationship exists between the first cleavage plane and the embryonic-abembryonic (Em-Ab) axis.

One year later the Zernicka-Goetz lab published that the first cleavage preferably passes through or close to the second polar body (PB2) (Plusa *et al.*, 2002). PB2 was defined as the marker for an animal pole of the zygote. This implies that the Em-Ab axis is orthogonal to a presumptive animal-vegetal (AV) axis. From transplantation experiments they concluded that the animal pole contains factors that regulate the orientation of the first cleavage division. However, in 2004 Hiiragi and Solter showed that the observed correlation between the first cleavage plane and PB2 was most likely not a result of regulated division (Hiiragi *et al.*, 2004). In contrast they observed that PB2 often moves towards the cleavage plane. This is in agreement with the observation of extensive cortical and cytoplasmic flows towards the cleavage plane during the first division (reviewed in Johnson, 2009). Furthermore, an earlier study by Zernicka-Goetz had shown that removal of either the presumptive animal or vegetal pole of the zygote, as marked by PB2, does not affect development (Zernicka-Goetz, 1998). Together, these results argue against a role for PB2 as a marker for the animal pole and invalidate the existence of an AV axis in the zygote. Notably, Hiiragi and Solter

observed a correlation between the first cleavage plane and the position of the pronuclei just before cleavage division (Hiiragi *et al.*, 2004).
One year later, Plusa et al. disputed their own finding by showing that an orthogonal relation between the first cleavage plane and the Em-Ab axis still exists when the orientation of the first cleavage is experimentally manipulated (Plusa *et al.*, 2005). This was achieved by changing the shape of the zygote, which aligns the cleavage furrow with the short axis of the zygote. Three later studies shed more light on the mechanism behind this process. They showed that a correlation exists between the shape of the ZP and the orientation of the first two cells after cell division on the one hand and the blastocyst axis on the other hand (Alarcon *et al.*, 2008; Kurotaki *et al.*, 2007; Motosugi *et al.*, 2005). In many embryos, the first two blastomeres enforce an ellipsoid shape on the ZP due to space constraints. The blastomeres position themselves along the long axis of the ZP. At the onset of cavitation, the embryo has not increased in size. The early embryo only undergoes cleavage divisions in the absence of cell growth. Therefore the ZP retains its oval shape (Alarcon *et al.*, 2008). It is only logical to assume that the blastocyst cavity will form on one side along the long axis where the most space is available and not along the short axis. This is supported by computer simulations of blastocysts formation (Honda *et al.*, 2008).
In summary, a likely explanation for a correlation between the orientation of the first cleavage plane and the Em-Ab axis exists of the following steps, driven by space constraints: i. orientation of the 2-cell blastomeres along the long axis of the ZP, ii. fixed ZP shape, iii. blastocyst cavity formation along the long axis of the ZP. Further support for this model comes from the fact that the apparent correlation between the first cleavage orientation and the Em-Ab axis seems to be lost in embryos that have a ZP in which no obvious long axis exists (Alarcon *et al.*, 2008; Honda *et al.*, 2008). Similarly, removal of the ZP at the 2-cell stage abolishes the apparent correlation between the first cleavage plane and the Em-Ab axis (Kurotaki *et al.*, 2007). The first cleavage division does therefore not seem to comply with the prepatterning requirement of a regulated division plane.

Fate of the 2-cell blastomeres

In embryos with an oval ZP shape, one would expect the 2-cell blastomeres to predominantly contribute to either the embryonic or the abembryonic side of the blastocyst if no cell mixing occurs before blastocyst formation. Alternatively, it is conceivable that the blastomeres mix during development without affecting the orientation of the blastocyst cavity due to the stability of ZP shape.
Two studies showed expansion of the 2-cell blastomeres with limited cell mixing (Piotrowska *et al.*, 2001; Plusa *et al.*, 2005). By labeling the membrane of the 2-cell blastomeres with different dyes, they showed that one blastomere predominantly contributes to the embryonic part of the embryo while the other contributes to the abembryonic part in more than 70% of embryos (Figure 5). This correlation was irrespective of whether the axis of cell division was natural (Piotrowska *et al.*, 2001) or experimentally induced (Plusa *et al.*, 2005). Together these results suggest expansion of the 2-cell blastomeres in the absence of extensive cell mixing.

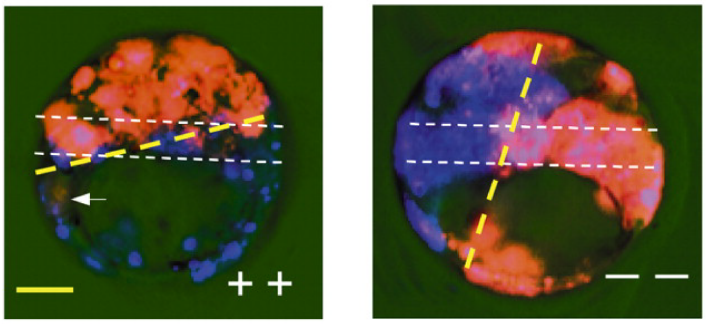

Figure 5. Correlation between the first cleavage division and the Em-Ab axis exists *in vivo*.
Blastomeres were labeled with DiI (red) and DiD (blue) at the 2-cell stage. The distribution of blastomere progeny was analyzed in the blastocyst stage. ++, strong axis correlation; --, no axis correlation. Both examples have been observed in several studies. However, different studies find different fractions of embryos in each class. Yellow dashed line, separation between the two cell clones; white dashed lines, boundary zone. Adapted from (Piotrowska *et al.*, 2001).

