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ESSENTIAL TRANSCRIPTION FACTORS FOR MOUSE BLASTOCYST STAGE EMBRYOS

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Abstract

Preimplantation is a process in which embryo is prepared for implantation to the wall of the uterus. This stage defines some morphological changes that occur in the first three days after fertilization during mouse development. It culminates in the generation of the blastocysts, which has a fluid-filled inner cavity and two distinct cell lineages. These two distinct cell lineages consist of trophectoderm (TE) and inner cell mass (ICM). While TE cells contribute to the placenta and the extraembryonic membranes and allow the blastocysts to implant in the mother's uterine wall, the pluripotent inner cell mass gives rise to the fetus. Some transcription factors such as Cdx2 and Oct4 (Pou5f1) have been identified in the mouse blastocyst to generate TE and ICM. Cdx2 is specifically expressed in TE and suppresses the expression of Oct4. Oct4, a key regulator of pluripotency, is strongly expressed in ICM and is essential for early lineage segregation. To present the localization of this transcription factors, we flushed 2 cell stage embryos from the oviducts and cultured to late blastocyst stage in medium. Samples were fixed and immunostained with mouse anti-Cdx2, goat anti-Pou5f1, then images were assessed using a fluorescence microscope to show the localization and presence of Cdx2 and Oct4 by immunocytochemistry. In this work, we demonstrated and reviewed the current knowledge on the Cdx2 and Oct4 in the formation of TE and ICM that is important for understanding the mechanisms of mouse embryo development.

Key words: CDX2, mouse blastocysts, OCT4, preimplantation, TE.

INTRODUCTION

Mammalian embryonic development is characterized by an initial preimplantation phase which extends from the fusion of the mammalian sperm and egg, namely fertilization and culminates in the generation of blastocysts. Fertilization and preimplantation development of eutherian embryos naturally occur in the oviduct of the mother and they can also be recapulated *in vitro* in a chemically defined culture medium. During the first three days of mouse embryogenesis, the zygote divides and changes morphologically, resulting in a blastocyst consisting of two distinct cell lineages: the inner cell mass (ICM) and the trophectoderm (TE). One or more cavities start to form and gradually expand between 16-32 cell stages to generate blastocyst, in which external cells become TE and internal cells become ICM (Marikawa Y et al., 2009; Marikawa Y et al., 2012). TE is a differentiated epithelium that is

responsible for implantation of the embryo into the mother's uterus and gives rise to placental tissues. The ICM is an undifferentiated mass of cells that will give rise to the whole embryo (Figure 1) (Bell C.E et al., 2013) .

In mouse, precursors of ICM and TE cell lineages begin to diverge very early in development and the position of the cells within the morula is important for this specification. After the fourth cleavage generates a total 16 blastomers, and this cleavage results in formation of two distinct populations of cells: the inner and outer cells (Kondratiuk I et al., 2012 ;Marikawa Y et al., 2009). According to the "Inside-Outside" Model, the cell fate in the blastocysts is established in the late morula and determined by its position; the outer cells are precursors of TE and inner cells become ICM (Kondratiuk I et al., 2012; Tarkowski et al., 1967). Another model for lineage determination is "Cell Polarity" Model, which suggests that cell fate is established at the eight cell stage, during the compaction. Another important point of this model is that cell fate is influenced by the polarity and this gives clues for the localization of the cells. A cell division parallel to the apical-basal axis (symmetric cleavage) produces two polar cells which occupy outside on the embryo and give rise to TE. A division perpendicular to the apical–basal axis (asymmetric cleavage) will produce one polar cell which is inherited to remain outside and becomes TE, and one apolar cell which stays inside and becomes ICM (Yamanaka et al., 2006). After the fifth cleavage, one or more cavities starts to form, which will gradually expand. At this point, embryo is named blastocyst (Figure 1). In addition to their polarity status, the inner and outer cells of mouse morula differ in the activity of specific genes (Kondratiuk I et al., 2012). And change in the cell fate determination at fifth cleavage is considered to be associated with an alteration in the expression of Caudal-like transcription factor (Cdx2) that is essential for normal TE development (Beck et al., 1995; Marikawa Y et al., 2009)). At around 8- to 16- cell stages, Cdx2 protein can be detectable in nuclei of all cells of the embryo. But the transition from 16- to 32- cell stage the level of Cdx-2 becomes stronger in the external blastomeres and weaker in the internal blastomeres. And Cdx2 protein is commited to external blastomers that will become TE cells at around 32 cell stage. (Marikawa Y et al., 2009). The other important gene is a POU domain trnscriptin factor, Oct4 (Yamanaka et al., 2006). Oct4 is the marker of pluripotent cells and is strongly expressed in ICM, while Cdx2 is exclusively expressed in TE (Stumpf et all. 2005; Szczepanska et al.,2011).

