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Full Length Research Paper

Non-intact zona improves development of murine preimplantation embryos transfected by an adenovirus vector

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The present study explored whether embryos could be transfected by the adenovirus-vector if the zona pellucida (ZP) was not completely removed. An adenovirus vector with green fluorescent protein (*pAd-GFP*) was used to transfect mouse non-intact zona zygotes (following partial removal of the ZP induced by pronase), zona-free and zona-intact embryos. Non-intact zona and zona-free embryos expressed GFP (confirmed with inverted fluorescence microscopy) after 48 h of culture. The transfection rate of non-intact zona group was up to 51% and the entire zona-free group was transfected. However, none of the zona-intact embryos was transfected. Regardless of whether non-intact zona embryos were transfected by *pAd-GFP,* their developmental rate (74.3 ± 2.4 and 69.2 ± 3.3% for non-transfected and transfected, respectively; mean ± SEM) was higher (P<0.05) than that of zona-free embryos without and with transfection (54.5 ± 4.3 and 46.7 ± 5.5%). Developmental potential of embryos was decreased for ZP-digestion (non-intact zona 71.8 ± 1.6%; zona-free 50.6 ± 2.2%, P<0.05) or pAd-GFP expression (non-transfected 64.4 ± 1.9%; transfected 56.0 ± 2.1%, P<0.05); therefore, ZP-digestion affected more intensely embryos development than pAd-GFP expression. In summary, non-intact zona murine embryos were readily transfected by the adenovirus-vector, and had much greater development potential than zona-free embryos. Although, the susceptibility of the ZP to digestion by pronase varied among embryos, on average, approximately 3.5 to 4.0 min of digestion resulted in partial removal of the ZP and promoted both transfection and satisfactory embryonic development. It is expected that this method could be used to increase the efficiency of generating transgenic animals.

Key words: Mouse, non-intact zona embryos, *adenovirus vector with green fluorescent protein (pAd-GFP)*, embryos development.

INTRODUCTION

Transgenesis in mammals is an important tool for recombinant protein production (Schnieke et al., 1997; Salamone et al., 2006) and for livestock improvement (Golovan et al., 2001). Presently, the challenge was to develop a highly efficient, low-cost technique for transgenesis. Virus-mediated transgenesis was more popular than microinjection because of greater efficiency and lower costs (Tsukui et al., 1996; Savatier et al., 1990; Di Fruscio et al., 1997). However, virus-mediated transgenesis was commonly required for removal of the zona pellucida (ZP) of early embryos (Tsukui et al., 1995; Gordon, 2002), which was unfavorable for development of embryos (Hiroshi et al., 1995; Ricardo et al., 2006),

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Abbreviations: ZP, Zona pellucida; **GFP,** green fluorescent protein; **BSA,** bovine serum albumin; **TNZE,** transfected non-intact zona embryos; **NTNZE,** non-transfected non-intact zona embryos; **ZFE,** zona-free embryos.

since the ZP had vital roles in preimplantation embryonic development *in vivo* (Bronson and McLaren, 1970; Modilinski, 1970). In this regard, the ZP could augment the tight junctions between blastomeres during compaction (Wassarman et al., 1999; Breed et al., 2002), protecting the early embryo from potential immunological rejection (Ozgur et al., 1998), and inhibiting their adhesion or fusion to the oviductal wall (Modilinski, 1970) or to other cells (Mintz, 1962). Further, it might act to protect against infiltration of leukocytes (Modilinski, 1970) and infection by bacterial or fungal agents in the genital tract (Singh, 1987). It is noteworthy that zona-free mouse embryos transferred to recipient mice at the one-, two- and four-cell stages did not implant (Modilinski, 1970), and developmental rates into fetuses that were carried to term were lower for zona-free than zona-intact embryos (Suzuki et al., 1987). Clearly, the ZP was important for developing zygotes into viable offspring. Although, virus vector transfection of zona-free zygotes had been used to produce transgenic animals (Tsukui et al., 1995), it was not efficient. Thus, optimization of this technique was clearly needed before it could be widely applied. If virus vectors could transfect zygotes with ZP, this would be an important technical innovation for introducing foreign genes into early embryos.

An adenovirus vector had been used to transfect boar sperm, and zona-free mouse embryos, and to generate transgenic offspring (Tohru et al., 1996; Farre et al., 1999). However, there were obviously no reports that an adenovirus vectors have transfected non-intact zona embryos (with the ZP incompletely removed). Therefore, the overall objectives of the present study was to explore whether embryos could be transfected by the adenovirus-vector if the ZP was not completely removed; whether the development of non-intact zona embryo was better than that of zona-free embryos. Some factors that produce non-intact zona embryos were determined and developmental potential of non-intact zona embryos was studied.