However, two other labs were not able to replicate these results. Alarcon and Marikawa did observe minimal cell mixing of the 2-cell blastomeres, but did not observe a correlation between the first cleavage plane and the Em-Ab poles in over 70% of embryos (Alarcon *et al.*, 2003; Alarcon *et al.*, 2005). This seems to be a result of rotation of the embryo within the ZP. Alternatively the difference may stem from variances in ZP shape between the embryos. If the embryos developed within a round ZP instead of an oval ZP this may well explain the random orientation of the blastocyst cavity (Alarcon *et al.*, 2005; Alarcon *et al.*, 2008). Interestingly, Chroscicka et al. did also not observe exclusive contribution of the first two blastomeres to either the embryonic or the abembryonic pole of the embryo, but for a different reason (Chroscicka *et al.*, 2004). In their study they showed that cell mixing occurs from the 8-cell stage onwards in about 30% of the embryos. Cell mixing will disrupt any correlation between the 2-cell blastomeres and the later embryonic and abembryonic cell populations. ZP shape and the extend of cell mixing may differ between mouse strains, explaining the differences in results obtained by different labs.

A later study gathered additional support for the hypothesis that progeny of the first two blastomeres occupies either the embryonic or the abembryonic pole (Piotrowska-Nitsche *et al.*, 2005b). A study of cleavage plane orientation in 2-cell blastomeres argued that these divisions often occur in a predictable pattern (Gardner, 2002). One cell would divide in parallel to the first cell division (meridionally, M) whereas the other would have a division plane perpendicular to that of the first cleavage division (equatorially, E) (Figure 6) (Gardner, 2002). Besides orientation, the first two divisions also differ in timing. It was observed that when the first blastomere divides meridionally and the second blastomere divides equatorially (ME division) (50% of embryos), the first dividing blastomere will contribute to the embryonic pole while the other blastomere will contribute to the abembryonic pole in about 80% of embryos (Piotrowska-Nitsche *et al.*, 2005b). This result suggests that the correlation between the first cleavage axis and the blastocyst cavity is not just determined by the ZP shape. Otherwise the blastocyst cavity would stochastically be located at the site of the first dividing cell in 50% of the embryos. However, others could not replicate these results (Alarcon *et al.*, 2008) and even showed that the first two cleavage divisions do not occur in a regulated orientation

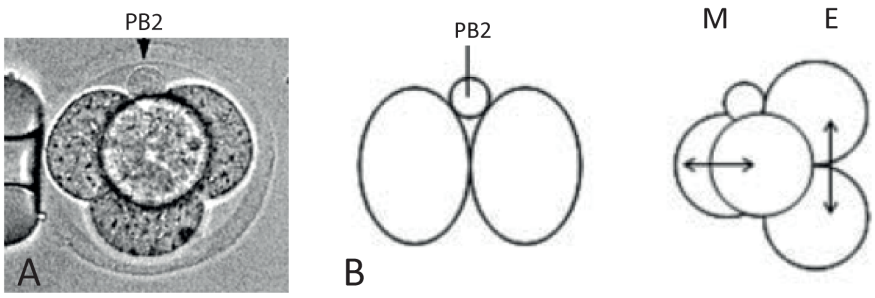

Figure 6. Division pattern of the first two blastomeres.
A. The blastomeres of most mouse embryos adopt a tetrahedral conformation at the 4-cell stage. **B.** The division pattern of the first two blastomeres has been categorized in meridional (M) and equatorial (E) in relation to the Em-Ab axis as specified by the second polar body (PB2). Adapted from Gardner (A) Alarcon & Marikawa 2008 (B).

but that the 4-cell blastomeres rotate after division due to the ZP shape (Louvet-Vallee *et al.*, 2005).
It is at this point difficult to conclude whether developmental bias exists between the first two blastomeres on the basis of the timing and plane of their division. Close comparison of experimental procedures might help solve this issue. However, it is clear that this model would not apply to all embryos. Furthermore different cleavage patterns do not seem to convey any developmental disadvantage (Piotrowska-Nitsche *et al.*, 2005b).

The second cell division and cell fate specification
Although a potential correlation between the first cell division and the Em-Ab axis would be an interesting phenomenon, it is not linked to the first cell fate specification between ICM and TE. The embryonic pole encompasses both ICM derived epiblast and TE derived polar trophectoderm. The abembryonic pole on the other hand consists of both TE derived mural trophectoderm and ICM derived primitive endoderm (Gardner, 2001). If potential maternal patterning factors are not distributed unequally by the first cell division, the second divisions may result in asymmetric distribution of these factors over the daughter cells (Figure 7). In that case 4-cell blastomeres may be subjected to developmental bias.
In 2003 Fujimori et al. stably labeled single blastomeres and their progeny from either the 2-cell or the 4-cell stage onwards by Cre-recombinase induced activation of transgenic β-galactosidase (Fujimori *et al.*, 2003). When they analyzed the progeny of labeled cells at E8.5, they did not observe dedicated contribution of 2-cell blastomeres to either TE or ICM. This finding is in agreement with the results in blastocysts as described in the previous paragraphs. However, looking at the progeny of $^{1}/_{4}$ blastomeres provided interesting additional information. 16 of 54 labeled 4-cell stage blastomeres exclusively contributed to TE derived tissues, whereas 4 out of 54 blastomeres contributed to ICM alone (Fujimori *et al.*, 2003). Exclusive contribution of a cell to TE can be achieved if an outside cell solely undergoes symmetric cell divisions. A pure ICM fate on the other hand can only result from asymmetric division followed by migration of the outer blastomere to the inside of the embryo. This latter process is surprising and expected to be much more rare, which explains the higher occurrence of TE-only blastomeres. The question remains whether this observation is based on chance or on biased development.

More support for exclusive contribution of one of the 4-cell blastomeres to TE comes from the experiments that displayed different developmental potentials of the 2-cell blastomeres based on orientation of the second cleavage division (Piotrowska-Nitsche *et al.*, 2005a; Piotrowska-Nitsche *et al.*, 2005b). Follow-up experiments from the same lab showed that, in case of an ME division pattern of the first two blastomeres, the two blastomeres from the second, equatorial division populate specific regions of the abembryonic pole. More precisely, close to 90% of cells derived from the vegetal-most blastomere (based on position of PB2) (Figure 7) were found in TE derived tissues (Piotrowska-Nitsche *et al.*, 2005a). Additionally, aggregates made of four vegetal-most E blastomeres had limited developmental potential. None of 22 aggregates developed to term (Piotrowska-Nitsche *et al.*, 2005a). Decreased developmental potential was not observed for homogeneous aggregates of M-division derived blastomeres (Piotrowska-Nitsche *et al.*, 2005a). These results could explain why dedicated contribution of a single 4-cell blastomere to either TE or ICM is only observed in a fraction of embryos (those with an ME division pattern) and only for some of the blastomeres (so far only demonstrated for the vegetal-most E blastomere). However, the results are controversial. Others disputed the use of PB2 as a marker for the animal pole (Hiiragi *et al.*, 2004), observed random orientation of the second division planes (Louvet-Vallee *et al.*, 2005) or did simply not observe similar bias in developmental potential when repeating the experiments (Alarcon *et al.*, 2008). In addition, the fact that a correlation between cell division orientation and cell fate was never observed in all embryos makes it unlikely that the first cell divisions are strictly regulated.

