Evaluation of morphokinetic characteristics of zona pellucida free mouse pre-implantation embryos using time-lapse monitoring system

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ABSTRACT The mammalian zygote cleaves and develops to blastocyst within the zona pellucida (ZP) in vivo. The presence or absence of ZP may affect the characteristics of the embryo, including blastomere alignment, cell-cell junction, and compaction. This study aimed to compare the morphokinetic characteristics of ZP-intact and ZP-free mouse pre-implantation embryos with a time-lapse monitoring system. Mouse 2-cell embryos were collected 1.5 days post coitum (dpc), and their ZPs were removed by treatment with acid Tyrode’s solution. All embryos were cultured in vitro up to the outgrowth stage at 7.5 dpc. In this study, ZP did not influence the cumulative times from 2-cell to further stages and blastulation. Interestingly, ZP-free embryos at 4-cell stage have three patterns of blastomere alignment according to the number of contact points between blastomeres. However, blastomere alignment did not lead to any differences in morphokinetic comparisons. Regardless of the presence or absence of ZP, embryos compacted after the 8-cell stage took shorter time to become blastocysts than embryos compacted pre-8-cell stage. Nevertheless, cell-cell junction proteins required for successful compaction were similarly expressed between ZP-intact and ZP-free embryos. ZP-intact embryos compacted post-8-cell stage had a higher rate of reaching blastocysts than compacted ZP-intact embryos before 8-cell stage, while the outgrowth/blastocyst rate was similar. In this study, the presence or absence of ZP did not influence embryonic development and expression of cell surface glycoproteins, whereas compaction timing may be one of the criteria for evaluating embryo quality. ZP-free embryos may become an alternative for overcoming cases with ZP problems in human ART programs.

KEY WORDS: timing of compaction, zona pellucida, developmental competence, time-lapse monitoring system

Introduction

The mammalian zygote cleaves and develops to the blastocyst stage within the zona pellucida (ZP) in vivo, coinciding with dynamic changes in morphology and gene expression (Govindasamy et al., 2019, Tosenberger et al., 2019). Before the blastocyst stage, blastomeres within the ZP are tightly bound together through a process known as compaction, and become individually indistinguishable (Cockburn and Rossant 2010, White et al., 2016). The compaction process usually occurs around the 8-cell stage in mice and humans. This process involves functional changes with expanding membrane channels and junctional formation serving as the intracellular communication pathway (Ma et al., 2009). E-cadherin/-catenin cell adhesion complexes appear at the time of...
compaction and facilitate the cells adhering to one another more tightly than previously.

The ZP is a thick extracellular elastic coat composed of long interconnected sulfated glycoprotein fibrils (Greve and Wassarman 1985). The ZP ultrastructure is net-like and permeable to large molecules, including some small viruses, through which they can gain entry into the embryo (Michelmann et al., 2007). The ZP has diverse biological functions, including protecting the oocyte from uncertain physical stresses during growth and ovulation, preventing not only polyspermic fertilization but also blastomere separation before compaction, protecting against viral and bacterial infections, creating a microenvironment through selective permeability to soluble factors in oviductal and uterine fluids, and others (Hunter 1976, Zusman et al., 1984, Suzuki et al., 1995, Van Soom et al., 2010). As aforementioned, physiological functions of ZP are essential for folliculogenesis and fertilization. However, it has been controversial in preimplantation development of mammalian embryos (Wassarman and Litscher 2008, Wassarman and Litscher 2012).

For successful implantation, the blastocyst must hatch from the ZP to invade the uterine endometrium. ZP hardening and thickening may occur as a consequence of in vitro fertilization-embryo transfer (IVF-ET), which may lead to low implantation and pregnancy outcomes (De Vos and Van Steirteghem 2000). Several studies have suggested ZP removal as an option to overcome fertilization and hatching failure due to an abnormal ZP structure in human embryos (Ueno et al., 2016, Nishio et al., 2006). However, procedures of ZP removal may damage the oocyte and affect the resulting rat embryo’s developmental potential (Okuyama and Funahashi 2012, Li et al., 2013). Despite this risk, Urman et al., reported that the absence of ZP on day 5 human embryos improved pregnancy rates in patients with poor IVF/ICSI (in vitro fertilization/intracytoplasmic sperm injection) prognosis (Urman et al., 2002). The roles of ZP in embryo fertilization, implantation, and development are still controversial within the field of human IVF-ET.

