

Roles of cell differentiation factors in preimplantation development of domestic animals

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Abstract. In mammalian embryos, the first visible differentiation event is the segregation of the inner cell mass (ICM) and trophectoderm (TE) during the transition from the morula to the blastocyst stage. The ICM, which is attached to the inside of the TE, develop into the fetus and extraembryonic tissues, while the TE, which is a single layer surrounding the fluid-filled cavity called the blastocoel, will provide extraembryonic structures such as the placenta. ICM/TE differentiation is regulated by the interaction between various transcriptional factors. However, little information is available on the segregation of the ICM and TE lineages in preimplantation embryos of domestic animals, such as cattle and pigs. This review focuses on the roles of cell differentiation factors that regulate the ICM/TE segregation of preimplantation bovine and porcine embryos. Understanding the mechanism of cell differentiation in early embryos is necessary to improve the *in vitro* production systems for bovine and porcine embryos.

Key words: Cattle, Embryo development, Gene expression, Inner cell mas (ICM)/ trophectoderm (TE) segregation, Pig
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Introduction

In vitro production (IVP) of bovine embryos, such as composite technologies of *in vitro* maturation (IVM) of oocytes, *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of embryos, has gained worldwide interest for their contribution to improving genetic gains in beef and dairy cattle. In 2016, approximately 1 million bovine IVP embryo transfers were completed worldwide (IETS Report, December 2017). Thus, IVP is already an important method in cattle production, and all indications are that this trend will continue [1]. However, pregnancy success in cattle after embryo transfer (ET) using IVP embryos is far from ideal. In bovine embryos obtained from IVP, a high rate of embryonic or fetal loss and the large offspring syndrome (LOS), the phenomenon of increased birth weight of newborn calves, has been consistently observed [1, 2]. On the other hand, pigs have attracted increasing attention as suitable sources for xenotransplantation, production of specific proteins by transgenesis, and biomedical models for studying human physiology and pathology. Successful piglet production from IVP embryos has accelerated progress in these areas. However, IVP of porcine embryos is still inefficient than that of other mammals, such as mice and cattle. This is attributed to low development rates to the blastocyst stage and the production of poor-quality blastocysts [3]. One of the reasons for the decreased development of bovine and porcine IVP systems

described above is limited knowledge of the molecular mechanisms involved in early embryonic development. Therefore, to improve the IVP systems for bovine and porcine embryos, it is important to focus on the molecular mechanisms underlying the regulation of early embryonic development.

Differentiation of unspecialized cells into other cell types is a crucial process of development. Thus, understanding the molecular mechanisms governing lineage segregation during early embryonic development is critical for elucidating fundamental developmental pathways. In early mammalian development, the first lineage segregation occurs during the transition from the morula to the blastocyst stage when blastomeres differentiate into the inner cell mass (ICM) and trophectoderm (TE). The ICM is a group of pluripotent cells attached to inside of the TE that gives rise to the embryonic tissue comprising the ectoderm, mesoderm, and endoderm [4]. In contrast, TE is a single layer of polarized cells surrounding the blastocoel, which gives rise to the embryonic portion of the placenta [5, 6]. The segregation of ICM and TE lineages is regulated by the interaction of various genes. In mouse embryos, the transcription factors, POU domain class 5 transcription factor 1 (Oct-4) and Caudal-related homeobox 2 (Cdx2) play pivotal roles in the segregation of the ICM and TE [7–9].

As described above, the molecular mechanisms that regulate the segregation of the ICM and TE lineages have been well characterized in mouse embryos. However, little information is available on the segregation of ICM and TE lineages in bovine and porcine embryos. Recently, some researchers reported that in contrast to mice, *OCT-4* expression does not appear to be restricted to the ICM, even in expanded blastocysts in pigs and cattle [10–13]. These findings led us to expect a difference in the molecular mechanisms that regulate the segregation of the ICM and TE lineages between species. This review focuses on the roles of cell differentiation factors in the