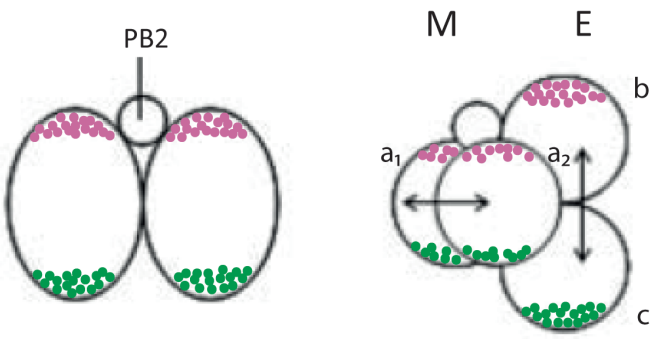

Figure 7. Asymmetric division of patterning factors at the 4-cell stage. Both 2-cell blastomeres contribute to ICM as well as TE. If patterning factors are asymmetrically distributed over the 2-cell blastomeres, a second, asymmetric division may create two unequal cells (b and c). According to a study by Piotrowska-Nitsche et al. (2005), the vegetal most blastomere (c), uniquely contributes to TE derived tissues in embryos where a meridional (M) division is followed by an equatorial (E) division. However, other labs have disputed this finding. Adapted from Alarcon & Marikawa 2008.

Candidate factors for prepatterned development

Prepatterning requires asymmetric distribution of maternally provided factors. The most efficient prepatterning factors would be transcription regulators of developmental pathways. Few copies of these factors would need to be segregated asymmetrically in order to produce a strong effect on cell fate. Considering a small number of molecules this may occur stochastically just as well as in a regulated manner. Conveniently, zygotic transcription is not activated until the late 2-cell stage (Hamatani *et al.*, 2004; Schultz, 2002). Gross transcriptional changes only occur after the second cell division (Wang *et*

al., 2004). This means that asymmetric segregation of maternal transcription master regulators would need to be established by the 4-cell stage latest.
So far, only few maternally provided factors have been described in mice (Li *et al.*, 2010). Most of these factors are required for general early development and are unlikely to function in either TE or ICM specification (Li *et al.*, 2010). Therefore limited options exist to perform candidate-based studies. Recent development of single cell profiling techniques allows a more unbiased search for candidate factors. Two studies have compared transcript profiles of the first mouse blastomeres. One study showed that significant differences in transcription profile between the first two blastomeres are sparse and small (Roberts *et al.*, 2011). Furthermore, transcripts that showed significant differential expression (around 1.4 fold difference) function in general processes and are thus unlikely to pattern specific cells. A second study showed that still no difference exists between the first three blastomeres (VerMilyea *et al.*, 2011). It would be interesting to see whether the same holds true at the 4-cell stage since this is when Piotrowska-Nitsche et al. observe differences in cell fate (Figure 7, blastomeres b & c) (Piotrowska-Nitsche *et al.*, 2005a). However, similar experiments on human blastomeres show no difference in transcriptional profile at the 8-cell stage, which is one cell cycle after zygotic genome activation (Galan *et al.*, 2010). It is important to keep in mind that transcriptional differences may be small and therefore undetectable by the current techniques. Furthermore, maternal patterning might be established through asymmetric distribution of proteins or even non-coding RNAs, both of which have not been profiled yet.

Regulative development

Regardless of whether prepatterning has a role in mouse embryonic development or not, it can be overruled by regulative development. This was clear very early on, when single blastomeres from the 2-cell embryo were shown to develop into healthy mice (Tarkowski *et al.*, 1967). This experiment can be compared to the natural occurrence of monozygotic twins, also in humans. For a long time, mouse embryos retain a high degree of developmental flexibility. Isolated and aggregated ICMs or TE cells can develop into blastocysts and even full grown embryos until just before the 32-cell stage and transplanted cells will adopt a fate according to their location (Rossant *et al.*, 1979; Rossant *et al.*, 1980; Spindle, 1978; Suwinska *et al.*, 2008).
The question remains whether all blastomeres can contribute to both ICM and TE or if these observations are a result of just a few blastomeres that retain developmental flexibility. The best way to approach this question is by studying the developmental potency of single blastomeres. Although 2-cell blastomeres can readily develop into healthy mouse, development of later stage blastomeres seems to face more impediments. Isolated blastomeres from 4-cell and 8-cell embryos were shown to develop into blastocysts consisting of both ICM and TE cells (Rossant, 1976; Tarkowski *et al.*, 1967). However, only one out of 13 4-cell blastomere derived blastocysts was able to develop into a healthy embryo *in utero* (Rossant, 1976). This is probably caused by low cell numbers when cavitation is initiated. Embryos derived from isolated blastomeres start cavitation around the same developmental time as unmanipulated embryos, when