MATERIALS AND METHODS

Recombinant adenovirus vector

The replication-defective adenovirus vector, pAd-GFP, was made as described by Han et al. (2007). In this vector, the green fluorescent protein *(GFP)* gene was driven by the human cytomegalovirus (CMV) early promoter; viral titers of the preparation were diluted in KSOM (Millipore, Billerica, MA, USA) which is consistent with the requirements of the experimental design. In brief, two primers (forward: 5'-tttaagcttaccatggtgagcaagggcgag-3'; reverse: 5'-cccgatatcttacttgtacagctcgtccatg- 3') were designed to amplify the 735-bp GFP gene from the pEGFP-N1 vector (Takara, Shiga, Japan). The PCR product was digested with HindIII and EcoRV enzymes (Takara). Following gel purification, the PCR fragment was cloned into the corresponding sites of the pShuttle-CMV vector (Stratagene, CA, US). The AdEasy vector (Stratagene, CA, US) containing the adenovirus type 5 genome deleted for the E1 and E3 regions was first transformed into the ultral-competent BJ5183 bacteria to obtain the AdEasy bacteria. The plasmid pShuttle-GFP was then transformed to the AdEasy bacteria, yielding the pAd-GFP recombinant adenovirus vector. The pAd-GFP vector was linearized with PacI and purified with commercial purification kits (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. Viral stocks were produced by transient transfection of HEK 293 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The final virus stock was stored at -70°C until use.

Production of mouse embryos

Kunming mice were obtained from the Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China), and maintained under conventional conditions in a 14:10 h light : dark cycle. Mature (7- to 8-weeks-old) female mice were superovulated with 8 IU of pregnant mare's serum gonadotropin (PMSG; Ningbo Second Hormone Factory, Ningbo, China; # P1000) intraperitoneally, and 48 h later by 8 IU of human chorionic gonadotropin (hCG; Ningbo Second Hormone Factory; #P5000) and immediately placed with Kunming males (9- to 10-weeks-old) to mate. The following morning (about 15 to 16 h after hCG injection), mating was documented by the presence of a vaginal plug. Mated females were killed by cervical dislocation, the abdomen was incised, and presumptive fertilized ova were recovered from the ampulla. They were incubated at 37°C for 5 min with 3 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, MO, USA; # H-3884) in KSOM medium for removal of cumulus cells and subsequently washed in KSOM until the embryos were devoid of cumulus cells.

ZP digestion, embryo culture and infection

Zygotes with a second polar body were digested in 0.3% Pronase E (Sigma Chemical Co.) in droplets of KSOM at 37°C for 2 to 6 min. These zygotes were quickly transferred to KSOM containing 25% fetal calf serum (FCS; Invitrogen, Carlsbad, CA, USA) to halt digestion. Based on microscopic examination, the ZP of some zygotes disappeared (designated zona-free embryos, Figure 2), whereas the ZP was damaged but still present; these embryos were designated non-intact zona embryos (NIZE, Figure 1). After digestion and evaluation, the embryos were washed two or three times in fetal calf serum (FCS)-free KSOM, and transferred into 45 µl microdrops of KSOM medium containing pAd-GFP vector (titer, 1 x 10^7 PFU/mL). Then, the microdrops were covered with mineral oil and incubated at 37°C in a 5% CO_2, for 6 h. Thereafter, the embryos were washed 3 to 5 times in KSOM (temperature, 37°C) containing 3% bovine serum albumin (BSA) and cultured in KSOM containing 3% BSA at 37°C in a 5% CO_2, for 48 to 50 h. The embryos were examined with epifluorescence microscopy (Nikon 120, Tokyo, Japan), and transfected efficiency was calculated according to the percentage of embryos expressing green fluorescent protein. Thereafter, embryonic development was assessed at 4 h intervals from 84 to 120 h after mating. Embryos with a blastocele occupying greater than half their volume was designated blastocysts (Figure 3A). Control embryos, which were zona-intact embryos (Figure 3A) were cultured in the same manner as zona-digested embryos.

Experimental design

Duration of ZP digestion

The duration of exposure to pronase for ZP removal was critical for subsequent development of embryos (Mintz, 1962; Trounson and Moore, 1974; Ji and Bavister, 2000). In previous studies, the