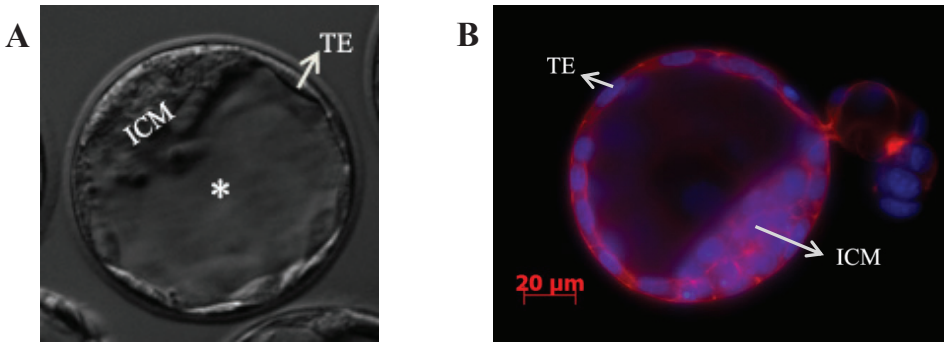

Figure 1. **A**: Image of a late stage blastocyst that is cultured in vitro. TE, trophectoderm; ICM, inner cell mass, (*), blastocyst cavity. **B:** Fluorescence microscopic images of a late blastocyst stage embryo that is stained for actin filaments (red) using fluorescently labeled phalloidin to label membranes. In the image actin filaments observed in cell-cell contact sites. Nuclei were stained with DAPI (blue). Scale bar =20 µm.

MATERIALS AND METHOD

Embryo Collection

Firstly, B6D2F1 female mice 6-8 weeks old, were induced to superovulate by intraperitoneally injections of 5 IU of equine chorionic gonadotropin (PMSG) and human chorionic gonadotropin (hCG) at 48 hours (h) apart. Female mice were mated overnight with fertile males of the same strain. Following morning, inseminated females were selected by the presence of vaginal plug. At 44 h after hCG injection, female mice were sacrificied by cervical dislocation and embryos were flushed from the dissected oviducts with FHM HEPES-buffered medium (MR-024-D;EMD Milipore) under the stereo-microscope. After that, embryos were cultured in 20µl drops of KSOM-AA medium (MR-121-D;EMD Milipore) under mineral oil in 3,5 cm plastic dishes at 37ºC in a 5% CO^2 humidified air incubator.

Immunofluorescent Staining

Embryos were fixed in 4% paraformaldehyde (PFA) solution in phosphate-buffered saline (PBS) for 30 minutes (min) at room temperature. Embryos were subsequently permeabilized in PBS containing 0.5% Triton X-100 for 15 min at room temperature. After blocking with 5% bovine serum albumin in PBS containing 0.1% Tween-20 (PBSw), samples were incubated in the primary antibody overnight at 4ºC and embryos were incubated in secondary antibody for 2-3 h at 25ºC. Primary antibodies used were, mouse anti-CDX2 (1:200; CDX2-88; BioGenex), goat anti-POU5F1 (1:200; N-19, #sc-8628; Santa Cruz Biotechnology), Secondary antibodies (1:1000; Life Technologies) used were conjugated with Alexa Fluor 546 namely rabbit anti-mouse, and conjugated with Alexa Fluor 488, namely rabbit anti-goat. F-actin filaments were visualized by adding phalloidin conjugated with Alexa 546 (Life Technologies) at a final concentration of