Embryo selection based on morphology remains the most common and generally accepted method in ART (Balaban et al., 2011). However, the spatial arrangement of the blastomeres within the embryos is not currently considered as a potential criterion for embryo selection (2011, Ebner et al., 2003). In human embryos, the arrangement of blastomeres at the 4-cell stage is associated with their developmental potential (Cauffman et al., 2014). Interestingly, the arrangement of blastomeres at the 4-cell stage may be changed by ZP removal. Unfortunately, the exact correlation between blastomere arrangement and embryonic development in ZP-intact and ZP-free embryos has not yet been fully investigated.

It has been found that compaction timing is a control of a major developmental transition in early mouse embryogenesis (Levy et al., 1986). Moreover, despite the development of a time-lapse monitoring system, few research groups have attempted to demonstrate the correlation between compaction timing and development (Iwata et al., 2014, Milewski et al., 2018). In addition, no study has yet to demonstrate the impact of compaction timing on murine embryo-

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**Fig. 1. Schematic illustration of this experiment.** Mouse 2-cell embryos were collected and their zona pellucida (ZP) was removed on 1.5 dpc. The patterns of blastomere alignment and stages of the compaction process were evaluated in both ZP-free and ZP-intact embryos. In vitro embryonic development was assessed by monitoring blastocyst formation and outgrowth from 1.5 to 7.5 dpc. Data were collected at the end of monitoring. Abbreviations: dpc, days post coitum; hCG, human chorionic gonadotropin; OG, outgrowth; PMSG, pregnant mare’s serum gonadotropin; ZP, zona pellucida.
Embryonic morphokinetics and development

Comparison of mean cumulative times and developmental competence according to the patterns of blastomere alignment at 4-cell stage embryos in ZP-free embryos

There were no statistical differences in the mean cumulative times from the 2-cell to OG stage among the three types of blastomere alignments at the 4-cell stage in ZP-free embryos (Table 2). The blastulation rates were similar between ZP-free embryos with four and more than four points of contact (P=0.15).

Comparison of mean cumulative times and developmental competence according to the timing of compaction in ZP-free and ZP-intact embryos

ZP-free and ZP-intact embryos were retrospectively assigned to two different groups based on the compaction timings (pre-8C; compaction before 8-cell versus post-8C; compaction after 8-cell). In ZP-free embryos, compaction before the 8-cell stage showed significantly longer mean cumulative times from the start of blastulation (tB) to the OG stage than embryos compacted after the 8-cell stage (Table 3, P < 0.01) in each developmental stage. Embryos compacted before the 8-cell stage also showed increased mean cumulative times from 2-cell to the morula, blastocyst, and

Results

Comparison of mean cumulative times and developmental competence according to the presence or absence of ZP

Mouse 2-cell embryos were cultured in vitro to the OG stage for six days (from 1.5 to 7.5 dpc), and the development rates of ZP-free and ZP-intact embryos were compared. There were no differences between the mean cumulative times for each developmental stage between the ZP-free and ZP-intact embryos (Table 1). The blastulation of ZP-free (89.2%, 91/102) and ZP-intact (85.2%, 92/108) embryos on 4.5 dpc was similar. However, the OG rate was significantly higher in ZP-free embryos at 88.2% (90/102), than in the ZP-intact embryos at 38.0% (41/108). The trophoblastic OG area was not significantly different between the ZP-free (11.8 ± 1.0 mm²) and ZP-intact (11.3 ± 0.5 mm²) embryos.

Patterns of blastomere alignment in ZP-free embryos at the 4-cell stage

ZP-free embryos have three (Fig. 3A, 22/102, 21.6%), four (Fig. 3B, 40/102, 39.2%), or ≥5 intercellular contact points at the 4-cell stage (Fig. 3C, 40/102 39.2%). Most embryos have more than three points of intercellular contact at the ZP-free 4-cell stage (Fig. 3D, 78.4%)
hatching blastocyst (HB) stage ($P < 0.05$) compared with embryos compacted after the 8-cell stage in the ZP-intact embryo.

In Table 4, ZP-intact embryos that compacted before the 8-cell stage showed significant decreased blastulation rate ($P < 0.05$), while there was no significance in before or after 8-cell stage compacted ZP-free embryos. However, the compaction timings did not affect the outgrowth/blastocyst rate in either ZP-free or ZP-intact embryos.

Furthermore, we observed the compaction process in both ZP-free and intact embryos according to their compaction timing shown before or after 8-cell stage. As shown in Table 5, compacted before the 8-cell stage of ZP-free embryos have showed the more incidence of incomplete compaction process than embryos compacted after the 8-cell stage ($P < 0.05$). It is suggested that timing of compaction process is important for completion in ZP-free embryos. Interestingly, the proportion of appearance compaction process was similar in ZP-intact embryos. The more tightly aligned blastomeres might affect the compaction process which affect the implantation potential.