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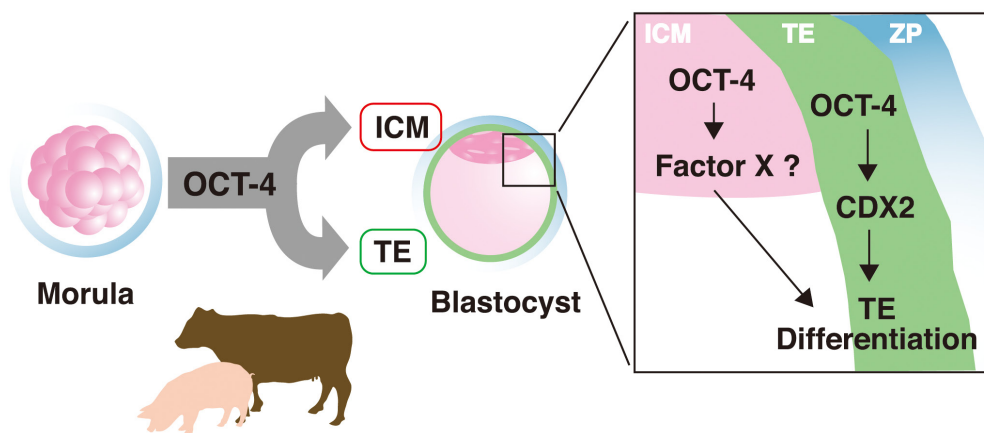


Fig. 1. OCT-4 and CDX2 are essential for development from the morula to the blastocyst stage and gene expression involved in the differentiation of ICM and TE lineages in bovine and porcine embryos. Especially, the continuous expression of OCT-4 in blastomeres is essential for TE formation in porcine embryos. The unknown factor(s) is indicated as Factor X.

preimplantation development of domestic animals by introducing several findings based on our studies [10, 14–20].

Roles of OCT-4 and CDX2 in Preimplantation Development of Bovine and Porcine Embryos

In murine embryos, the transcription factors Oct-4 and Cdx2 are necessary for the segregation and function of ICM and TE lineages. Oct-4, which is exclusively expressed in ICM after blastocoel formation, is required to maintain cell pluripotency and normal differentiation into the epiblast [7, 21]. Conversely, Cdx2 is a TE-specific transcription factor required for correct cell-fate specification and TE differentiation [9]. Murine embryos lacking Oct-4 or Cdx2 expression still form ICM or TE, respectively [9, 22, 23]. These findings indicate that both Oct-4 and Cdx2 function in the differentiation of ICM and TE after blastocyst formation. First, we demonstrated the differences in expression levels of several genes, including *OCT-4* and *CDX2*, between cells of the ICM and TE lineages in bovine and porcine embryos [10, 17]. The levels of *OCT-4* mRNA in the ICM of bovine and porcine blastocysts were higher than those in TE. In contrast, the levels of *CDX2* expression in bovine and porcine TE lineages were higher than those in ICM lineages. Thus, we concluded that OCT-4 and CDX2 might control the differentiation of ICM and TE in bovine and porcine embryos. However, OCT-4 expression was detected in both ICM and TE in cattle and pigs, even at the expanded blastocyst stage [10–13, 17, 24]. Although these observations suggest a distinct role of OCT-4 during early development in bovine and porcine embryos, little is known about the functions of OCT-4 and CDX2 during the embryonic development of domestic animals.

To elucidate the functions of OCT-4 and CDX2 during early development in bovine and porcine embryos, we performed OCT-4 and CDX2 downregulation using RNA interference [15, 18, 19]. We injected OCT-4- or CDX2-specific short interfering RNAs (siRNAs) into bovine or porcine zygotes. The blastocyst development rate in OCT-4-downregulated bovine and porcine embryos was lower

than that in uninjected or control siRNA-injected embryos [18, 19]. Gene expression analysis revealed decreased *CDX2* and *fibroblast growth factor 4 (FGF4)* expression in OCT-4-downregulated bovine embryos [19]. In murine embryos, FGF4 is highly expressed in the ICM and epiblast and activates the expression of FGF receptor 2 in the TE lineage [25, 26]. The FGF4 signaling pathway is required to maintain the proliferation of TE cells [27–29]. Furthermore, CDX2-downregulated bovine embryos developed to the blastocyst stage; however, in most cases, blastocoel formation was delayed [19]. In addition, we constructed chimeric embryos comprising blastomeres that either expressed *OCT-4* normally or showed downregulated *OCT-4* expression by co-injection of OCT-4-siRNA and tetramethylrhodamine isothiocyanate (TRITC)-dextran conjugate (Dx) into one blastomere in 2- to 4-cell stage porcine embryos [15]. In control embryos, co-injected with control siRNA and Dx, Dx-positive cells contributed to the TE lineage in almost all the blastocysts examined. In contrast, Dx-positive cells derived from a blastomere co-injected with OCT-4-siRNA and Dx degenerated in almost half the blastocysts. This was probably due to the inability of these cells to differentiate into the TE lineage.

