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Allocation of inner cells to epiblast vs primitive endoderm in the mouse embryo is biased but not determined by the round of asymmetric divisions (8→16- and 16→32-cells)

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ABSTRACT

The epiblast (EPI) and the primitive endoderm (PE), which constitute foundations for the future embryo body and yolk sac, build respectively deep and surface layers of the inner cell mass (ICM) of the blastocyst. Before reaching their target localization within the ICM, the PE and EPI precursor cells, which display distinct lineage-specific markers, are intermingled randomly. Since the ICM cells are produced in two successive rounds of asymmetric divisions at the 8→16 (primary inner cells) and 16→32 cell stage (secondary inner cells) it has been suggested that the fate of inner cells (decision to become EPI or PE) may depend on the time of their origin. Our method of dual labeling of embryos allowed us to distinguish between primary and secondary inner cells contributing ultimately to ICM. Our results show that the presence of two generations of inner cells in the 32-cell stage embryo is the source of heterogeneity within the ICM. We found some bias concerning the level of *Fgf4* and *Fgfr2* expression between primary and secondary inner cells, resulting from the distinct number of cells expressing these genes. Analysis of experimental aggregates constructed using different ratios of inner cells surrounded by outer cells revealed that the fate of cells does not depend exclusively on the timing of their generation, but also on the number of cells generated in each wave of asymmetric division. Taking together, the observed regulatory mechanism adjusting the proportion of outer to inner cells within the embryo may be mediated by FGF signaling.

Introduction

The first cell fate decision in mouse embryonic development results in the formation of the inner cell mass (ICM) and the first extraembryonic tissue—trophectoderm (TE), which gives rise to the embryonic part of the placenta and to trophoblast giant cells. Separation of these two cell populations is initiated by two successive waves of asymmetric (differentiative) divisions occurring at the 8→16- and 16→32-cell stages (4th and 5th cleavage division) (Fleming, 1987; Johnson and Ziomek, 1981; Sutherland et al., 1990). In addition, in some embryos, there is a third round of asymmetric divisions at the 32→64-cell stage (Morris et al., 2010). Shortly before implantation ICM cells differentiate into two subpopulations: the epiblast (EPI) – a source of cells of the future definite embryo and the primitive endoderm (PE), which contributes to the endoderm layer of extraembryonic tissue – the yolk sac.

PE and EPI lineage determination was initially thought to be dependent on the outer or inner position of cells in the ICM (Becker et al., 1992; Dziadek, 1979; Martin and Evans, 1975). However, recent studies showed that the fate of cells in ICM is largely determined prior to positioning of the PE at the blastocyst cavity-facing surface of the ICM. It has been shown that EPI and PE cell progenitors with specific molecular 'identity' (i.e., expressing lineage-specific markers) are scattered within ICM in a random 'salt and pepper' pattern and are localized in both deeper and surface compartments of the early ICM (Chazaud et al., 2006; Meilhac et al., 2009; Plusa et al., 2008). Later in development, just before implantation, these two populations sort out to become the distinct layers: a monolayer of PE cells facing the blastocyst cavity and EPI occupying an inside position.

The source of heterogeneity of ICM cells and the mechanisms of their interaction leading to the segregation of EPI and PE progenitors and their allocation into appropriate layers remain obscure. Until the 64-cell blastocyst stage, expression of Cdx2, Gata6 and Nanog transcription factors, which are specific for TE, PE and EPI, respectively, overlaps in all cells of the embryo (Dietrich and Hiiragi, 2007; Plusa et al., 2008). As development progresses,