**Localization of cell-cell contact molecules, β-catenin, and E-cadherin, in compacting embryos**

Fig. 4 shows immunofluorescence images for the β-catenin and E-cadherin of compacting embryos. β-catenin and E-cadherin were expressed and localized on the surface of each blastomere in both ZP-intact and ZP-free embryos. However, the compaction timings did not affect the localization of these molecules.

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**Table 2**

| Developmental stage | Pattern of blastomeres alignment (n=102) | P value |
|---------------------|------------------------------------------|---------|
|                     | 3-point of contact | 4-points of contact | ≥ 5-points of contact |
| Post-hCG (n)        | Post-hCG           | Post-hCG           |                      |
| t2 (from hCG)       | 46.0 ± 0.0 (22)    | 46.0 ± 0.0 (40)    | 46.0 ± 0.0 (40)      |
| t3                  | 53.6 ± 3.5 (22)    | 54.1 ± 5.1 (40)    | 52.5 ± 3.7 (40)      |
| t4                  | 54.8 ± 3.7 (22)    | 54.9 ± 5.3 (40)    | 53.4 ± 3.8 (40)      |
| t6                  | 67.5 ± 6.0 (21)    | 68.0 ± 6.2 (40)    | 66.3 ± 5.6 (35)      |
| t8                  | 70.2 ± 5.9 (21)    | 69.2 ± 6.3 (39)    | 67.4 ± 5.7 (35)      |
| Comp                | 73.9 ± 6.8 (21)    | 72.7 ± 5.7 (39)    | 71.0 ± 5.3 (35)      |
| tMo                 | 80.2 ± 6.4 (20)    | 80.0 ± 8.9 (39)    | 76.3 ± 5.6 (34)      |
| tSB                 | 93.5 ± 7.1 (20)    | 94.5 ± 7.7 (38)    | 90.9 ± 5.3 (33)      |
| tB                  | 98.6 ± 1.6 (20)    | 100.0 ± 8.2 (38)   | 95.7 ± 5.6 (33)      |
| tOG                 | 158.8 ± 14.8 (20)  | 159.0 ± 23.6 (38)  | 158.9 ± 13.5 (33)    |

Data represent as a mean ± SD. t2, t3, t4, t6, t8, time to 2, 3, 4, 6, 8 cells divisions, respectively; Comp; starting compaction; tMo, morula formation; tSB, starting blastulation; tB, expanded blastocyst stage; tOG, blastocyst outgrowth.

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**Table 3**

| Developmental stage | Pattern of blastomeres alignment (n=102) | P value |
|---------------------|------------------------------------------|---------|
|                     | ZP-free embryos                          | ZP-intact embryos |
|                     | Compaction before 8C stage (n=18)        | Compaction before 8C stage (n=17) | Compaction after 8C stage (n=76) | Compaction after 8C stage (n=77) |
| tMo                 | 84.6 ± 7.4                               | 80.7 ± 7.4 | 77.4 ± 5.3* |
| tSB                 | 99.5 ± 8.2                               | 97.2 ± 8.3 | 92.7 ± 7.0* |
| tB                  | 104.9 ± 8.2                              | 104.3 ± 8.5 | 97.5 ± 7.5* |
| tHB                 | -                                       | 116.6 ± 14.7 | 107.8 ± 13.0* |
| tOG                 | 171.3 ± 15.5                             | 172.1 ± 20.1 | 163.4 ± 12.3 |

Data represent as a mean ± SD. 8C, 8-cell; tMo, morula formation; tSB, starting blastulation; tB, expanded blastocyst stage; tHB, hatching blastocyst; tOG, blastocyst outgrowth. Asterisk (*) indicates a statistical significant difference by one-way ANOVA and Student’s t-test ($P < 0.05$).