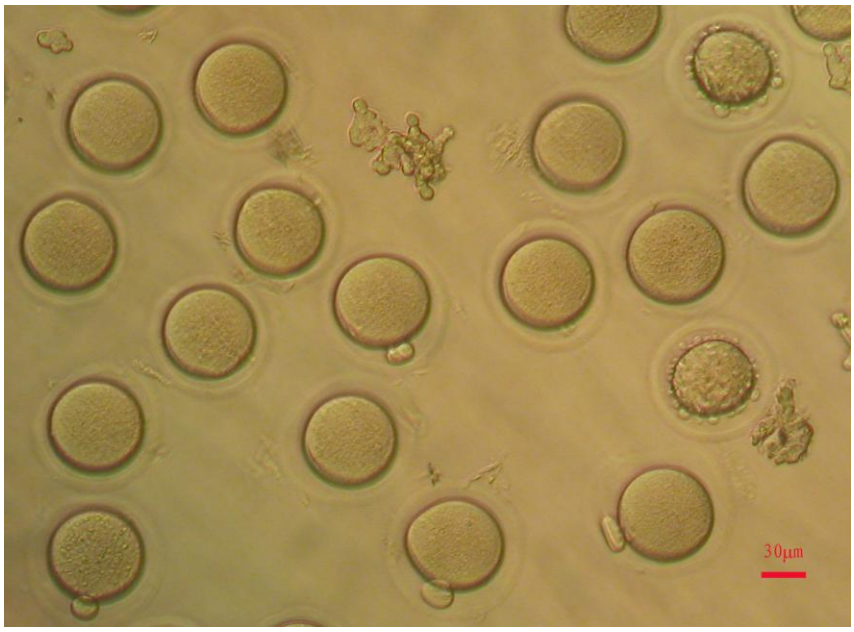


Figure 1. The zygote of non-intact zona embryos under epifluorescence microscope (Nikon 120, Tokyo, Japan) (10×10).

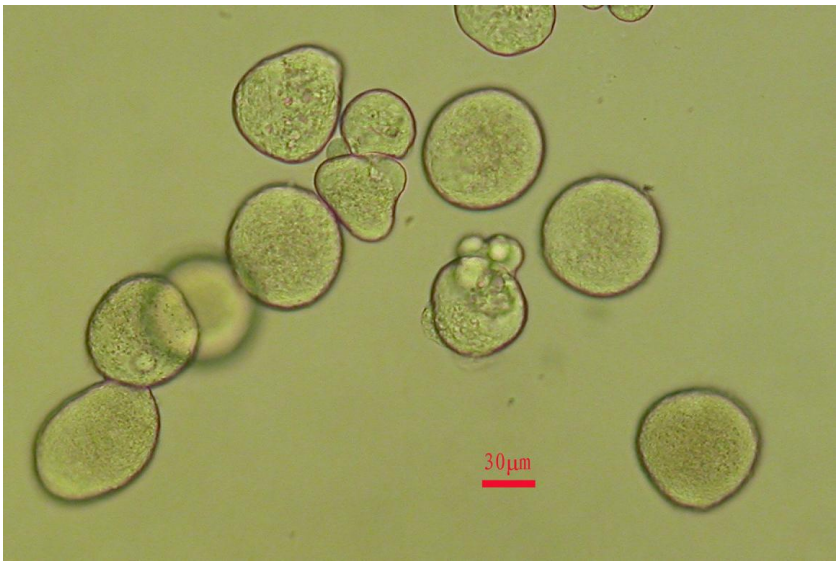


Figure 2. The zygote of zona-free embryos under epifluorescence microscope (Nikon 120, Tokyo, Japan) (10×10).

duration of exposure to pronase required to remove the zona was as short as 3 to 5 s (Ji and Bavister, 2000) or as long as 5 to 10 min (Hiroshi et al., 1995). In the present study, zygotes with a second polar body were digested with 0.3% Pronase E, and zona digestion was monitored microscopically. In Experiment 1, when the first zona-free embryo was observed, the digestion reaction was immediately terminated and digestion time was recorded. In Experiment 2, the digestion reaction was terminated as soon as all embryos had lost their ZP, and digestion time was recorded. The embryos of Experiment 1 or 2 were transferred into KSOM droplets containing pAd-GFP and cultured at 37°C in 5% CO_2, for 48 to 50 h, and transfected efficiency and cleavage rate was recorded. Culture was continued for 45 to 60 h, and the percentages of morulas (or blastocysts) and morphologically abnormal embryos were determined.

Improvement of transfection efficiency for non-intact zona embryos

Zygotes with a second polar body were digested with 0.3% Pronase E at 37°C; the duration of digestion ranged from 2 to 6 min, in

Table 1. Determination of duration of zona pellucidua digestion and its effects on embryonic development in mice (Experiments 1 and 2).

Experiment	Duration of digestion (min)	Transfection (%)	Cleavage (%)	Morulas (or blastocysts) (%)	Abnormal embryos (%)
1	2.0±0.41	17.3±2.2[a]	87.1±1.4	68.9±1.2[a]	0
2	6.0±0.32	89.5±1.3[b]	83.4±5.3	39.2±2.3[b]	25.3±3.3

Each mean is from four times replication, and each replication used ~50 embryos. The data is mean ± SEM except for digestion time (mean ± SD). [a, b]Within a column, values without a common superscript differ (P< 0.05). Transfection (%) = no. of transfected embryos/no. of cultured embryos; cleavage (%) = no. of two-cell embryos / no. of cultured embryos.

increments of 0.5 min. In other words, the zygotes were transferred into the pronase E solution and kept for 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 or 6.0 min, resulting in nine test groups. Groups of embryos were transferred into KSOM droplets containing pAd-GFP and cultured at 37°C in a 5% CO_2, for 48 to 50 h, and infection efficiency rate was determined using an inverted fluorescence microscope. Embryos were cultured for an additional 45 to 60 h, and percentage forming morulas (or blastocysts) was determined.