We summarized both OCT-4 and CDX2 roles in the early embryo development of domestic animals in Fig. 1. Our results indicate that 1) OCT-4 and CDX2 are essential for early development and gene expression involved in the differentiation of ICM and TE lineages in bovine and porcine embryos, and 2) the continuous expression of OCT-4 in blastomeres is essential for TE formation in porcine embryos.

Roles of Several Factors Involved in The Hippo Pathway in Preimplantation Development of Porcine Embryos

In mice, TEA domain family transcription factor 4 (Tea4) is detected in nuclei from the 4-cell to the blastocyst stage [30]. Murine embryos lacking *Tea4* expression fail to form a blastocoel and do not express *Cdx2* [30–32]. Furthermore, expression of Oct-4 and SRY-related HMG-box gene 2 (Sox2) was induced in these Tea4-

deficient embryos [32, 33]. Therefore, Tead4 is a critical factor in TE segregation in murine embryos. In ICM progenitor cells, which are inside of embryos, the Hippo pathway is active, inducing cytoplasmic restriction of Yes-associated protein 1 (Yap1) via phosphorylation [34]. Contrastingly, the Hippo pathway is weakly activated in TE progenitor cells, which are outside of embryos [34]. In the outer cells, nuclear accumulation of Yap1 leads to form Tead4-Yap1 complex, and the complex induces *Cdx2* expression [34]. Thus, Tead4 regulates the segregation of the TE lineage through the expression of *Cdx2* in murine embryos. In murine embryos, *Cdx2* mutation leads to failure of TE maintenance [9, 35, 36], whereas *CDX2*-downregulated embryos of pigs and cows are able to develop normally to the blastocyst stage and form TE [19, 37, 38]. Furthermore, *CDX2*- and *TEAD4*-specific localization in the TE lineage starts from the ovoid stage, the next stage of the blastocyst [17]. On the other hand, *TEAD4* expression in porcine embryos has been observed from 4-cell stage, and *TEAD4* expression has been observed in both ICM and TE regions at the blastocyst stage [17, 39]. These results suggest that *TEAD4* controls the preimplantation development of porcine embryos through the expression of a specific factor other than *CDX2*. Therefore, we assessed *TEAD4* expression at both mRNA and protein levels in porcine preimplantation embryos and performed *TEAD4* knockdown to investigate *TEAD4* function during the early development of porcine embryos [16]. Nuclear localization of *TEAD4* protein was detected at the 16-cell stage, as well as at subsequent developmental stages. In porcine embryos injected with *TEAD4* siRNA, transformation from morula to blastocyst was inhibited. Although *TEAD4* downregulation did not affect the expression levels of *OCT-4*, transcription of *SOX2* was detected at high levels in *TEAD4*-downregulated embryos. It is possible that *TEAD4* contributes to blastocyst formation in porcine embryos through downregulation of *SOX2* expression.

The Hippo pathway controls various cellular events such as cell proliferation, differentiation, and cell death [40, 41]. Thus, this mechanism is one of the main regulators of ICM/TE lineage divergence in murine embryos [34, 42]. Activation of the Hippo pathway depends on cell position in preimplantation embryos. In the inner cells of murine morulae, the Hippo pathway is activated, allowing the activation of large tumor suppressor 1/2 (Lats1/2) kinases to phosphorylate Yap1 for preventing its nuclear accumulation [34, 42]. As a result, inhabitation of Yap1 transition into the nucleus occurs, and Yap 1 cannot bind to Tead4 which is a promoting factor for *Cdx2* expression. The complex of Tead4 and Cdx2 is essential for TE segregation [30–32, 34, 43]. Thus, in the absence of Tead4-Yap1 activity, ICM-induced genes such as Oct-4 and Sox2 are expressed in the inner cells [33, 40, 44–46]. On the other hand, the Hippo pathway is repressed and Lats1/2 is not activated in the outer cells. Thus, Yap1 is not phosphorylated and can be transferred into the nucleus. Consequently, it is indicated clearly that Yap1 binds to Tead4, and drives *Cdx2* expression is one of very important event for TE segregation [34]. As described above, we have demonstrated that blastocyst formation was inhibited in *TEAD4*-downregulated porcine embryos, and *SOX2* transcript levels were increased in these embryos [16]. These findings suggest that *TEAD4* is needed for TE segregation in porcine embryos and the Hippo pathway controls this mechanism in porcine preimplantation embryos, similar to mice. Therefore, we investigated in order to elucidate the roles of YAP1