they consist of fewer cells. More detailed study of the blastocysts showed that they often contain very few or even no ICM cells (Rossant, 1976; Tarkowski *et al.*, 1967).
To solve this problem, Tarkowski et al. developed a method to support development of single blastomeres (Tarkowski *et al.*, 2005). They fused blastomeres together to obtain tetraploid cells. These cells can contribute to extraembryonic tissues and support embryonic development, but will not contribute to the embryo proper. When either $^{1}/_{4}$ or $^{2}/_{8}$ blastomeres were aggregated with tetraploid cells, these blastomeres could develop into healthy mice (Tarkowski *et al.*, 2005). Furthermore, the blastomeres contributed to both TE and ICM derived tissues (Tarkowski *et al.*, 2005). The same was shown for both inner and outer cells of morula-stage embryos (Tarkowski *et al.*, 2010). This apparent developmental flexibility was lost at the 32-cell stage just like in the aggregation experiments (Rossant *et al.*, 1979; Rossant *et al.*, 1980; Spindle, 1978). Sporadically, $^{1}/_{32}$ blastomeres contributed to all tissues of the embryo, but these embryos never developed to term (Tarkowski *et al.*, 2010).
These experiments unveil a high degree of developmental flexibility. However, development rates of single blastomeres are much lower than those of unmanipulated embryos. Although multiplets have been obtained, development of all blastomeres to healthy mice has not been observed beyond the 2-cell stage. Therefore these experiments are not sufficient to answer the question whether all blastomeres retain equal developmental potential. A recent study indicates that this may not be the case. Grabarek et al. showed differential developmental potential of respectively EPI and PE precursors in the ICM (Grabarek *et al.*, 2012). EPI precursors less frequently contribute to TE derived tissues than PE precursors in transplantation experiments. This may be a result of their relatively low sensitivity to extracellular signaling (Grabarek *et al.*, 2012).

3. Cdx-2 reveals a complex signaling network underlying TE specification

Regardless of whether differentiation between ICM and TE is biased or purely regulative, proteins lie at the basis of this process. To better understand the mechanisms of cell fate specification, we can study the proteins that are involved. Cdx-2 is a well-studied transcription factor that is involved in TE specification.

Cdx-2 is a TE specific transcription factor

Cdx-2 is a member of the homeobox transcription factor family. Just like Hox genes, Cdx genes are expressed in a specific portion of the embryo and are required for its differentiation. Initial studies on Cdx-2 focused on its role in caudal patterning of amongst others the hindgut (Beck *et al.*, 1995). Already then it was observed that Cdx-2 is first expressed during early embryonic development and that its expression becomes confined to the TE by the time of implantation (Beck *et al.*, 1995). Subsequent studies of homozygous *Cdx-2* mutants showed lethality around the implantation stage (Chawengsaksophak *et al.*, 1997; Chawengsaksophak *et al.*, 2004). Implantation can be rescued by aggregating *Cdx-2*-/- cells with *Cdx-2* heterozygous tetraploid fusions

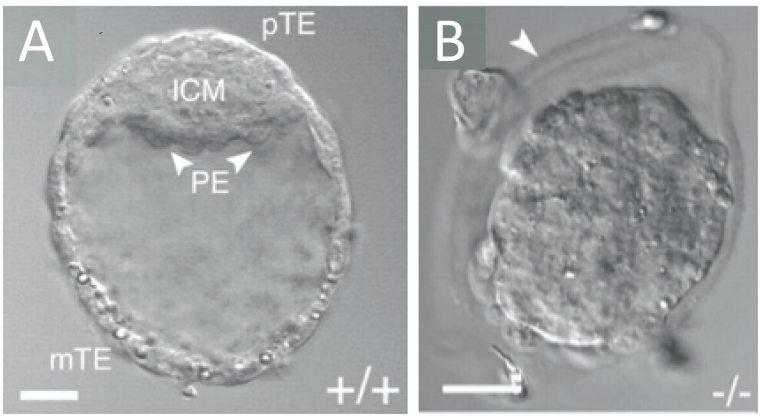

Figure 8. Phenotype *Cdx-2* mutant.
DIC image of a wild type (**A**) and a *Cdx-2* homozygous mutant embryo (**B**). Cdx-2$^{-/-}$ embryos initiate blastocyst formation, but are not able to maintain the blastocyst cavity and collapse. Adapted from (Strumpf *et al.*, 2005).

(Chawengsaksophak *et al.*, 2004). This indicates that *Cdx-2-/-* embryos have a specific defect in TE formation. Indeed, TE development was shown to be defective in homozygous mutant embryos (Strumpf *et al.*, 2005). The importance of Cdx-2 as a TE specific transcription factor is further supported by the fact that Cdx-2 regulates the expression of many TE specific genes (Kidder *et al.*, 2010). The best know example of a Cdx-2 target is the transcription factor Eomes (Ciruna *et al.*, 1999; Russ *et al.*, 2000).
Although mutants initially show epithelialization of outer blastomeres and initiate cavitation, the blastocyst cavity will collapse shortly after formation and the epithelial-like appearance of the outer cells will disappear (Figure 8) (Strumpf *et al.*, 2005). This coincides with and may be due to dissociation of adherens and tight junctions (Strumpf *et al.*, 2005). Together these results show that Cdx-2 is required for TE maintenance but not initiation. Overexpression of Cdx-2 in embryonic stem cells (ESCs) and in ICM cells induces differentiation to TE, showing that *Cdx-2* expression is also sufficient to induce TE differentiation (Niwa *et al.*, 2005; Tolkunova *et al.*, 2006). However, maintenance of the induced TE state requires additional factors (Tolkunova *et al.*, 2006).

Cdx-2 expression pattern
At E3.5 Cdx-2 protein expression is confined to nuclei of the outer blastocyst cells, as can be expected for a transcription factor with an important role in TE specification (Figure 9). More interesting is its expression pattern before this time. It might tell us something about the first moment cells commit to a TE fate. Clear Cdx-2 protein expression comes up at the 8-cell stage after compaction (Figure 9) (Dietrich *et al.*, 2007; Ralston *et al.*, 2008). At this point protein expression levels vary greatly per cell. Upon the formation of inside cells, Cdx-2 expression is highest among the cells on the outside of the embryo (Figure 9). The apical domain, which is uniquely present in outside cells, seems to have a role in this observed difference in Cdx-2 expression between inside and outside cells (Dietrich *et al.*, 2007; Stephenson *et al.*, 2010). Over the course of development, Cdx-2 expression levels in outside cells will increase, while expression in inside cells decreases and then completely disappears (Dietrich *et al.*, 2007). It has been suggested that cells that express higher levels of Cdx-2 undergo more symmetric divisions and therefore make a greater contribution to TE (Jedrusik *et al.*, 2008). However no lineage tracing experiments have been performed to show that this actually happens in the unmanipulated situation.

