MINI-REVIEW: DEVELOPMENTS IN REPRODUCTIVE BIOLOGY AND MEDICINE

Cell identity in the preimplantation mammalian embryo: an epigenetic perspective from the mouse

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The early preimplantation mouse embryo is a unique system where it is possible to explore the foundations of totipotency and differentiation. Following fertilization, a single cell, the zygote, will give rise to all tissues of the organism. The first signs of differentiation in the embryo are evident at the blastocyst stage with the formation of the trophectoderm, a differentiated tissue that envelopes the inner cell mass. The question of when and how the cells start to be different from each other in the embryo is central to developmental biology: as cell fate decisions are undertaken, loss of totipotency comes about. Although the blastomeres of the preimplantation embryo are totipotent, as the embryo develops some differences appear to develop between them which are, at least partially, related to the epigenetic information of each of these cells. The hypothesis of epigenetic asymmetries acting as driver for lineage allocation is presented. Although there are now some indications that epigenetic mechanisms are involved in cell fate determination, much work is needed to discover how such mechanisms are set in play upon fertilization and how they are transmitted through cell division. These considerations are further discussed in the context of preimplantation genetic diagnosis: does it matter to the embryo which cell is used for genetic diagnosis? The exquisite complexity and richness of chromatin-regulated events in the early embryo will certainly be the subject of exciting research in the future.

Keywords: cell fate; epigenetics; histone methylation; mouse embryo; pluripotency

Embryonic development starts from a single cell, the zygote. In this cell, the two gametes convey and contribute information to start a new developmental programme. The formation of the newly fertilized zygote constitutes therefore the climax of totipotency because of the resulting zygote's inherent ability to produce all cell types in a new organism.

In the mammalian embryo, the first differentiative event occurs as inner cells form upon cell division at the 8-cell stage. As a result of this division, an 'inner' and an 'outer' population of cells can be distinguished in the 16-cell stage embryo, which will respectively occupy different positions in the morula (Johnson and Ziomek, 1981). Much work has been done in trying to understand how cell polarity develops in the inner and outer cells, but I will not deal with this topic here and instead will refer the reader to an excellent review