and LATS2 in porcine preimplantation development [14]. In pigs, both *YAP1* and *LATS2* mRNA expressed higher levels in *in vitro* matured oocytes and 1-cell stage embryos, and decreased gradually with embryo development. Furthermore, we demonstrated YAP1 nuclear localization in porcine morula and blastocyst embryos. Interestingly, downregulation of either *YAP1* or *LATS2* by specific siRNA injection into 1-cell stage porcine embryos inhibited early development and affected the expression levels of *OCT-4* and *SOX2*. Therefore, we concluded that YAP1 and LATS2 are essential for porcine preimplantation development, and it is possible that the Hippo pathway has important roles in porcine ICM/TE segregation as murine preimplantation embryos [14].

We summarized the roles of several factors involved in the Hippo pathway in porcine embryos based on our findings in Fig. 2. Our results indicate the importance of *TEAD4* and regulation by YAP1 and LATS2, which are main components of the Hippo pathway, in early development and gene expression involved in the differentiation of ICM and TE lineages in porcine embryos.

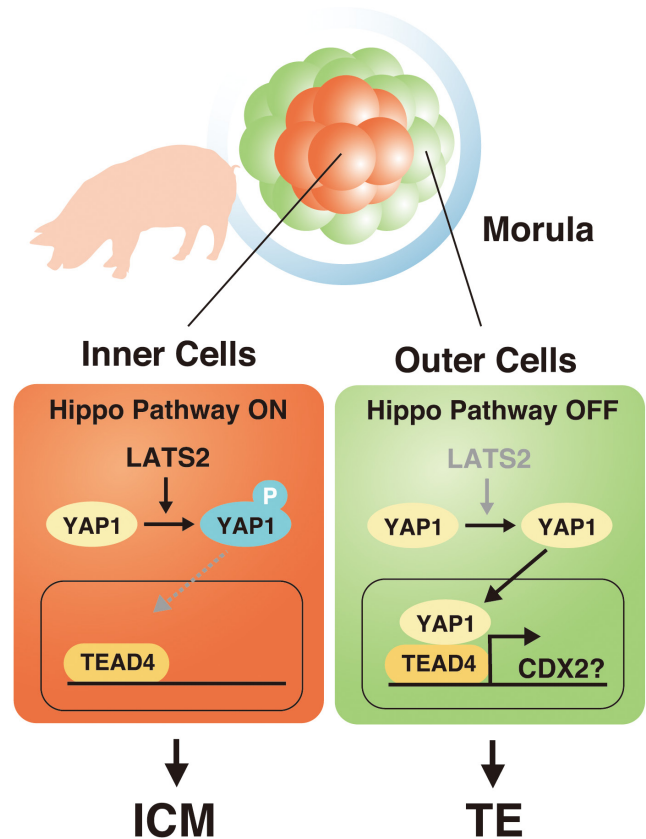


Fig. 2. A model of ICM/TE segregation mechanism in porcine embryos. Our results indicate the importance of *TEAD4* and regulation by YAP1 and LATS2, which are the main components of the Hippo pathway, in early development and gene expression involved in the differentiation of ICM and TE lineages in porcine embryos.

Conclusions and Perspective

Our results obtained from artificial downregulation of specific gene expression by RNA interference in early embryos indicated clearly a regulatory mechanism for the cell differentiation of ICM and TE lineages in domestic animals. Our observations led us to confirm some differences in the molecular mechanisms that regulate the segregation of the ICM and TE lineages between species. Further analyses of the expression of other factors and regulation mechanisms of these factors in the cell differentiation of preimplantation embryos are required. Furthermore, it is well known that bovine and porcine embryos elongate after the blastocyst stage. Therefore, to obtain a better understanding of the molecular mechanism responsible for the segregation of the ICM and TE lineages in domestic animal embryos, it is necessary to study changes in the expression of such genes during preimplantation development including the elongation stage.

Our findings and future analyses should be available for improving IVP systems for bovine and porcine embryos. For example, one of the causes of LOS, which is serious problems after ET of bovine embryos obtained from the IVP system, is aberrant gene expression status in these embryos [1, 2]. However, there are no suitable markers for evaluation of epigenetic status in bovine IVP embryos. We expect that the gene expression levels or status of several factors involved in the differentiation of ICM and TE lineages in bovine embryos will be valuable for assessment of bovine IVP embryos. To avoid the incidence of LOS offspring in the IVP system, we have approached the establishment of evaluation methods for bovine IVP embryos using gene expression analysis of these factors.

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