Developmental potential of transfected non-intact zona embryos

The highest rate of transfected non-intact zona embryos (TNZE) occurred after digestion of the ZP for 3.5 to 4.0 min. The following experiments were designed to determine the developmental potential of TNZE. The embryos were randomly divided into two groups: 1) a control group of intact-zona embryos; and 2) a group of embryos digested in 0.3% pronase for 3.5 min at 37°C; these were subsequently classified as non-intact zona and zona-free embryos. All embryos were transfected and cultured under the same conditions. Percentages of embryos undergoing cleavage and those forming a morula or blastocysts were recorded. In addition, percentages of morulas and blastocysts of non-intact zona embryos with or without green fluorescent protein were assessed under an inverted fluorescence microscope.

Zona pellucida digestion or pAd-GFP expression damage developmental potential of embryos

A two-factor crossover test was applied in the experimental design. The two-factors that affected developmental potential of embryos were ZP absence (presence) and GFP expressed (unexpressed). Embryos were digested in 0.3% pronase at 37°C for 3.5 min and divided into non-intact zona and zona-free embryos. Non-intact zona embryos were randomly separated into two groups; one group was transfected and cultured, whereas the other group was cultured without being transfected; zona-free embryos received the same treatment as non-intact zona embryos. All embryos were cultured under the same conditions, and percentages of cleavage and morulas (or blastocysts) were counted.

Statistical analyses

Data from all experiments were normally distributed (Kolgomorov-Smirnov test) and they had homogeneity of variance (Bartlett's test). Comparisons of two groups were done with an equal variance *t*-test for independent samples, whereas comparisons of multiple groups were done with ANOVA (with a Duncan's test to locate significant differences). All analyses were performed using SPSS®, Version 13.0 for Windows software (SPSS, Chicago, IL, USA). The level of significance was set at $P < 0.05$.

RESULTS

Determination of digestion duration of ZP

The duration for transfection of non-intact zona embryos was about 2 to 6 min distention time (Table 1). Transfection efficiency was lower in Experiment 1 than in Experiment 2 (P<0.05), but the rate of morula (or blastocysts) formation was significantly higher in Experiment 1 than in Experiment 2. In Experiment 1, 17% of non-intact zona embryos were transfected. Furthermore, developmental potentiality of non-intact zona embryos was significantly higher than that of zona-free embryos. In addition, morphologically abnormal embryos were only present in the zona-free embryos (Table 1).

Improvement of transfection efficiency for non-intact zona embryos

The percentage of transfected non-intact zona embryos (TNZE) increased when the digestion interval was up to 4.0 min, but developmental rate decreased (Table 2). The percentage of TNZE did not differ between digestion intervals of 3.5 or 4.0 min, but the percentage of each was higher (P<0.05) than that of other groups. Consequently, a digestion interval of 3.5 to 4.0 min was used for subsequent experiments. Moreover, the rate of morula (or blastocysts) production of non-intact zona and zona-free embryos both decreased as the duration of zona digestion increased, although the rate of morula (or blastocysts) formation for TNZE significantly decreased only when the duration of digestion exceeded 4.0 min (Table 2).

Developmental potential of transfected non-intact zona embryos

Zona-intact embryos were not transfected by pAd-GFP; however, approximately 46% of non-intact zona embryos were transfected with pAd-GFP, whereas 100% of

Table 2. The effect of duration of pronase digestion on transfection efficiency and embryonic development in mice.

Duration (min)	NTNZE (%)	TNZE (%)	ZFE (%)	Development of TNZE (%)	Development of TZFE (%)	Development of TTE (%)
2.0	82.2±0.51[a]	14.8±0.65[a]	3.0±0.75[a]	69.7±0.74[a]	54.3±0.55[a]	11.9±0.83[a]
2.5	58.3±1.12[b]	21.3±0.66[b]	20.4±0.67[b]	70.1±0.78[a]	51.6±0.58[b]	25.5±0.76[b]
3.0	36.7±2.35[c]	32.6±1.56[c]	30.7±0.86[c]	69.8±0.67[a]	48.5±0.49[c]	37.0±0.67[c]
3.5	9.3±3.32[d]	47.0±2.31[d]	43.7±0.94[d]	69.5±0.89[a]	45.4±0.53[d]	54.5±0.89[d]
4.0	0	51.6±2.24[d]	48.4±1.23[e]	64.6±1.21[b]	45.6±0.54[d]	53.4±0.88[d]
4.5	0	37.2±2.92[c]	62.8±1.12[f]	63.5±1.18[b]	42.5±1.21d[e]	48.3±1.56[e]
5.0	0	15.5±2.94[a]	84.5±2.31[g]	58.5±1.04[c]	40.3±1.02[e]	43.1±1.54[f]
5.5	0	2.8±0.12[e]	97.2±1.98[h]	56.8±1.12[c]	39.8±1.12[e]	40.3±1.45[g]
6.0	0	0	100	0	38.7±2.11[e]	38.7±1.43[g]