published elsewhere (Johnson and McConnell, 2004). The inner cells will develop into the inner cell mass (ICM) and the outer layer of cells will differentiate into the trophectoderm (Tarkowski and Wroblewska, 1967; Ziomek and Johnson, 1982). Morphologically, however, the first overt signs of differentiation are evident only at the blastocyst stage, with the formation of the trophectoderm, which is the first differentiated tissue to form as an epithelial layer that envelops the ICM (Fig. 1A). The latter, in contrast to the trophectoderm, retains its pluripotent character and the ability to self renew. While the ICM will give rise to the embryo proper, the trophectoderm will give rise to the extraembryonic tissues that will support development of the embryo during gestation. The ICM will also give rise to yet another extraembryonic tissue, the primitive endoderm, which is first visible as a cuboidal layer of
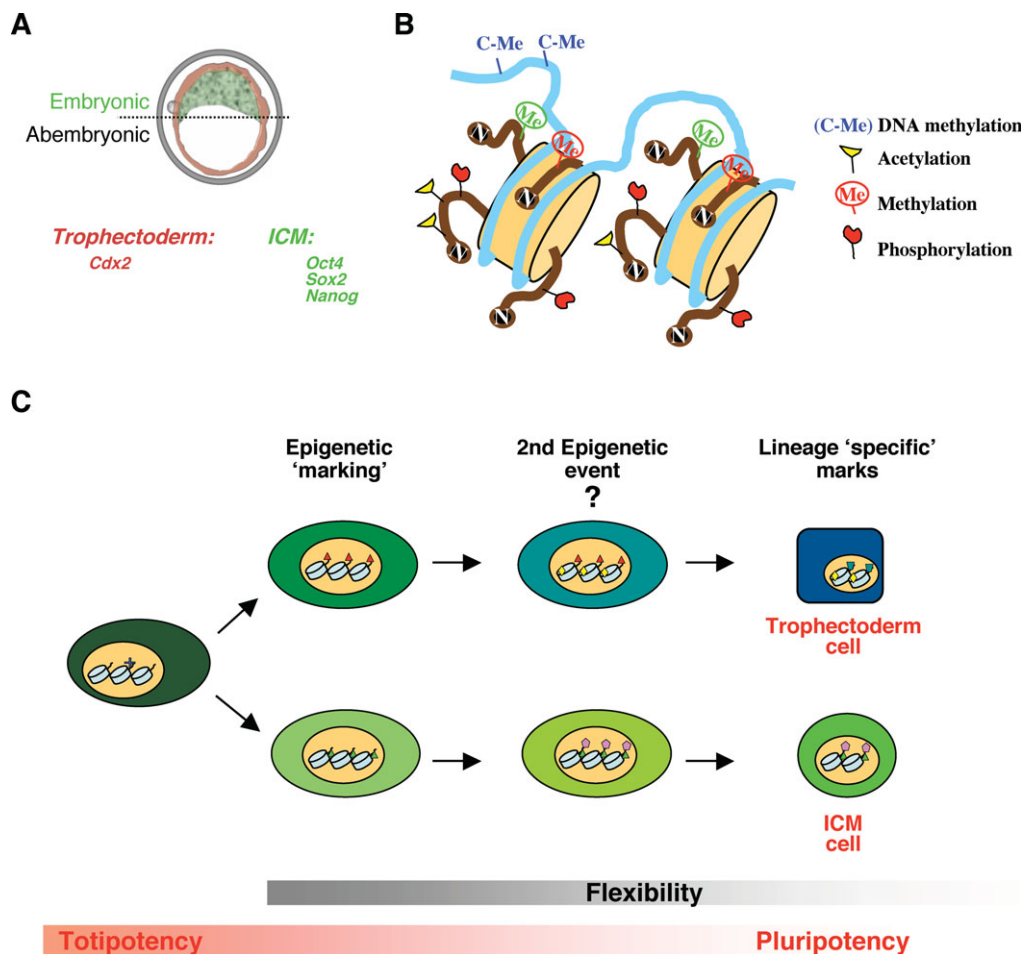
Figure 1: Cell lineages of the mammalian blastocyst and epigenetic marking
(**A**) Representation of the lineages in the mammalian blastocyst on the third day of gestation (E3.5). The blastocyst is composed of two distinct populations of cells: the trophectoderm (red) and the inner cell mass (ICM, green), which display molecular identity and epigenetic asymmetries. The embryonic-abembryonic regions (dotted line) of the blastocyst are determined by the position of the ICM, which lies within the embryonic region of the blastocyst. (**B**) Diagram illustrating some of the epigenetic marks. The DNA wrapped around the nucleosome (beige cylinders) is shown as light blue. The DNA is subject to DNA methylation, which constitutes one of the main epigenetic players. The core histones (beige) that form the nucleosome can be covalently modified (by acetylation, methylation, phosphorylation), particularly on their N-terminal tails. Each of these marks can have an effect on how the information contained in the DNA is read by modulating downstream events such as transcriptional activation or repression. For example, histone methylation (Me) can have a positive effect on transcription (green) or a repressive one (red). The marks can be present in different combinations and may change during the cell cycle. (**C**) Model for epigenetic marking and lineage allocation. In this model, an epigenetic mark would be laid down in a given cell during development. There could be other epigenetic event(s) that reinforce and/or are influenced by the first marking event. Cumulatively, this could result in determination of the fate of that cell towards a lineage in the blastocyst. The lineage specific marks could stabilize such cell identity and might be necessary for further differentiation. Alternatively, the acquisition of these epigenetic marks could be the result of cell fate determination. One should also consider that not only the nature of the mark would be important, but also the different regions of the chromatin that would be affected by such marks. Because the cells in the preimplantation embryo are totipotent and because the chromatin will still need to be dynamically remodelled during subsequent development, flexibility should be an important component of epigenetic mechanisms taking place during early development. As cell fate decisions are taken, a concomitant loss of totipotency takes place