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**Table 4**

| Developmental stage | Pattern of blastomeres alignment (n=102) | P value |
|---------------------|------------------------------------------|---------|
|                     | ZP-free embryos                          | ZP-intact embryos |
|                     | Compaction before 8C stage (n=18)        | Compaction before 8C stage (n=17) | Compaction after 8C stage (n=76) | Compaction after 8C stage (n=77) |
| BL rate (%)         | 17/18 (94.4)                             | 15/17 (88.2)* | 77/77 (100.0)* |
| OG/BL rate (%)      | 17/17 (100.0)                            | 7/15 (46.7) | 34/77 (44.2) |

Data represent as a mean ± SD. 8C, 8-cell; BL, blastocyst; OG, outgrowth; OG/BL, proportion of embryos developing to the outgrowth stage per blastocysts. Asterisk (*) indicates a statistical significant difference by Chi-square test or Fisher’s exact test ($P < 0.05$).
was observed only in the margin of the blastomeres. However, there was no differential finding between these two groups. These results demonstrate that the presence or absence of ZP does not have any detrimental effect on the embryo compaction process.

**Comparison of implantation potential after ZP-free or -intact embryos transferred**

Table 6 shows *in utero* implantation rate of ZP-free or intact embryos transferred. Even though no significant difference was observed between ZP-free and ZP-intact embryos, the implantation ability in the ZP-free embryos was slightly increased compared with the presence of ZP in the mouse embryo (P > 0.05). ZP removal might not affect the implantation potential *in utero*.

**Discussion**

Zona pellucida has important functions during sperm-egg recognition, binding, *in vivo* fertilization, and preimplantation embryonic development. For successful implantation, embryos need to be hatched-out from the ZP and implanted on the maternal uterine endometrium (Tu et al., 2014). Even though some of these functions are not essential during *in vitro* culture, a suboptimal *in vitro* culture condition may also affect ZP flexibility and elasticity, and consequently decrease *in vitro* hatching rates. Removal of ZP is an alternative method to prevent side effects derived from changes in ZP by using chemical reagents and enzymes (acid Tyrode’s solution or pronase), laser, or other techniques. (Trounson and Moore 1974, Ji and Bavister 2000, Wu et al., 2004, Park et al., 2014) Several studies have shown that ZP-free embryos can be cultured without compromising blastocyst formation rates, quality, and even epigenetic alterations. (Ribas et al., 2006, Lagutina et al., 2007, Ueno et al., 2014, Bodri et al., 2015) Moreover, a few cases have reported the successful pregnancy and delivery of ZP-free embryos in the human-assisted reproductive techniques (ART) program (Shu et al., 2010, Stanger et al., 2001, Ueno et al., 2014). In this study, we found that ZP removal from mouse embryos at the 2-cell stage did not decrease the developmental competence to the blastocyst and outgrowth stage embryos. The *in vivo* implantation rate was comparable between ZP-free and intact embryos (Table 6, p=0.115).

The ZP protects the integrity of the pre-compaction embryo during embryonic development. Suzuki et al., reported that the contact pattern in ZP-free embryos might affect their...
developmental competence (Suzuki et al., 1995). This conclusion was drawn from observing the blastomeres of ZP-intact and ZP-free embryos, where the former appeared to be more tightly packed than the latter (Katayama et al., 2010). Interestingly, Liu et al., demonstrated that embryos with fewer than six intercellular contact points at the end of the four-cell stage showed compromised subsequent development and reduced implantation potential in human IVF (Liu et al., 2015). The most of ZP-intact embryos have a tetrahedral shape with more than four intercellular contact points (Cauffman et al., 2014), but the number of intercellular contact points varied in ZP-free embryos. About 20% of ZP-free embryos have decreased contact points of blastomeres. ZP removal interrupts the increase in cell-cell contact resulting in the loss of the tetrahedral structure in ZP-free embryos. However, losing their tetrahedral shape in ZP-free conditions did not alter their developmental competence with unchanged localization of E-cadherin and β-catenin in morula which are known to be involved in the cell-junctional organization during embryonic development as shown in Fig. 4. (De Vries et al., 2004)

The compaction process is an essential step during pre-implantation embryo development. When embryos are compacted before reaching the 8-cell stage, they might fail to develop into further stages (Alikani et al., 2000). Iwata et al., reported that early compaction before the 8-cell stages with cytokinetic defects might be a cause of aberrant pre-implantation embryonic development in humans (Iwata, Yumoto, Sugishima, Mizoguchi, Kai, Iba and Mio 2014). During the compaction process, the cell boundaries progressively disappear until the embryo is fully compacted. However, in the fair quality of morula, the cell boundaries reappear with a few small cells or fragments, resulting in incomplete compaction. We observed that the occurrence of the incomplete compaction process increased in embryos that compacted before the 8-cell stage (Table 5). Previous studies by Wrenzycki et al., and Rizos et al., demonstrate that intercellular structures are altered in in vitro produced embryos, most likely due to a reduction in the expression of genes responsible for compaction and cell-cell adhesion, such as CX43 (gap junction alpha-1 protein) and CDH1 (cadherin-1), respectively (Wrenzycki et al., 1996, Rizos et al., 2003). As shown in Table 5, incomplete compaction process was the more observed in ZP-free embryos, while ZP-intact embryos showed similar rate. This result indicates expression of other junctional proteins might be affect depend on their intercellular structures during cleavage stage. Similarly occurred in about 20% of both ZP-free and intact embryos, which may have not changed genes related junctional proteins by ZP removal.