Each mean is from four times of replication and each used ~80 embryos (the data are mean ± SEM). [a-g]Within a column, values without a common superscript differ (P< 0.05). NTNZE (%) = Non-transfected non-intact zona embryo / embryos digested; TNZE (%) = transfected non-intact zona embryo / embryos digested; ZFE (%) = zona-free embryos/embryos digested; development of TNZE (%) = morulas of transfectious non-intact zona embryos / transfectious non-intact zona embryos; development of TZFE (%) = morulas (or blastocysts) of transfectious zona-free embryos / free-zona embryos.

zona-free embryos were transfected with pAd-GFP (Tables 2 and 3 and Figure 3B). The rate of morula (or blastocysts) formation for TNZE was significantly higher than that of zona-free embryos, but lower than that of zona-intact embryos and non-transfected non-intact zona embryos (NTNZE); and there were no difference between zona-intact embryos and NTNZE (P>0.05). Therefore, developmental potential of TNZE exceeded that of zona-free embryos (P<0.05), but remained lower than that of zona-intact embryos. Moreover, cleavage rate was not significantly different among groups, and only zona-free embryos had morphological abnormalities (Figure 3A). Therefore, the presence of a partial zona pellucida was beneficial for embryonic development.

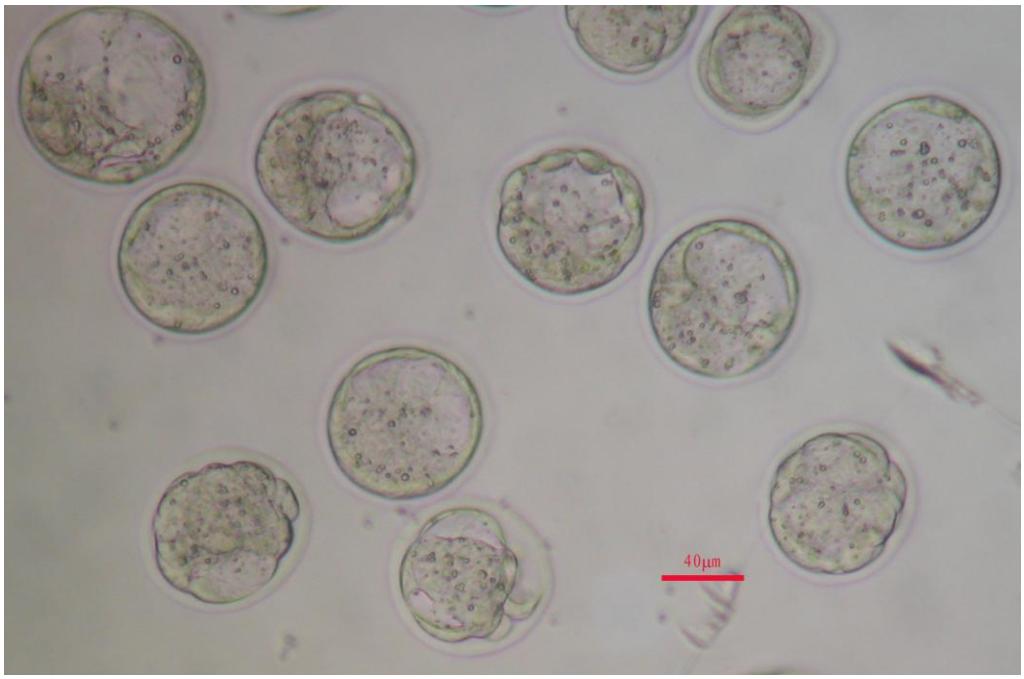
Zona pellucida removal or pAd-GFP expression damage developmental potential of embryos

Zona pellucida digestion and pAd-GFP expression might reduce developmental potential of embryos (Table 4). The morulas (or blastocysts) rate of non-transfected non-intact embryos were higher than that of transfected non-intact embryos (P<0.05), and the morulas (or blastocysts) rate of non-transfected zona-free embryos were higher than that of transfected zona-free embryos (P<0.05). Furthermore, all non-transfected embryos had significantly better embryonic development than all transfected embryos (P<0.05). Moreover, all non-intact zona embryos had better embryo development than all zona-free embryos (P<0.05). The difference of embryo development rate between non-intact zona embryos and zona-free embryos were greater than that between non-transfected embryos and transfected embryos (P<0.05). Therefore, ZP-digestion was more detrimental to embryos developmental potential than pAd-GFP expression. Moreover, ZP-digestion and pAd-GFP expression had interactions on the damage potential of embryonic development (P<0.05). However, the cleavage rate was not significantly different among groups.