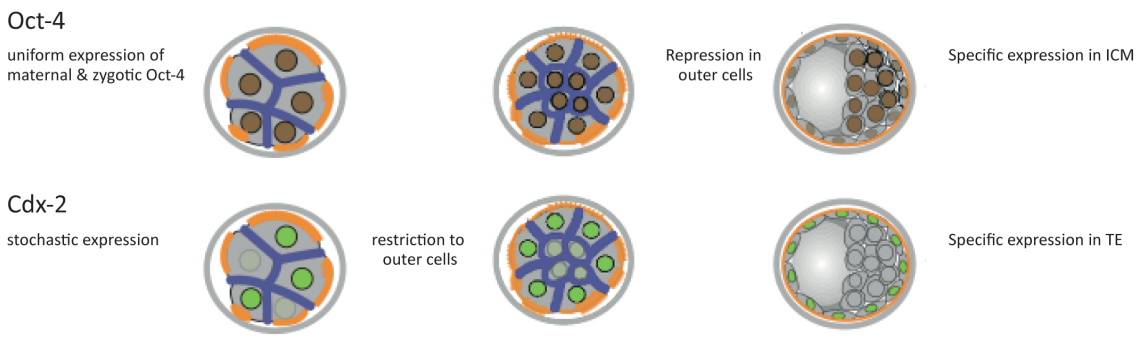

Figure 9. Dynamics of Cdx-2 and Oct-4 expression.
Model of the expression patterns of Oct-4 and Cdx-2. Oct-4 is initially expressed in all nuclei, but becomes confined to the ICM. Cdx-2 initially displays random expression, but becomes confined to the TE. Negative feedback between the two transcription factors reinforces their expression domains. Adapted from (Rossant *et al.*, 2009).

The first studies on Cdx-2 expression did not detect Cdx-2 protein prior to the 8-cell stage. This argues against a role for Cdx-2 in prepatterning. In 2006, a controversial paper was published that claimed Cdx-2 protein was already present at the 2-cell stage and that expression levels were even higher in one of the two nuclei of the first blastomeres (Deb *et al.*, 2006). Not much later the paper turned out to contain manipulated data and was retracted (Kennedy, 2006; Vogel, 2006). Interestingly, two labs recently published observations of maternal *Cdx-2* mRNA in the oocyte and subsequent cleavage stages of the embryo (Jedrusik *et al.*, 2010; Wu *et al.*, 2010). However, both labs came to different conclusions regarding its importance. Jedrusik et al. observed more severe phenotypes in *Cdx-2*-/- embryos after knock-down of maternally provided mRNA (Jedrusik *et al.*, 2010). In contrast, Wu et al. were not able to replicate these results (Wu *et al.*, 2010). The value of both studies has been debated (Bruce, 2011; Wu *et al.*, 2011) and future studies will need to shed more light on this issue. Of note, neither study was able to detect Cdx-2 protein expression until the 8-cell stage, when the zygotic genome is already fully active. However, this may be a result of insufficient antibody sensitivity. Protein expression levels are worth analyzing further, for example by single-cell mass spectrometry (Rubakhin *et al.*, 2011). In the light of potential Cdx-2 mediated cell fate bias prior to compaction, it will also be interesting to visualize single *Cdx-2* mRNA molecules and follow the fate of cells in which these are present (Raj *et al.*, 2008; Santangelo *et al.*, 2009).

Regulation of Cdx-2 expression

Over the course of development, Cdx-2 expression gets confined to the TE. A complex signaling network lies on the basis of this process. This ensures robust establishment and maintenance of the Cdx-2 expression domain.

A balance between TE Cdx-2 and ICM Oct-4

While TE is characterized by Cdx-2 expression, the POU domain transcription factor Oct-4 is required for ICM specification. Homozygous mutants for *Oct-4* reach the blastocyst

stage, but their ICM shows properties of TE differentiation and embryos die around the time of implantation (Nichols *et al.*, 1998).
Oct-4 expression becomes restricted to the ICM during blastocyst development (Figure 9) (Palmieri *et al.*, 1994). Since Oct4 and Cdx-2 occupy opposing expression domains in the blastocyst, it was hypothesized that the two transcription factors regulate each other's expression. This hypothesis was supported by the observation that Oct-4 is still expressed in the outside cells of *Cdx-2*-/- blastocysts by the time that wild type control embryos already showed ICM-restricted expression of Oct-4 (Strumpf *et al.*, 2005). Conversely, *Oct-4* mutation results in aberrant expression of Cdx-2 in the ICM (Nichols *et al.*, 1998). Indeed, Cdx-2 and Oct-4 were shown to downregulate each other's expression in ES cells (Figure 10) (Niwa *et al.*, 2005).
The initial paper spoke about a direct mechanism for reciprocal downregulation of Cdx-2 and Oct-4 in which the two transcription factors physically interact (Niwa *et al.*, 2005). However, Cdx-2 expression is induced in the presence of Oct-4 and Oct-4 expression is not downregulated in TE until about 24 hours after restriction of Cdx-2 expression (Dietrich *et al.*, 2007; Szczepanska *et al.*, 2011). Considering the short half-life of Oct-4 of approximately six hours, it is unlikely that Cdx-2 represses *Oct-4* expression through a direct mechanism, although *Oct-4* mRNA could in theory persist (Nichols *et al.*, 1998). Recently, a repression mechanism has been suggested that involves cooperation of Cdx-2 with Brg1, a chromatin-modifying enzyme (Wang *et al.*, 2010). In this paper, recruitment of Cdx-2 and Brg1 to the *Oct-4* promoter was delayed by 24 hours after *Cdx-2* induction. This could explain the late repression of Oct-4 in TE. Furthermore, a recent paper showed that not the presence of Oct-4 in a cell is important for cell fate specification, but its dynamics (Plachta *et al.*, 2011). Oct-4 appeared to be transported out of the nucleus of TE precursor cells more quickly than in ICM cells because of reduced interaction with the DNA and therefore possibly reduced functionality.