cells lining the blastocoelic cavity on the fourth day of development. The trophectoderm and the ICM each display molecular identity, which is reflected in part by the expression of specific genes that are, for the ICM, involved in its specification and the maintenance of pluripotency (such as Nanog and Oct4) or, for the trophectoderm, that are required for its differentiation (such as Cdx2) (Palmieri *et al*., 1994; Nichols *et al*., 1998; Chambers *et al*., 2003; Mitsui *et al*., 2003; Strumpf *et al*., 2005). The bifurcation of these two lineages is complete at the late blastocyst stage. At this stage, cells from the ICM do

no longer have the potential to form trophectoderm derivates *in vivo* upon transplantation (Rossant and Lis, 1979), indicating that these cells have lost their totipotency and that lineage allocation has definitely occurred.

Investigations during recent years have recreated an interest in whether the blastomeres of the mammalian embryo are truly alike throughout preimplantation development before the first differentiative division mentioned above. In other words, whether they acquire a 'fate' or whether they start to differ from each other prior to their spatial 'inner/outer' allocation

within the embryo upon the formation of the morula. Of course, if this were to be the case, the big challenge would be to ascribe molecular mechanisms to these processes.

The question of when and how the cells start to be different from each other is not a trivial one. In particular, because as the first cell fate decisions are undertaken, a concomitant loss of totipotency occurs. The developmental time window when this first cell fate decision occurs comprises a number of epigenetic events (Morgan *et al.*, 2005; Surani *et al.*, 2007). These events include the reprogramming of the parental chromatin. Whether such epigenetic events are the cause or the consequence of reprogramming remains an exciting open question, but it is probably a combination of the two. Moreover, the two lineages of the blastocyst exhibit some epigenetic asymmetries.

This mini-review is divided in two parts, the first one will deal with the main epigenetic mechanisms that are known to occur during mammalian preimplantation development. The second one will give an overview on data obtained through experimental embryology manipulations and lineage tracing observations to study cell fate in the early mouse embryo.

Epigenetic mechanisms in the preimplantation embryo

In general terms, epigenetic mechanisms include DNA methylation, covalent modification of histones, chromatin remodelling and histone replacement through incorporation of the so-called histone variants (Fig. 1B). Histone marks have emerged as one of the main players involved in epigenetic mechanisms (Kouzarides, 2007). Histone modifications can be highly dynamic, or have a function in epigenetic memory. Although it is still unclear whether they are the actors of the epigenetic information or the epigenetic information itself, it is evident that covalent modifications of histones are essential components of the epigenome.

Histones can be modified by a number of enzymes that mediate methylation, acetylation, phosphorylation, ubiquitynation and ADP-ribosylation of specific amino acid residues (reviewed in Kouzarides, 2007). By and large, the highest density of modifications so far described occurs in histone H3, particularly on its tail. The effects of these modifications on the chromatin and on cellular processes are very diverse, and a modification of the same residue can even have opposite effects depending on the type of modification. For example, trimethylation of H3K9 is considered as a repressive mark, whereas acetylation of the same lysine has a positive effect on transcription (Bannister and Kouzarides, 2005). Likewise, methylation of arginine residues can have a positive effect on transcription (Chen *et al.*, 1999) or a repressive effect (Pal *et al.*, 2004), depending both on the targeted residue and on whether the methylation is symmetric or asymmetric. For some of the modified residues, there is a very clear view of the outcome of an eventual modification: H3K9me3 creates a specific docking site for the heterochromatin protein 1 (HP1), which subsequently recruits the H3K9 methyltransferase Su(var)3-9 and reinforces an autoregulatory loop for heterochromatin formation and maintenance (Bannister *et al.*, 2001; Lachner *et al.*, 2001; Nakayama *et al.*, 2001).