DISCUSSION

The primary objective of the present study was to discover whether non-intact zona embryos could be transfected by an adenovirus-vector. In these study, the adenovirus vector transfected non-intact zona embryos; these non-intact zona embryos were obtained with digestion times of 2 to 6 min in culture medium containing 0.3% pronase E, and the proportion of transfected embryos increased as the duration of digestion increased. Moreover, zona-free embryos were all transfected (Figure 4A and B), whereas none of the zona-intact embryos was transfected (Figure 3A and B). In addition, ZP digestion and pAd-GFP expression suppressed developmental potential of embryos.

In a previous report, the embryos of ZP completely removed could only be transfected by vector of adenovirus (Tsukui et al., 1995), but the embryos of the ZP incompletely removed could be transfected by pAd-GFP in this study. The difference of external embryo morphology was not easy to be distinguished between both non-intact zona embryos and zona-intact embryo with an ordinary optical microscope (Figures 3A and 5A, a) because non-intact zona embryos and zona-intact embryos were all surrounded by the ZP. Nevertheless, after embryos was transfected by pAd-GFP, non-intact zona embryos had a green fluorescent (Figure 5B), whereas zona-intact embryo had no green fluorescent (Figure 3B). We thought that non-intact zona embryos could be transfected by pAd-GFP because of the special structure of the ZP. The ultrastructural studies had confirmed the three-dimensional, highly ordered

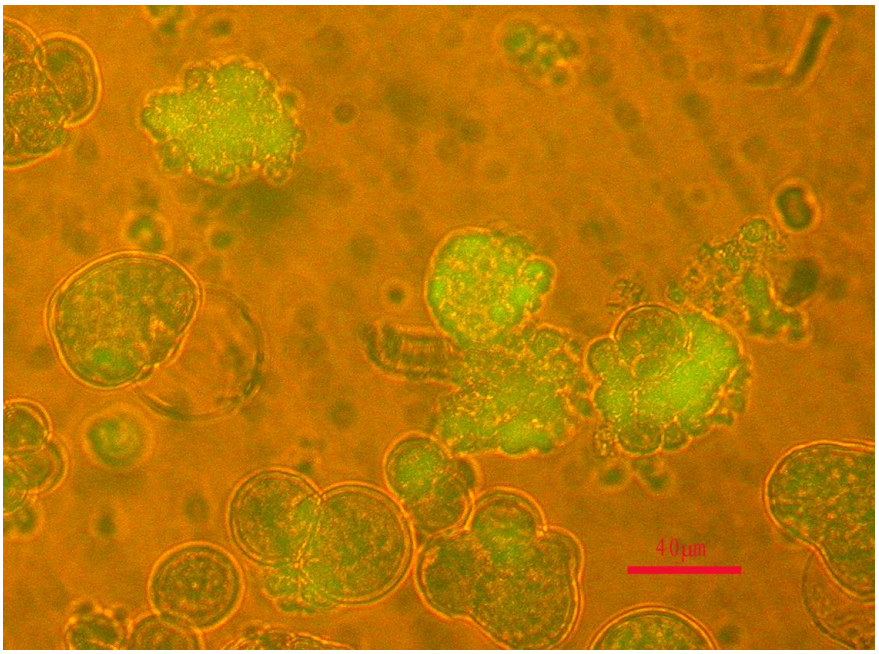


(A)

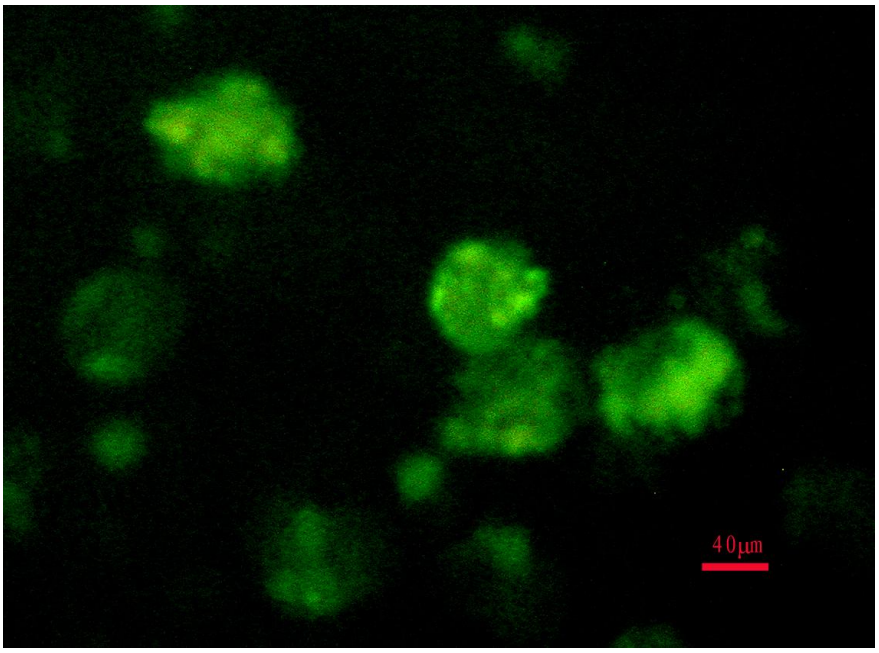
(B)

