博 士 論 文 の 要 約

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Study on the role of zona pellucida in pre- and post-implantation development of mouse embryos

(マウス胚の着床前後の発生における透明帯の役割に関する研究)

The zona pellucida (ZP) plays various roles in embryonic development both *in vivo* and *in vitro*. The use of assisted zona hatching by physical cutting or enzyme-treated partial thinning of ZP is widely used for improving the pregnancy rate of human and bovine embryos after embryo transfer (ET). Recently, the zona pellucida removal (ZPR) is also applied for the early embryonic cleavage stage and is mostly used for blastomere separation for producing identical multiplets such as twins and quadruplets combined with genomic analysis, somatic cell nuclear transfer clones, chimeric animals, and improving the cytoplasmic fragmentation embryos. In the past many studies, ZF embryos after ZPR lose the cell-cell contact and show the flat blastomere structure compared with zona intact (ZI) embryos which show the 3-dimensional (3D) blastomere formation with limited development. Despite the overall need for ZPR for reproductive research, little is known about the effect of ZPR on pre-implantation development, differentiation, and post-implantation development including gene expression. In this study, I investigated the effect of ZPR in the pre- and post-implantation development of mouse embryos.

1. Establishment of the customized WOW (cWOW) system for zona free (ZF) mouse embryos

A previous study has shown that in human embryos, removal of the ZP and culturing in the WOW system for ZF embryos could reduce the cytoplasmic fragmentation, which may provide the major breakthrough needed for those patients who have difficulty obtaining quality embryos. However, a previous study reported cellular association at the ZF 4-cell stage embryos importantly affects the further differentiation of ICM and TE when using

multiple systems influencing subsequent embryonic development in mice. To date, several culture systems for ZF embryos have been developed including glass oviducts, and WOW, all designed to improve outcomes. In humans, although in vitro culture of embryos is an important step in assisted reproduction, the more culture system currently used in the research or commercial is Well of the Well (WOW). Despite the size and shape of the original WOW providing a practical and useful solution to improve the overall quality of cultured embryos, three-dimensional (3D) surfaces are currently used instead of the large flat bottom and microdroplets. Also, these microwells are designed for optical clarity rather than for the needs of ZI embryos, especially not for ZF embryos. Therefore, a culture system is required for supporting the development of mouse ZF embryos.

In this experiment, I investigated the optimized *in vitro* culture system for zona free mouse embryos by comparing the ordinary flat microdroplet culture, commercially available Well of Well (WOW) individual culture well system used for human and bovine embryos, and customized WOW with deep and small well suitable for mouse embryos with small size (cWOW). The cWOW system significantly improved the compaction process and blastocyst rate compared with the microdroplets system and commercial WOW *in vitro*. These results suggest the suitable well size for embryo size could be one of the important factors to keep the 3-dimensional embryo structure for further development and differentiation.

2. Effect of zona pellucida removal (ZPR) on the pre-implantation development in mice

After fertilization, the 2-cell embryos have totipotency and exhibit cellular plasticity at the 4-cell stage. Subsequently, the embryos undergo multiple cleavages to initiate compaction. After compaction, the first cell lineage segregation occurs at the time of blastocyst formation. Most of the outer cells form the trophectoderm (TE), whereas the inner cells become the inner-cell mass (ICM) in blastocysts, which is referred to as the first cell fate decision. When mouse embryos develop to d4.5, the cells located in the inner layer of the ICM become epiblast (EPI), and the cells located in the blastocoel become primitive endoderm (PE). ICM develops into the entire fetus, whereas TE forms the fetal portion of the placenta. During embryonic development, all transcription factors act as a network that influences and interact with each other, which is essential for the specification of distinct cell types. It is well known that the key regulators, OCT4, SOX2 and NANOG are essential for the formation and maintenance of ICM during mouse embryonic development as well as pluripotent embryonic

stem cells (ESCs) self-renewal. *Sox17* is an important marker of the PE and its derivatives, which are involved in developmental processes. In addition, the regulatory region of SOX21 is the target of OCT4 binding, and its expression is regulated by SOX2. SOX21 is involved in ESCs differentiation and the inhibition of *Cdx2* expression. Moreover, CDX2 a well-known transcription factor expressed specifically in TE, which represses the expression of the pluripotency regulator *Oct4* and vice versa.

The ZP plays diverse roles in embryonic development, both *in vivo* and *in vitro*. Although preimplantation development of ZF embryos has been investigated, little is known about the role of the ZP in early embryonic development and gene expression.

In this experiment, I investigated the effect of ZPR on pre-implantation development and gene expression of mouse embryos. After ZPR was performed, zona free embryos showed a faster compaction speed than control embryos. Expression analysis of inner cell mass (ICM)- and trophectoderm (TE)- specific genes has revealed that the expression of ICM-related genes was increased both in ZF-morula and blastocyst, whereas expression of TE-related genes was decreased by ZPR. After immunodetection, an increase in ICM- specific protein (OCT4) and a decrease of TE-specific protein (CDX2) were consistent with gene expression patterns in ZF embryos. The total cell number was significantly decreased in ZF embryos compared with ZI embryos, which was caused by decreasing in TE cell numbers.

3. Effect of zona pellucida removal (ZPR) on the post-implantation development in mice

The process of implantation includes three phases: apposition, attachment, and penetration. During implantation embryonic development, the polar TE proliferates and differentiates into the ExE and the EPC, which will contribute to the part of the placental tissues. On the other hand, a mural TE differentiates into primary trophoblast giant cell (TGC), which are necessary for embryonic implantation.