33 nM in the secondary antibody solution to visualize cell membranes. Stained samples were mounted in ProLong Gold antifade reagent containing 4',6'-diamidino-2-phenylindole (DAPI; Life Technologies) (Laeno AMA, 2013).

Microscopy and Image Analysis

Embryos were imaged using an Axiovert 200 fluorescence microscope (Carl Zeiss). Blastocysts were placed in the KSOM-AA drop and images were captured using Axiocam MRm digital camera, which was controlled by the Axio Vision software (Carl Zeiss).

RESULTS AND DISCUSSIONS

Cdx2 expression is known as a marker of TE and TE precursors (Stupf et al.,2005) and is absent from ICM in mouse blastocysts. (Stupf et al.,2005; Niwa et al., 2005). Oct-4 is a marker of pluripotent cells and is also expressed in TE cells of mouse blastocysts (Dietrich et al.,2007).

We examined the expression of Cdx2 and Oct4 in blastocyst stage embryos. Initially, embryos were incubated until blastocyst stage and were imaged using an fluorescence microscope. As expected, Cdx2 was localized exclusively to the nuclei of TE cells, while Oct4 was expressed and localized in nuclei of all cells in mouse blastocyst. We also stained blastocysts using two transcription factors (Cdx2 and Oct4) to confirm that Cdx2 can be used as a specific marker for trophectoderm cells. We did not observe any colocalization on ICM cells that showed us Cdx2 is TE specific. In blastocyst stage embryos, ICM cells exhibited strong nuclear Oct4 staining while TE cells are weakly stained.

The Cdx2 is one of the earliest transcription factors that is observed during lineage determination essential for formation and maintenance of the TE lineage in mouse blastocysts (Niwa et al., 2005; Sritanaudomchai H et al., 2009). In Cdx2, knockout embryos are capable of forming compaction, blastomere polarization and blastocyst cavity, showing that epithelization of TE is independent of Cdx2 (Marikawa Y et al., 2012, Stupf et al.,2005). At later stages, the fact that Cdx2 null embryos lose the TE and fail to sustain the blastocyst cavity shows that Cdx2 may be essential for the maintenance of epithelial integrity during this process (Marikawa Y et al., 2009; Marikawa Y et al., 2012).