Our study compared spent time to develop into the blastocyst and OG stages according to compaction process before or after 8-cell stage. We found that the embryos that reached compaction before the 8-cell stage took longer time to develop into the blastocyst and OG stages compared to embryos with compaction after the 8-cell stage in both ZP-free and ZP-intact groups. Despite the sample size of outgrowth rate and compaction patterns was too small to be used in comparisons, only less than 20% (18/102 and 17/108) of embryos developed into blastocysts in ZP-free and intact groups. The compaction process is the one of important morphokinetic parameters to choose good quality of embryos for better clinical outcomes.

In present study, we tried to demonstrate the effects of morphokinetics including the timing of compaction and presence or absence of ZP during embryogenesis on the implantation process using embryos transfer. However, we only showed the implantation abilities of the embryos regardless of the presence or absence of ZP. Because the number of embryos compacted before 8-cell stage was not enough to make any significance. In the next study, implantation abilities of embryos by compaction timing would be demonstrated to give us more insights for clinical applications.

Conclusions

Collectively, our study monitored and evaluated the in vitro developmental competence of embryos with or without ZP using a time-lapse monitoring system. Our results demonstrate that the modification of ZP could be useful for various purposes without decreasing the developmental competence of mouse 2-cell embryos. Furthermore, we hypothesize that the appearance of compaction after the 8-cell stage in ZP-intact mouse embryos may be a positive indicator of developmental competence and pregnancy rate with the appropriate timing of compaction.

Materials and Methods

The overall scheme of this study is presented in Fig. 1.

Hormonal stimulation

Experimental animal protocols were approved by the Eulji University Institutional Animal Care and Use Committee (EUIACUC 12-19). Outbred ICR (Institute of Cancer Research) female mice (6 to 8 weeks old) were superovulated by the intraperitoneal injection of 5 IU serum gonadotropin from a pregnant mare (PMSG; Sigma, USA) followed by injection with 5 IU human chorionic gonadotropin (hCG; Sigma) 46 h later. Superovulated female mice were then mated with sexually mature males (8 to 20 weeks

### TABLE 5

| Patterns of compaction process in ZP-free and intact embryos | ZP-free embryos | ZP-intact embryos |
|------------------------------------------------------------|----------------|------------------|
| Before 8C stage (n=18) After 8C stage (n=76) | 16.7% (n=3) 60.5% (n=46) | 35.3% (n=6) 42.9% (n=33) |
| Before 8C stage (n=17) After 8C stage (n=77) | 16.7% (n=3) 26.3% (n=20) | 29.4% (n=5) 35.4% (n=27) |
| Fully compaction (normal) | 66.7%* (n=12) | 35.3% (n=6) 22.1% (n=17) |

### TABLE 6

| Implantation rates in ZP-free or -intact embryo transferred mice | ZP-free embryos | ZP-intact embryos |
|---------------------------------------------------------------|----------------|------------------|
| Implantation rate on 7.5 dpc | 63.3 ± 20.3% (n=6) | 43.3 ± 20.3% (n=6) |
old) and euthanized via cervical dislocation 46 h after hCG injection. The morning of the vaginal plugging day was designated as 0.5 days post coitum (dpc). In this study, ten independent experiments were carried out to monitor embryonic development using a time-lapse monitoring system. A total of 210 embryos, including 102 ZP-free and 108 ZP-intact embryos, were used in this study.

**Embryo culture and the preparation of ZP-free embryos**

Mouse 2-cell embryos were collected by oviduct flushing on 1.5 dpc and cultured in Quinn’s Advantage Blastocyst Medium (QABM; SAGE/Origio, Denmark) containing serum protein substitute (SPS; SAGE/Origio) in 5% CO2 at 37°C. The ZP of two-cell embryos was removed by treatment with acid Tyrode's solution (Sigma, USA) and immediately washed with QABM, then transferred into a WOW (well-of-well) dish for time-lapse monitoring. For the outgrowth (OG) of blastocysts, the culture media was replaced with Dulbecco’s modified Eagle medium (DMEM; Welgene, Korea) containing 10% fetal bovine serum on the third day of in vitro culture (4.5 dpc). The outgrowth of blastocysts was monitored for three days (from 4.5 dpc to 7.5 dpc).