Figure 3. A. The morula or blastocysts of zona-intact embryos. The embryos were observed under natural light and blue light (490 nm) under epifluorescence microscope (Nikon 120, Tokyo, Japan) (20×10). When the natural light and blue light of microscope were simultaneously turned on, the embryo was observed. **B.** The zona-intact embryos were observed under only blue light (490 nm) with epifluorescence microscope (Nikon 120, Tokyo, Japan) (20×10). When the natural light was turned off (only blue was turn on), nothing was observed and It shows that zona-intact embryos cannot be transfected.

architecture of the ZP of mammalian embryos or oocytes (Familiari et al., 1992; Green, 1997; Wassarman et al., 1999, 2004; Wassarman, 2002; Oehninger, 2003). The ZP consisted of a paracrystalline, three-dimensional



(A)



(B)

Figure 4. A. The morula or blastocysts of zona-free embryos. The embryos were observed under natural light and blue light (490 nm) under epifluorescence microscope (Nikon 120, Tokyo, Japan) (20×10). When the natural light and blue light of microscope were simultaneously turned on, the embryo was observed as glow green. B. The zona-free embryos were observed under only blue light (490 nm) with epifluorescence microscope (Nikon 120, Tokyo, Japan) (20×10). When the natural light was turned off (only blue was turn on), all embryos were observed as strong green fluorescence.

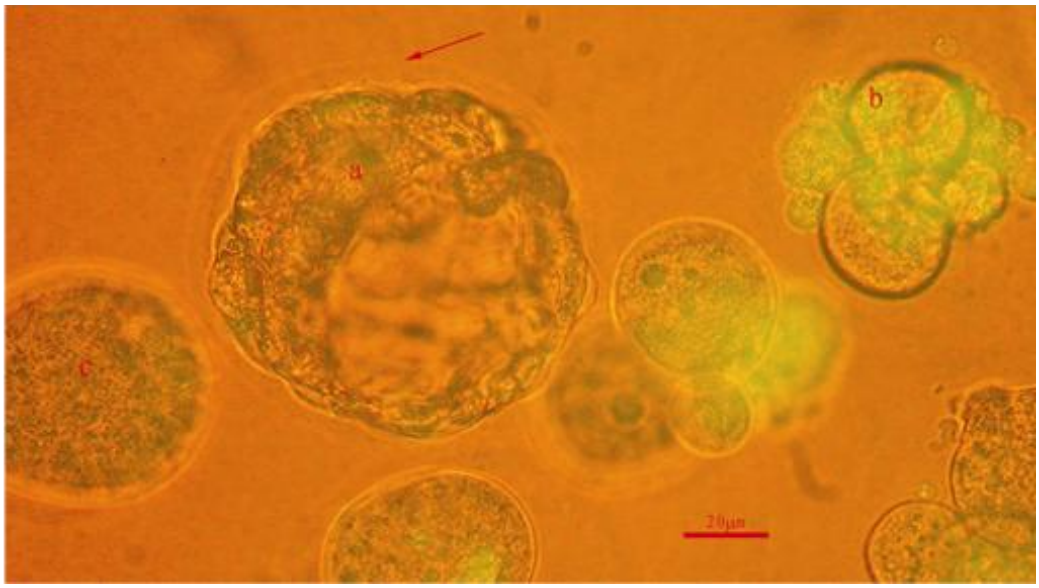
network composed of heterodimeric filaments of ZP_2 and ZP_3 proteins, cross-linked by ZP_1 proteins (Wassarman, 1988; Wassarman et al., 2004). Therefore, we had a novel hypothesis of ZP structural models that could