The levels of regulation of epigenetic events in the preimplantation mouse embryo are multiple. They include the regulation of the subcellular localization of DNA methyltransferase activity, highlighted by the cytoplasmic retention of Dnmt1o (Carlson *et al.*, 1992); the exclusion of a particular histone modification from the chromatin, which is exemplified by the lack of detection of H3K9me3 in the paternal pronucleus after fertilization resulting in an asymmetry of histone marks between the two pronuclei (Arney *et al.*, 2002; Santos *et al.*, 2005), the differential incorporation of chaperons and histone variants in the parental chromatin (van der Heijden *et al.*, 2005; Torres-Padilla *et al.*, 2006) and the acquisition of highly specific histone variants in the gametes (Clarke *et al.*, 1992; Tanaka *et al.*, 2001; Govin *et al.*, 2007). Further, the maternal and paternal pronuclei exhibit different patterns of global DNA methylation: while the paternal pronucleus is rapidly demethylated—presumably through an active mechanism—right after fertilization, the maternal pronucleus is only passively demethylated through the subsequent rounds of replication and cell division that follow the first mitosis of the embryo (Mayer *et al.*, 2000). Moreover, while the centromeric and pericentric paternal chromatin remain DNA methylated, the maternal DNA loses methylation in such regions (Rougier *et al.*, 1998).

The changes in the levels of DNA methylation as development proceeds in the preimplantation embryo are dynamic. Global levels of DNA methylation have been analysed by immunofluorescence, bisulphate sequencing and restriction digestion (Rougier *et al.*, 1998; Mayer *et al.*, 2000; Santos *et al.*, 2002; Aranyi and Paldi, 2006). Bisulphate sequencing and restriction digestion have also been used to analyse the methylation status of repeat sequences (such as L1 and IAP repeats) and some single-copy sequences (such as actin) (Howlett and Reik, 1991; Oswald *et al.*, 2000). These studies have revealed that although global levels of DNA methylation decrease until the blastocyst stage, changes in DNA methylation do not occur to the same extent on all genes. Remarkably, imprinted genes (such as H19) and some repeat sequences (such as IAPs) do not undergo demethylation (Tremblay *et al.*, 1997).

During early stages of development, decisions involved in cell fate determination and pluripotency have to be assumed. These processes require the chromatin to be dynamically remodelled to ensure its plasticity. This implies that the mechanisms involved in regulation of chromatin structure need to ensure stability across generations and cell division, but they also need to be flexible (Reik, 2007). The double nature of a covalent modification either on histones and/or on the DNA as dynamic (because in principle it can be added and removed) and at the same time its potential ability to propagate a memory, fits well with these aforementioned needs. Moreover, in keeping with the importance of epigenetic mechanisms during early development, the possibility for an epigenetic mark(s) underlying these phenomena appears very attractive.

Concerning the epigenetic asymmetries of the ICM and the trophectoderm, the ICM displays, in global terms, higher levels of DNA methylation compared with the trophectoderm (Dean *et al.*, 2001; Santos *et al.*, 2002). Specific histone

marks such as trimethylation of lysines 9 and 27 of histone H3 (H3K9me3 and H3K27me3, respectively) are enriched in the ICM compared with the trophectoderm (Erhardt *et al.*, 2003). Likewise, the trophectoderm retains an imprinted form of X inactivation, where the paternal X chromosome is silenced (Heard and Disteche, 2006). This is in contrast to the ICM, where there is reactivation of the inactive X chromosome and a subsequent round of inactivation occurs at random in which either the maternal or the paternal chromosome is inactivated (Mak *et al.*, 2004; Okamoto *et al.*, 2004).

The aforementioned epigenetic asymmetries of the two lineages of the blastocyst are evident once lineage allocation has taken place and might reinforce their molecular identity. However, epigenetic asymmetry could also act as a *driver* for lineage allocation, in which case, the former would precede the latter (Fig. 1C). This constitutes a fascinating current working hypothesis.