Cross regulation
The reciprocal repression of Cdx-2 and Oct-4 exemplifies the importance of cross regulation in specification of TE and ICM. Cross regulation ensures robustness of cell fate specification in the embryo by enhancing subtle differences in gene expression between blastomeres. Initial differences can either be prepatterned, stochastic, or based on signaling due to cell polarity or position.
The most direct way in which *Cdx-2* expression is maintained is by positive autoregulation (Figure 10). The promoter of *Cdx-2* contains Cdx-2 responsive elements (Chen *et al.*, 2009; Saegusa *et al.*, 2007). Indeed, Cdx-2 is recruited to its own promoter and upregulates its expression by 4-6 fold (Saegusa *et al.*, 2007).
In addition, *Cdx-2* expression is stabilized by several other TE specific transcription factors that either function downstream or in parallel of Cdx-2 to establish and maintain TE fate. The Elf5 transcription factor functions downstream of Cdx-2 in TE specification (Ng *et al.*, 2008). *Elf5* activity is regulated by DNA methylation of its promoter. Methylation levels of the *Elf5* promoter are high in ICM and low in TE due to Cdx-2 signaling. In turn, Elf5 binds to the *Cdx-2* promoter and enhances its expression, creating a positive feedback loop (Figure 10) (Ng *et al.*, 2008). Two TE specific transcription

factors that are upregulated independently of Cdx-2 are TCFAP2C/AP-2γ and Gata3 (Home *et al.*, 2009; Kuckenberg *et al.*, 2010; Ralston *et al.*, 2010). Both transcription factors upregulate *Cdx-2* expression (Figure 10) (Home *et al.*, 2009; Kuckenberg *et al.*, 2010).

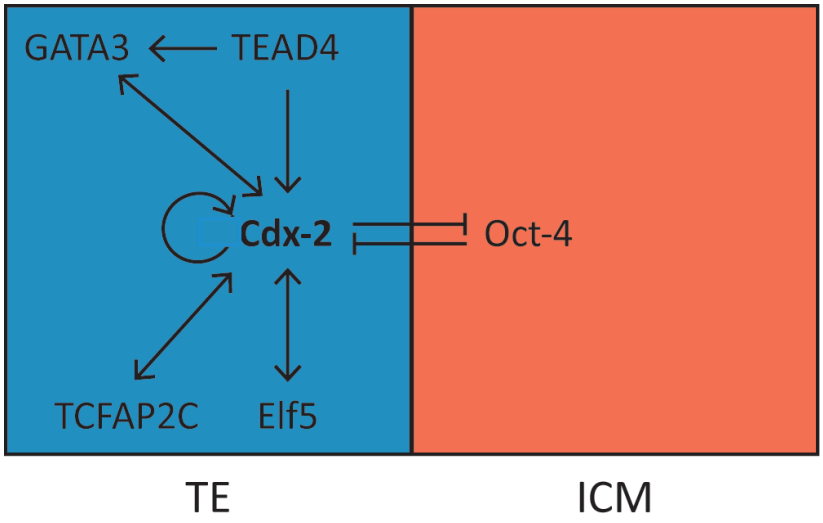

Figure 10. Simplified model of Cdx-2 expression regulation.
TEAD4 is the most upstream factor regulating Cdx-2 expression known to date. Cdx-2 expression is reinforced by autoregulation and positive feedback by several other transcription factors. Interaction between Oct-4 and Cdx-2 ensures that Cdx-2 expression becomes confined to the TE.

Upstream of Cdx-2

The question remains which cue initially induces upregulation of Cdx-2 expression in outside blastomeres specifically. Cdx-2 levels are upregulated in the outer cells of the ICM after removal of the TE (Suwinska *et al.*, 2008; Tarkowski *et al.*, 2010), a process that requires cell polarization (Dietrich *et al.*, 2007; Stephenson *et al.*, 2010). This tells us that, regardless of potential prepatterning or stochastic effects, cell polarization and cell position are important cues for Cdx-2 upregulation. Two lines of study provide mechanistic support for this finding: i. the role of PAR proteins in Cdx-2 expression, ii. the recently discovered involvement of the Hippo pathway in Cdx-2 upregulation. Activity of this pathway has both been implicated to be regulated by cell-cell contact and by the apical domain of outside cells.

PAR proteins are important regulators of cell asymmetry and asymmetric division in many organisms (Bastock *et al.*, 2008; Munro *et al.*, 2004). In the mouse embryo PAR homologs become asymmetrically distributed by the 8-cell stage (Vinot *et al.*, 2005). This coincides with the first observations of Cdx-2 expression. Indeed, the apical domain has been implicated in Cdx-2 expression regulation (Stephenson *et al.*, 2010). Interestingly, a recent paper reported a link between the apically localized mouse Par6 homolog Pard6b and Cdx-2 expression (Alarcon, 2010). *Cdx-2* expression was reduced in *Pard6b* mutants. This result provides a link between establishment of the apical domain, which will be inherited by TE blastomeres, and *Cdx-2* expression.

Discovery of the involvement of the Hippo pathway (Figure 11) in TE specification provided an even more exciting link between cell position and the regulation of *Cdx-2* (Nishioka *et al.*, 2008; Nishioka *et al.*, 2009; Yagi *et al.*, 2007; Zhao *et al.*, 2007). TEAD4 is a transcription factor of the Hippo pathway. *Tead4* homozygous mutant embryos show defective TE differentiation (Nishioka *et al.*, 2008; Yagi *et al.*, 2007). The *Tead4*-/- phenotype is more severe than the *Cdx-2*-/- phenotype. While *Cdx-2* mutant embryos initiate blastocyst formation, a blastocyst cavity is never observed in *Tead4* mutants (Nishioka *et al.*, 2008; Yagi *et al.*, 2007). This led to the hypothesis that TEAD4 regulates

TE specification upstream of Cdx-2. Indeed, Cdx-2 expression is abolished in *Tead4*-/- embryos (Nishioka *et al.*, 2008; Yagi *et al.*, 2007). In addition, the expression of several other TE specific factors is affected in *Tead4*-/- embryos (Nishioka *et al.*, 2008; Ralston *et al.*, 2010; Yagi *et al.*, 2007). The expression of some of these factors, like Gata3, is regulated independently of Cdx-2 (Figure 10) (Ralston *et al.*, 2010). TEAD4 is thus not only required for the induction of Cdx-2 expression in TE, but regulates TE specification in additional ways.