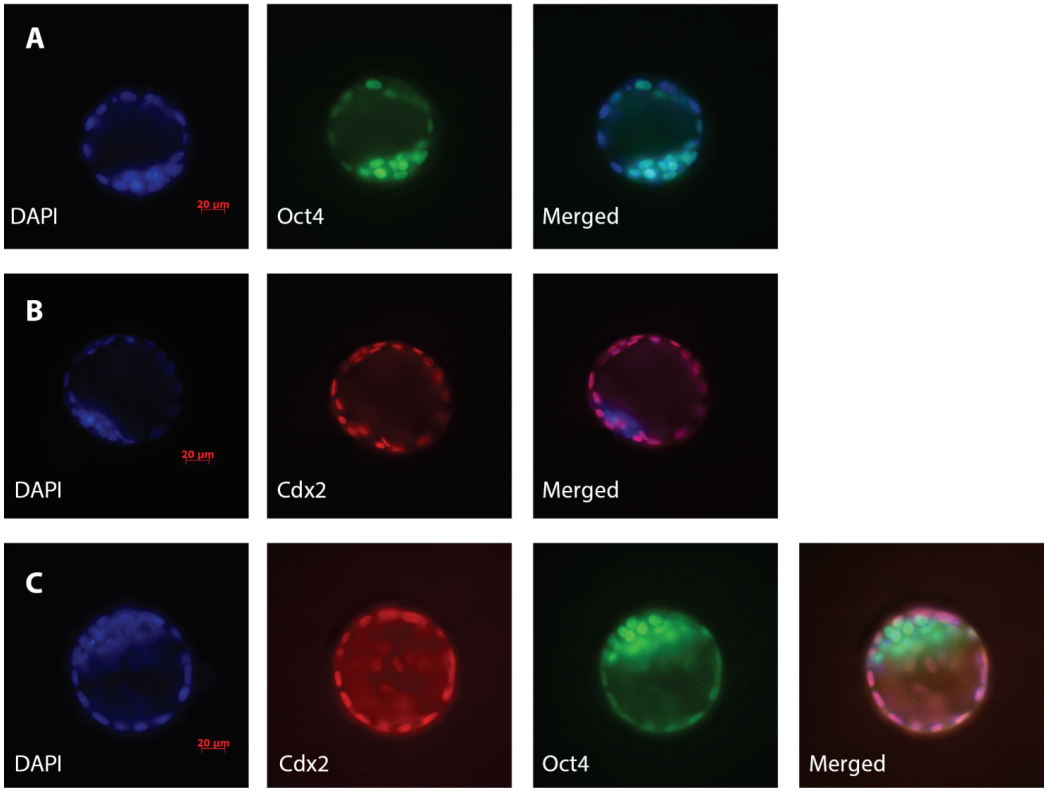

Figure 2. **A**. Fluorescence microscopic images of a late blastocyst stage embryos that is stained for nuclei with DAPI (blue), and for Oct4 protein with a specific antibody (green). **B.** Fluorescence microscopic images of a late blastocyst stage embryos that is stained for nuclei with DAPI (blue) and for Cdx-2 protein with a specific antibody (red). **C.** Fluorescence microscopic images of late blastocysts immunostained for Cdx-2 (red) and Oct-4 (green) proteins**.** Nuclei were stained with DAPI (blue). Scale bar =20 µm

Loss of Cdx-2 results in ectopic expression of ICM markers (Pou5f1 and Nanog) in the TE and an inability to maintain TE development (Stumpf et al., 2005, Stephenson RO., 2010). The early restriction of Cdx2 expression, along with its role in inhibiting the expression of ICM-specific transcription factors in the TE indicate that Cdx2 is an essential factor for the divergence of TE and ICM lineages (Stephenson RO., 2010). Although Cdx2 is required for the maintenance of TE, it is dispensable for the formation and maintenance of ICM (Marikawa Y et al., 2009). It has been suggested that Cdx2 expression is regulated by Tead4 (TEA-domain transcription factor) and Yap (Nishioka et al., 2008, 2009; Yagi et al., 2007). Tead4 is expressed in all cells of the embryo during mouse preimplantation development. Yap, which is co-activator partner of Tead4, is localized in the nucleus, and is restricted in developing TE, thus restricting CDX2 expression to outer cells of TE (Stephenson RO., 2010; Nishioka et al.,, 2009).

Oct4 is one of the cell fate determination factors during preimplantation development. It has been shown that Oct4 is essential to prevent ICM from diverting towards the TE lineage (Marikawa Y et al., 2009). Some transcriptional targets of Oct4 are Fgf4 gene and Nanaog gene. Nanog is a homeobox transcription factor that is essential for the

maintenance of pluripotency (Chambers I et al., 2003; Mitsui K et al., 2003).

CONCLUSIONS

Blastocyst stage is critical for implantation and maintaining the pregnancy. Implantation is also critical to the survival and development of the early embryo. A complication in this stage of development will lead to defective implantation and pregnancy. Our study demonstrated and reviewed that the transcriptional factors Cdx2 and Oct4 are essential and critical regulators of cell lineage determination and these factors are important to understand mechanisims of mouse embryo development.

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