The ZPR procedure is currently used more often in human ET, which is performed at the blastocyst stage, as an assisted technology (AT) to the abnormal or thickened ZP that affects the implantation. Other research on ZP thickness, as an important factor influencing the implantation and pregnancy rates, has been conducted in humans. However, the effects of ZPR at the early stage are unclear. After ZPR at the 2-cell stage, there was a significant reduction in the TCN and a significant effect of ICM/TE differentiation at the blastocyst stage

during early embryonic development, and now, it is unclear whether ZPR will continue to have subsequent effects on post-implantation development in mice, so I mainly performed ET experiments to explore the effects of ZPR during post-implantation development.

In this experiment, I investigated the effect of ZPR on the post-implantation development of mouse embryos. After ET of the early blastocyst (E3.5) to each uterine horn (left/right) of recipient female mice at 2.5 days of pseudo-pregnancy, a lower rate of implantation and a lower number of live fetuses were observed in ZF embryos than in ZI embryos. There was no significant difference in fetal weights at E17.5 (17.5 days after ET). However, the placental weight of ZF embryos was significantly higher than that in ZI embryos. After collecting total RNA from placentas, RNA-seq analysis was performed. A total of 473 differentially expressed genes (DEGs) were significantly altered, including 279-upregulated and 194 down-regulated DEGs, which influenced more on the biological processes. Overall results suggest that ZPR affects post-implantation development, especially affecting placental growth.

4. **Effect of ZPR on the blastomere allocation at the early stage and subsequent development and gene expression**

A currently unresolved and critical study during mammalian development is when cells begin to differ from each other and whether these initial differences play any role in cell fate specification. In many experimental models, the initiation of cell-fate specification arises from heterogeneity between each blastomere, and the first cell-fate specification in the mammalian embryo leads to the separation of embryonic and extra-embryonic lineages. The embryonic lineage is pluripotent and will give rise to the fetus, whereas the extra-embryonic lineage will differentiate into the supporting structures, placenta, and yolk sac, which are essential for embryo implantation and fetal development. How and when these lineages begin to segregate in morphologically homogeneous cells is difficult to address. From reports of the previous studies, it appeared that the embryo had the regulatory ability to compensate for changes in cellular arrangement, and thus cells of early mouse embryos were thought to be identical in their ability to produce embryonic or extra-embryonic lineages. However, recent research has suggested that the blastomeres at the 4-cell stage become heterogeneous, exhibiting differences in developmental fate and potential and the activity of specific cell-fate regulators.

In this experiment, I investigated the effect of blastomere structure at the 4-cell stage due to

the loss of the ZP protection and the morphological changes from a 3D structure to a two-dimensional (2D) structure on the subsequent development and gene expression. The blastomere allocation of ZF 4-cell stage embryos was significantly different compared with that of ZI embryos, which were classified into four types (3P, 4P, 5P, 6P) based on the number of blastomere attachment surface sites. In contrast, most ZI embryos showed two types (5P and 6P), and 3P and 4P types were not observed. There was no significant difference in the blastocyst rates of ZF embryos showing 3P, 4P, 5P, and 6P types compared with ZI embryos. The total cell number (TCN) of the blastocyst developed from the 3P type was significantly lower than that of the ZI and other types, whereas the TCN of the 4P, 5P, and 6P types was not significantly different compared with the ZI blastocysts. In addition, the expression of Carm1 mRNA and protein, which has been recently discovered as a transcriptional regulator for differentiation, was significantly higher in ZF 4-cell embryos than in ZI embryos. At the blastocyst stage, the expression of DNA methylation-related genes (*Dnmt1, Uhrf1, Dnmt3a,* and *Dnmt3b*) was also significantly decreased in the ZF embryos compared with ZI embryos. These results suggest that the ZPR at the 2-cell stage affects the morphological changes of blastomere allocation in 4-cell embryos associated with TCN of the blastocyst and the expression of DNA methylation-related genes.

5. Establishment and evaluation of a new ZPR method for the development of ZF embryos

ZPR protocols are commonly performed as a single use of acid Tyrode's solution or pronase on their own with both options being reasonably popular due to their simplicity and relative cost-effectiveness. Given this, several efficient ZPR protocols have been developed for a variety of species, including humans, cows, and pigs with concentration or treatment time by considering the suitable chemical composition of each ZP. However, some chemical (i.e., acid Tyrode's solution or pronase) treatments used for ZPR can reduce developmental competence due to their toxicity with a long exposure time for complete ZPR to prevent insufficient digestion, or excessive digestion. This means that there is still a need for a stable, high efficiency, and low toxic ZPR protocol to produce embryos suitable for ART and research into embryonic development and differentiation.

In this experiment, I developed a new ZPR protocol by combining acid Tyrode's solution

and proteinase K treatment and subsequent culture in the cWOW system. Although acid Tyrode's solution treatment is commonly used for ZPR and reduces ZPR time, I found that the acid Tyrode's solution has detrimental effects on blastomere morphology such as wrinkled surface and on developmental competence with induction of apoptosis in the blastocyst. Besides, proteinase K treatment, which is also used for ZPR, increased ZPR time and significantly decreased the blastocyst rate, but did not increase apoptotic cell numbers or induce the expression of apoptosis-related genes. In contrast, a serial combined treatment (two-step method) significantly reduced ZPR time and improved blastocyst rate by increasing the TCN and reducing the apoptotic cell numbers. These results suggest that a new ZPR protocol is beneficial for reducing the toxicity of ZF embryo development and its quality.

In conclusion, I elucidated the effects of ZPR by acid Tyrode's solution not only keeps the blastomere structure for development but also affects the gene expression and differentiation of pre- and post- implantation development. In addition, suitable culture systems or reconstruction of blastomere structure for mouse ZF embryos will contribute to the development of assisted reproductive technology in the future.