The exclusive involvement of TEAD4 in TE specification was initially difficult to explain since the protein is expressed in both inside and outside cells. However, it was shown that TEAD4 mediated transcription activation requires its binding to a co-activator, Yap (Nishioka *et al.*, 2009). Yap is phosphorylated in inside cells by the Hippo pathway kinase Lats (Nishioka *et al.*, 2009). Phosphorylation results in its sequestration in the cytoplasm, thus depleting TEAD4 of its co-activator (Figure 11) (Nishioka *et al.*, 2009; Zhao *et al.*, 2007). Several lines of evidence suggest that Yap phosphorylation occurs in response to cell-cell contact (Wada *et al.*, 2011; Zhao *et al.*, 2007). This could explain its exclusive phosphorylation in inside cells (Nishioka *et al.*, 2009). However, the apical domain has also been implicated in Hippo pathway regulation (Halder *et al.*, 2011). Together these results provide strong support for the importance of regulative cell fate specification in the differentiation between TE and ICM.

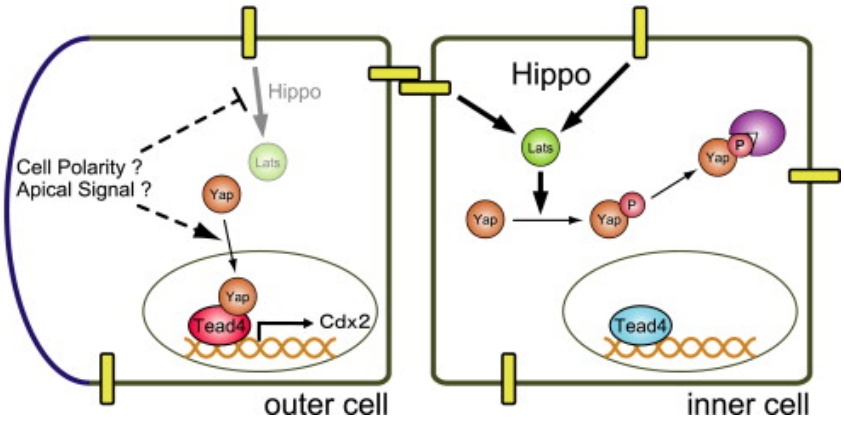

Figure 11. Regulation of Cdx-2 expression by the Hippo pathway.
In outside cells, Hippo signaling is inactive. Yap and TEAD4 form a transcription-activating complex in the nucleus and induce Cdx-2 expression. In inside cells Hippo signaling is active. Lats kinase phosphorylates Yap. Yap-P is sequestrated in the cytoplasm. In lack of its co-activator, Tead4 cannot activate Cdx-2 expression. Taken from Nishioka et al. 2009.

4. Discussion

The first cell fate specification event during mouse development is the differentiation between TE and ICM. TE will form the extraembryonic tissues, while ICM will contribute to both the embryo proper and extraembryonic tissues. This differentiation process is highly regulative. Complex signaling networks underlie the cell fate specification event, as exemplified by the contribution of Cdx-2.

Although the highly regulative nature of the separation between ICM and TE is generally accepted, a potential role for prepatterning is subject to debate. Study of the molecular

mechanisms behind the first differentiation step can shed more light on the issue. Cdx-2 has been shown to be important for TE maintenance, but is not involved in its initial establishment. The question remains which factors are responsible for the very first expression induction of Cdx-2 before the formation of inside and outside cells. Study of this question may lead us to find new factors involved in the separation of ICM and TE.

Regulative vs prepatterned development

The unique regulative nature of mouse development is undisputed. However, one of the key questions in the field is whether mouse development in addition contains a component of prepatterning or bias. One lab argues that the fate of some blastomeres is set at the 4-cell stage in a subset of embryos (Piotrowska-Nitsche *et al.*, 2005a). Their arguments are under debate. Other labs were not able confirm their results (Alarcon *et al.*, 2008; Hiiragi *et al.*, 2004; Louvet-Vallee *et al.*, 2005). Additionally, single cell transcription profiling does not support a difference between the blastomeres up to at least the 3-cell stage (Roberts *et al.*, 2011; VerMilyea *et al.*, 2011). Furthermore prepatterning was never observed in all embryos (Piotrowska-Nitsche *et al.*, 2005a). From these results I conclude that there is currently not sufficient proof to support a role for prepatterning in mouse development. I believe that lineage-tracing experiments in unmanipulated embryos are the most informative method for future studies because of the regulative nature of mouse development. Any experimental manipulation of mouse embryos can disrupt potential prepatterning. These experiments could be combined with visualization of candidate patterning factors. Asymmetric distribution of specific factors during early stages of development, linked to a predictable cell fate, would provide the strongest proof for the existence of prepatterning.

There is a reasonable explanation why regulative development is unique to early mammalian development. TE differentiation is only observed in mammals, where it had to be established in order to enable *in utero* development. This could explain why the differentiation process is different from the first differentiation steps in other animals. Starting from the EPI, which will form the embryo proper, mammalian development actually shows much resemblance to that of other organisms. From the time of gastrulation, development of the mammalian EPI and development of non-mammalian species show a high degree of similarity (O'Farrell *et al.*, 2004).

Regulative development may have additional importance in humans. Even if the human embryo is split in two parts during early development, two healthy monozygotic twins can develop (Gilbert). Moreover, early stage human embryos often contain aneuploidic blastomeres (van Echten-Arends *et al.*, 2011). These blastomeres cannot contribute to a healthy embryo and will undergo apoptosis. Regulative development ensures that cell loss in the human embryo does not affect development. However, these phenomena may have evolved in the presence of regulative development, rather than be causal to it.