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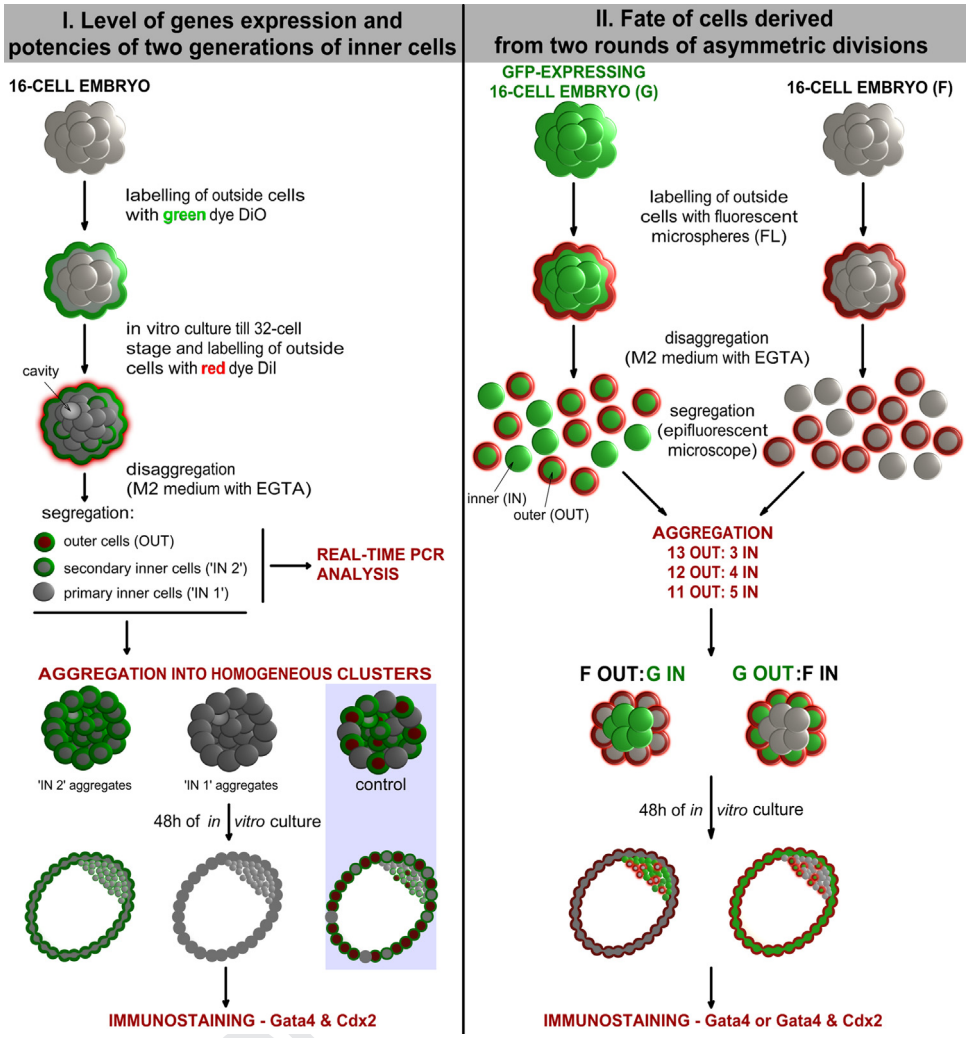

Fig. 1. Scheme of experiments (G—blastomeres derived from GFP-expressing embryos; F—blastomeres derived from GFP-negative embryos; OUT—outer blastomeres; IN—inner blastomeres).

reciprocal inhibition between these proteins leads to mutually exclusive expression and commitment of cells to differentiate in particular directions (Chazaud et al., 2006; Dietrich and Hiiragi, 2007; Guo et al., 2010; Kurimoto et al., 2006; Plusa et al., 2008; Strumpf et al., 2005). However, information when the final decision of EPI versus PE specification takes place and what are the factors responsible for this process is still missing. The study of Chisholm and Houliston (1987) began a discussion about the role of the time of inner cell generation in respect to whether a cell's fate was to differentiate into PE or retain pluripotency and contribute to the EPI. Inner cells directed to the nascent ICM are formed during the 4th and 5th cleavage division, i.e., at the 1st and 2nd wave of asymmetric divisions (Fleming, 1987; Pedersen et al., 1986) and are known as the primary and secondary inner cells, respectively. Analysis of the localization of cytokeratin filaments, which are a common feature of extraembryonic lineages (Brulet et al., 1980; Duprey et al., 1985), showed that the subpopulation of ICM cells possessing these filaments was almost exclusively derived from the 2nd wave of asymmetric division. This led to a hypothesis that PE cells originate from secondary inner cells formed during cleavage between 16 to 32-cell stage (Chisholm and Houliston, 1987). Two research groups have attempted to address this hypothesis using different experimental approaches and have presented partially contradictory results. Using time-lapse imaging to trace all cells of the embryo from the 8-cell to the late blastocyst stage, Morris et al. (2010) confirmed that the inner cells, from the 1st round of asymmetric divisions, are biased to generate EPI while cells internalized by the 2nd round have a preference to become PE. On the other hand, Yamanaka et al. (2010), who determined the division pattern of a single labeled blastomere in 8-cell embryo and traced its progeny after implantation, did not report any correlation between the cell origin and its fate linking it rather with the activity of fibroblast growth factor (Fgf)/mitogen-activated protein kinase (MAP kinase) pathway. There are several lines of evidence that formation of PE depends on Fgf-mediated activation of growth factor receptor bound protein 2 (Grb2) followed by the activation of MAP kinase signal transduction pathway (Chazaud et al., 2006; Frankenberg et al., 2011; Grabarek et al., 2012; Nichols et al., 2009; Yamanaka et al., 2010). However, the recent study reported the requirement of Fgf4 signaling not for the initiation of PE formation, but for the maintenance of PE cells in a subset of the ICM and establishment of a salt-and-pepper distribution of EPI and PE progenitors (Kang et al., 2013). Accordingly, one may presume that a combination of the previous cleavage history of blastomeres and their mutual interactions mediated by Fgf signaling may be responsible for the differentiation of cells within the ICM into PE and EPI lineages.

In the context of the apparently contradictory results of the earlier studies, we decided to study this problem by combining experimental and molecular approaches. First, we aimed to

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