**The time-lapse monitoring of embryo culture**

The in vitro embryonic development from 2-cell stage to OG was monitored using the Primo Vision time-lapse system (Vitrolife, Sweden) (Fig. 2). The camera for the time-lapse microscope was set to take a single image every 30 min for six days. Stages of embryo development were classified as 3-cell (t3), 4-cell (t4), 6-cell (t6), 8-cell (t8), morula (M0), blastocyst formation (IB), expanded blastocyst (TEB), blastocyst hatching (THB), and OG (IOG), as previously described (Minasi et al., 2016).

**The evaluation of developmental competence and outgrowth between ZP-free and ZP-intact embryos**

A total of 102 ZP-free and 108 ZP-intact embryos were observed in ten independent experiments and analyzed for this study. A comparative study by morphokinetics, including the mean cumulative times between cleavage stages, the blastulation rate on 4.5 dpc, and the proportion of embryos developing to outgrowth per blastocyst (OG/BL), was performed between ZP-free and ZP-intact embryos. In addition, the area of trophoblastic OG was measured on 7.5 dpc using Image J software (National Institutes of Health, USA).

**The classification and evaluation by intercellular contact points of ZP-free embryos at the 4-cell stage**

On 2.5 dpc, ZP-free embryos at the 4-cell stage were classified into three types according to the number of intercellular contacts. Embryos with three or more points of contact were classified, as shown in Fig. 3. Mean cumulative times and the rates of blastulation and OG were compared among these three types of blastomere alignments in ZP-free embryos at the 4-cell stage.

**Evaluation of developmental competence and OG according to the appearance of the compaction process in ZP-free and ZP-intact embryos**

ZP-free and ZP-intact embryos were assigned into two groups via time-lapse monitoring, based on compaction timing; embryos with compaction before the 8-cell stage and embryos with compaction after the 8-cell stage. The mean cumulative times from compaction to blastocyst were analyzed and the rates of blastulation and OG were compared between groups.

**The localization of β-catenin and E-cadherin in compacting embryos by immunocytochemistry**

To investigate the properties of ZP on the embryo compaction process, either ZP-intact or ZP-free two-cell embryos were cultured for 36-40 h. The compacting embryos were then fixed with 4% paraformaldehyde (Biosesang, Korea) for 30 min at room temperature (RT). Fixed embryos were washed with 0.1% PVA/PBS (Welgene, Korea) and permeabilized with 0.5% Triton X-100 for 30 min. After permeabilization, blocking was processed by incubating the embryos with 3% BSA/PBS for 30 min. The embryos were then incubated with either β-catenin (1:100, 712700, Invitrogen, CA, US) or E-cadherin (1:100, 3195s, Cell Signaling, MA, US) at 4°C overnight. The target proteins and nuclei were visualized by adding goat anti-rabbit Alexa Fluor 488 antibody (1:100, A11088, Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, CA, US), respectively, for one hour at RT. The images were obtained using confocal microscopy (Leica TCS SP8, Wetzlar, Germany).

**Evaluation of in vivo implantation potential in ZP-free and ZP-intact embryos**

To investigate the properties of ZP on the implantation potential, either ZP-intact or ZP-free two-cell embryos were transferred on 3.5 dpc to the contralateral uterine horn of pseudopregnant recipients after mating with vasectomized male. Transferred mice were sacrificed and implantations in uterine horns were assessed after 5 days.

**Statistical analysis**

The statistical significance of the two-group comparisons was analyzed by Student’s t-test or Fisher’s exact test. One-way analysis of variance or a chi-square test was performed for multiple comparisons. P values <0.05 were considered statistically significant.

**Declarations**

Ethics approval and consent to participate

Experimental animal protocols were approved by the Eulji University Institutional Animal Care and Use Committee (EUIACUC 12-19). All authors confirmed that all experiments were performed in accordance with relevant guidelines and regulations.

Availability of data and material

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

The author(s) declare no conflict of interest.

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**Authors’ contributions**

Kim, J: conception, experiment, writing, revision. Lee, J: conception, experiment, writing, revision. Choi, YJ: conception and experiment. Lee, J: conception, experiment, writing, revision. Lee, TB: data acquisition and analysis. Jun, JH: conception, writing, revision, correspondence.

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