Initiation of Cdx-2 expression

To get a better understanding of mouse embryonic development, researchers have begun to study specific factors that may regulate cell fate specification. One of the best-

studied factors in mouse development is the TE specific homeobox family transcription factor Cdx-2. From the onset of blastocyst formation, polarity proteins and the Hippo pathway regulate Cdx-2 expression. However, it is still unclear whether the Hippo pathway regulates Cdx-2 expression at compaction, before the separation of inside and outside cells.

Although recent studies showed that low levels of *Cdx-2* mRNA might be maternally provided, the significance of its early presence is under debate (Bruce, 2011; Jedrusik *et al.*, 2010; Wu *et al.*, 2010; Wu *et al.*, 2011). Low levels of maternal Cdx-2 provide an interesting opportunity for prepatterned development. Few molecules of Cdx-2 mRNA or protein could be distributed unequally during the first cleavage divisions either stochastically or in a regulated manner. Expression regulation of *Cdx-2* involves strong positive feedback to its own expression (Chen *et al.*, 2009; Saegusa *et al.*, 2007). If the few Cdx-2 molecules that are present in the zygote are unequally distributed by the time zygotic transcription is activated, this can result in strong expression in single cells. Indeed, Cdx-2 expression levels vary greatly between cells at the 8-cell stage (Dietrich *et al.*, 2007; Ralston *et al.*, 2008). Labeling of individual *Cdx-2* mRNAs should provide more insight into their distribution prior to genome activation (Raj *et al.*, 2008). Interestingly, one study found that cells that overexpress Cdx-2 are more prone to undergo symmetric divisions and are therefore more likely to contribute to TE (Jedrusik *et al.*, 2008). This mechanism could describe a connection between maternally provided Cdx-2 mRNA and cell fate at later stages. An important follow-up study would be to correlate endogenous Cdx-2 expression levels at the 8-cell stage with cell fate in the blastocyst by cell tracing.

Cells that express high levels of Cdx-2 at the 8-cell stage or later stages, also express high levels of the TE factor Gata-3 (Ralston *et al.*, 2010). This implies that a common transcription regulator other than Cdx-2 drives expression of both Cdx-2 and Gata-3 during early development. A correlation between cell fate and Cdx-2 expression levels may thus stem from differential expression of a transcription factor that regulates Cdx-2 expression. This makes sense given the fact that Cdx-2 protein is not required for initial TE specification (Strumpf *et al.*, 2005). TEAD4 is a good candidate as it has been shown to regulate both Cdx-2 and Gata-3 expression at later stages during development (Ralston *et al.*, 2010; Yagi *et al.*, 2007).

To learn more about the nature of the initial cue for TE specification, it would be interesting to perform transcription profiling of cells that express high levels of Cdx-2 and Gata-3 and compare these to blastomeres that contain low levels of both proteins. However, it should be kept in mind that protein location may be more important as exemplified by the role of Yap (Nishioka *et al.*, 2009) and Oct-4 (Plachta *et al.*, 2011) subcellular dynamics in gene expression regulation.

Other factors in TE specification

Cdx-2 mutants initiate cavitation and thus TE specification (Strumpf *et al.*, 2005). Therefore Cdx-2 can not be the driving factor behind this process. Several approaches can be used to discover novel factors involved in early cell fate specification. These include: i. protein profiling, ii. gene trap, iii. profiling of non-coding RNAs, iv. chromatin profiling.

Thus far studies have focused on transcript profiling in early blastomeres. However, transcript levels may not always reflect translation levels. Protein profiling may provide additional information. Mass spectrometry is a powerful method to identify large numbers of proteins in an unbiased manner, i.e. without the need for candidate genes. Mass spectrometry profiling could be performed on separate blastomeres before the 8-cell stage in order to unveil any potential prepattering signal or on isolated samples of TE and ICM to discover new factors involved in specification of these cell types (Rubakhin *et al.*, 2011).

A more specific way to uncover novel TE or ICM specifying proteins is by gene trap. For this technique a fluorescent protein is randomly inserted into the genome. If it is inserted in a transcribed gene, the gene's promoter may regulate its expression. In that case, promoter activity will be visualized by expression of the fluorescent protein. If the targeted protein is specifically expressed in TE or ICM, sequencing can be used to unveil the identity of this potentially novel cell fate specifying factor. Furthermore the dynamics of protein expression during pre-implantation can be studied.

In recent years, the importance of epigenetics in cell fate specification has emerged. Besides proteins, non-coding RNAs and chromatin modifications regulate gene transcription and translation. Studies in zebrafish have already shown that non-coding RNAs play an important role during development and are often maternally provided (Pauli *et al.*, 2011). To my knowledge neither short nor long non-coding RNAs have been profiled in the early mouse embryo. This may provide novel insights.

Genome-wide studies of chromatin modifications have unveiled that many modifications are either specifically associated with transcriptionally active or silenced genes (Barski *et al.*, 2007; Ernst *et al.*, 2010; Filion *et al.*, 2010; Wang *et al.*, 2008). Chromatin modifications can therefore teach us which genes are actively transcribed. Chromatin modifications may precede differences in transcript profile and may thus provide an earlier readout of cell fate specification.

The dynamics of chromatin modifications during early cell fate specification have already been studied to some extend in the mouse embryo. A study by the Zernicka-Goetz lab even unveiled a difference in chromatin modifications between the blastomeres of the 4-cell embryo (Torres-Padilla *et al.*, 2007). High levels of H3R26me seem to predispose for an ICM fate. However, further research on this topic is required to elucidate the role of epigenetic modifications in cell fate specification.

Another study looked at the distribution of H3K4me2 and H3K27me3 in the mouse embryo before and after blastocyst formation (Alder *et al.*, 2010). This study showed that genes that become repressed during TE differentiation, acquire an additional silencing mark. It would be interesting to map more chromatin modifications on a genome wide scale in a wide range of developmental stages.

In conclusion, I believe that studies that are not candidate-based may be very useful in finding novel factors involved in the differentiation between ICM and TE. The importance of novel candidates can be studied by a combination of fluorescent tagging and cell tracing experiments as well as by knockout studies.

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