Development of cell identity in the mouse embryo

From experimental embryology, we have learnt from pioneer experiments performed in the 50's that after mechanical separation of the blastomeres of a 2-cell stage embryo and transfer into foster mothers, each of these two cells gives rise to an adult mouse (Tarkowski, 1959). This indicates that mouse embryos are very flexible in what people have referred to as developmental potential. Derivation of twins from mouse blastomeres at later stages of development (e.g. 4-cell stage or later) has not been possible. Although this has been linked to the low number of cells present in the resulting embryos and hence their inability to form an ICM with a normal cell number, rather than to their developmental potential or identity (Tarkowski and Wroblewska, 1967; Rossant, 1976). Indeed, when random single 4- and 8-cell stage blastomeres are aggregated with 'carrier' blastomeres, their progeny is able to contribute to all the tissues of the embryo (Kelly, 1977; Tarkowski *et al.*, 2005), and in this sense, the blastomeres were considered to be totipotent. So, what are these carrier cells providing? Is it a simple matter of cell number? and/or of an appropriate environment? It is also possible however that some subtle intrinsic differences of these blastomeres might be masked by the limitations of the outcome of transplantation procedures, given that the results of these studies are very often far from 100% and the transplantation efficiency is never absolute. Whether this is solely related to technical difficulties linked to these challenging manipulations or to an intrinsic property of specific blastomeres of the embryo is impossible to ascertain.

Despite a controversial viewpoint on whether there is any polarity in the early mouse embryo or not, most reports coincide with the interpretation that a blastomere at the 2-cell stage contributes to both the ICM and the trophectoderm (reviewed in Edwards and Beard, 1997; Zernicka-Goetz, 2006). The suggestion of a given blastomere at the 2-cell stage having a 'preferential' fate towards either of the blastocyst lineage is not resolved and some researchers have suggested that a slight, but distinct difference in the fate of 2-cell stage blastomeres might be disturbed by experimental

manipulations (Alarcon and Marikawa, 2005; Hiiragi *et al.*, 2006). The role of extrinsic factors to the embryo, such as the shape of the zona pellucida, in axis specification of the blastocyst is also a matter of controversy (Gardner, 2007; Kurotaki *et al.*, 2007). However, there are some indications that a bias for a blastomere to contribute to a given region of the embryo in the blastocyst could already exist at the 4-cell stage (Fujimori *et al.*, 2003; Piotrowska-Nitsche and Zernicka-Goetz, 2005). These conclusions are mostly based on lineage tracing experiments of labelled blastomeres, and their degree of invasiveness is debatable.

Some groups have used the plane of division in relation to the animal–vegetal axis of the embryo as a sort of guideline to distinguish and characterize blastomeres according to their cleavage plane. By convention, the animal pole is demarcated by the position of the second polar body (which is extruded after resumption of meiosis II upon fertilization) and hence the vegetal pole lies on the opposite side (Fig. 2A). The division from the 2- to the 4-cell stage would segregate for the first time the 'animal' and the 'vegetal' components of the zygote if it occurs equatorially, that is, perpendicular to the animal–vegetal axis of the conceptus (Gardner, 2002). Thus, whereas a cell that derives from a meridional division (parallel to the animal–vegetal axis) inherits both components, an equatorial division gives rise to an 'animal' and a 'vegetal' blastomere (Fig. 2B). By looking into the plane of division and the order at which this division occurs from the 2- to the 4-cell stage and subsequent lineage tracing, a subgroup of embryos was identified where it is possible, with a relatively high probability, to predict the future position of the blastomeres in the blastocyst (Piotrowska-Nitsche *et al.*, 2005). This group of embryos are referred to as ME embryos (for Meridional– Equatorial, reflecting the type of cleavage plane and order of
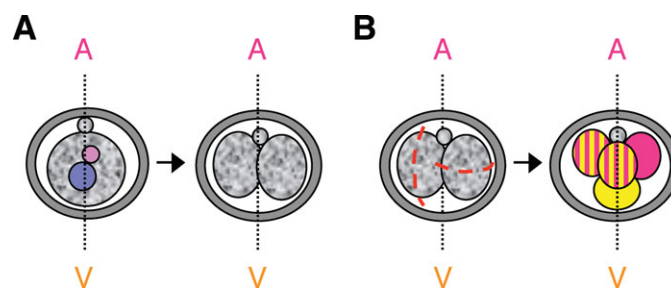

Figure 2: Blastomere division planes according to the animal-vegetal axis in the embryo

(**A**) The animal–vegetal (A–V) axis of the preimplantation embryo is demarcated, by convention, by the position of the second polar body, which marks the animal pole. The opposite side to the site of extrusion of the polar body is, by default, the vegetal pole of the embryo. (**B**) The division pattern from 2-to- 4-cell stage of a typical embryo that undergoes one meridional (M) and one equatorial (E) division (ME embryo) is represented. The cleavage plane is depicted by a red dashed line (embryo on the left). The blastomere that divides earlier is represented on the left. A Meridional division has a cleavage plane that is parallel to the A–V axis of the embryo and hence gives rise to two cells containing both 'animal' and 'vegetal' components (two cells with pink and yellow motifs on the embryo depicted on the right). In contrast, when a 2-cell stage blastomere divides equatorially, a segregation of the 'animal' and 'vegetal' cytoplasm occurs and follows derivation of an 'animal' (pink) and a 'vegetal' (yellow) blastomere

division that generated them (Fig. 2B) (Piotrowska-Nitsche et al., 2005; Piotrowska-Nitsche and Zernicka-Goetz, 2005). Although the ME type embryos constitute only a small part (20%) of a complete litter, they provide a very nice system where it is possible to explore the foundations of differentiation in the embryo. Indeed, the 'vegetal' blastomere would most often populate the abembryonic region of the blastocyst, which contains mainly mural trophectoderm.

In looking for epigenetic marks that could be involved in an eventual cell fate decision of the blastomeres of these ME embryos, it was found that the 'vegetal' blastomere displays the lowest levels of dimethylated arginine 26 of H3 (H3R26me2). If H3R26me2 participates in lineage allocation, one might predict that modulating the levels of histone arginine methylation, would have an effect over cell fate. Overexpression of the histone methyltransferase that methylates this residue on H3, PRMT4/CARM1, into individual blastomeres not only induced upregulation of Nanog and Sox2, but also resulted in an almost complete allocation of these blastomeres into the ICM compartment (Torres-Padilla et al., 2007).

It is interesting to note that in the mouse, the differences described in histone H3 arginine methylation appear at the 4-cell stage (Torres-Padilla et al., 2007), that is. as early as one cell cycle after the major wave of embryonic genome activation occurs (Schultz, 2002; Hamatani et al., 2004), which suggests that these events might be, at least in part, linked to the transcriptional programme of the embryo. The developmental stage at which genome activation occurs in other mammalian species varies considerably: it takes place at the 1-to- 2-cell stage in mice, the 4-to- 8-cell stage in cows and humans, and the 8-to- 16-cell stage in sheep and rabbits (Schultz and Heyner, 1992). Would this anticipate a different timing for an eventual 'cell fate path' for other species? Normal fertile adults can be derived from single blastomeres from 2-, 4- and 8-cell stage embryos in the rabbit, sheep and cattle (Moore et al., 1968; Willadsen, 1981; Willadsen and Polge, 1981). Thus, these species indeed support blastomere isolation and further development at later stages than the mouse does.

Transplantation of isolated 4-cell blastomeres into morula stage embryos has demonstrated that the blastomeres at the 4-cell stage are totipotent (Kelly, 1977). In this context, it is important to note that aggregating the 'vegetal' cell from ME embryos to form chimeric embryos, showed that this cell is able to contribute to all tissues in the embryo. However, aggregating the same blastomere with other 'vegetal' blastomeres from ME embryos exclusively, results in a failure to proceed through development (Piotrowska-Nitsche et al., 2005). Thus, the environment where the blastomeres develop is crucial for the success of the embryo throughout development, and in a 'normal' situation, where the embryo has not been perturbed and a given cell develops in its niche, some differences appear to develop, which are, at least partially, related to the epigenetic information of each of them (Torres-Padilla et al., 2007). If epigenetic asymmetries of the early embryo are related to lineage allocation, it is still uncertain whether they are a cause or a consequence for lineage choice. Also, it remains unknown whether such epigenetic asymmetries

would affect only particular regions of the genome. For example, whether genes involved in specification of the ICM such as Sox2 and Nanog would all be targeted by the same epigenetic marks in the same blastomere or whether such marks would vary among genes and/or among blastomeres. Further, are 'inner' and 'outer' cells at the 16-cell stage distinguishable in terms of their chromatin landscapes?

The experiments showing that blastomeres are able to respond to the overexpression of a histone modifier and change their fate, indicate that these cells have not yet acquired a 'fix' destiny, but that they can still be responsive to some kind of signals. These experiments have an important impact on showing that manipulating the epigenetic information can affect cell fate in the preimplantation embryo, in line with the importance of epigenetic mechanisms being crucial for early development. Moreover, these results do illustrate that such cells can still be flexible and accommodate themselves after a perturbing event (in this case, overexpression of a histone methyltransferase and the downstream effects on the information that is. imparted through specific histone modifications).

These studies have originated some interest from the part of the medical community, particularly, in the context of preimplantation genetic diagnosis (PGD) (Goldman, 2007). Does it matter to the embryo which cell is used for genetic diagnosis? Might the death of one of these cells have an effect on subsequent development? This question is equally valid on the impact of cell loss upon cryopreservation (Cohen et al., 2007). Although these are very delicate questions with very likely no easy answer, from the perspective of the mouse embryo, at least four things are to be considered. A tendency for a blastomere of some 4-cell stage embryos to contribute to a given region of the embryo has been documented. Second, the blastomeres in the 4-cell stage show clear differences not only in the levels of histone methylation, but also in their transcriptional activiy when they develop without being perturbed. However (third), the cells undergo a redirection of cell fate when a histone methyltransferase is overexpressed, indicating that they can readapt. Finally, the environment in which cells develop seems to be crucial for completing development and somehow the remaining cells in the embryo could compensate provided they are somehow different from each other. It is also important to note that a 4-cell stage mouse embryo might correspond to a very different developmental stage than a 4-cell embryo in other mammalian species, as illustrated by the differences in the onset of genome activation between them. Indeed, PGD is most often performed at the 8-cell stage and some reports document a better rate of development when 6-to- 9-cell stage embryos are diagnosed, as opposed to 3-to- 4-cell stage embryos (Wang et al., 2007). Moreover, the effects of in vitro fertilization procedures and culture on embryonic development are also extremely important, as they have been shown to alter epigenetic information in the mouse (Li et al., 2005).

As a final consideration, I would like to leave the reader with an open perspective of some ongoing questions in the field. Although there are strong indications that epigenetic mechanisms are involved in cell fate determination, we are still far from establishing a direct link between an epigenetic mark(s)

and the derivation of a particular cell lineage in the embryo. Much work is still to be done to determine how these mechanisms are set in play upon fertilization and how they are transmitted during subsequent cleavage stages. Also, what other epigenetic marks contribute to the inheritability of cell fate decisions? How do these marks relate to and influence each other? Are different lineage-specific genes marked by a different combination(s) of epigenetic marks? Do the marking of these genes occur at different stages of development? It is also tempting to expand these notions into the stem cell field and question whether these mechanisms would also underlie the intrinsic self renewal ability of adult stem cells and their potential to differentiate into other cell types. The exquisite complexity and richness of chromatin-regulated events in the early embryo will certainly be the subject of exciting research in the future.

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