explain non-intact zona embryos being transfected by adenovirus-vector. We proposed that the ZP consisted of glycoproteins filaments; some glycoproteins filaments were digestible in solution containing pronases, whereas others were indigestible in solution containing pronases. The indigestible glycoproteins constituted the framework of the ZP and each other cross-linked a permeation network around embryos, whereas the digestible glycoproteins (acting as 'filler') bond to the mesh constituted indigestible glycoprotein. The indigestible glycoprotein and digestible glycoprotein constituted a permeable structure of ZP that allowed penetration of small molecules, but concurrently prevented the passage of macromolecules (viruses). It should be emphasized that indigestible glycoprotein stability was relative since the zona-intact embryos would become zona-free embryos if digestion time was sufficient (Tables 1 and 2). Therefore, we inferred that the non-intact zona embryos could be transfected by pAd-GFP because the digestible glycoproteins in the ZP were digested by pronase, whereas the indigestible glycoproteins remained because of insufficiency of digestion time. Consequently, the difference of external morphology was difficultly distinguished between both non-intact zona embryos and zona-intact embryo with an ordinary optical microscope (Figures 3A and 5A, a). When non-intact zona embryos were incubated with pAd-GFP, the pAd-GFP readily passed through the mesh and caught adenovirus receptor in the membrane of the embryo (Gordon, 2002; Nemerow and Steward, 1999). Then, adenovirus receptor mediated the pAd-GFP into the embryonic cells and pAd-GFP expressed green fluorescent protein. Perhaps, the main ingredient of digestible glycoproteins was derived from sperm-egg binding material since the molecules of cortical reaction protected embryos from entry of foreign genes (Camaioni et al., 1992; Zani et al., 1995; Spadafora, 1998). Moreover, the ZP susceptibility to pronase had apparent differences probably due to genetic differences or fertilization (Prasad et al., 2000). For example, some embryos were infected after they were digested for only 2 min, whereas others could be infected after they were digested for 6 min (Table 1). Similarly, some embryonic ZP disappeared after 2 min of digestion, whereas the ZP of other embryos disappeared only after 5 min of digestion (Table 2).

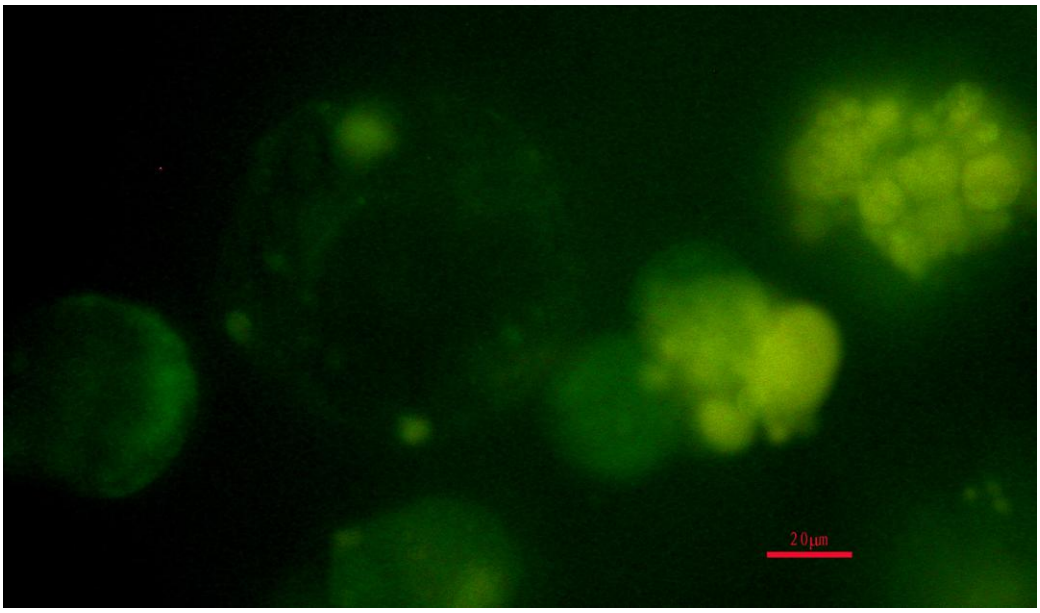
Although, many methods (Acidified Tyrode' solution, Laser assisted, micromanipulator assisted, and so on) could remove the ZP and acquire zona-free embryos, they hardly obtained the non-intact zona embryo. The unpublished data in our laboratory showed that only pronase could produce the ZP structure of non-intact zona embryos (Figure 5A, a and 5B). Additionally, acidified Tyrode' solution could obtain non-intact zona embryo but it was difficult to control precisely the digestion process (our laboratory, unpublished). Therefore, we utilized pronase digest ZP, acquired non-intact zona embryo, and mainly explored its digestion time. The pronase concentration had been studied in our laboratory and the data showed that 0.3% was the best operation concentration for non-intact zona embryos (unpublished). The duration of exposure to pronase for ZP removal was critical for subsequent embryonic development (Mintz, 1962; Trounson and Moore, 1974; Ji and Bavister, 2000). Although, there were differences among embryos in their resistance to pronase digestion, the largest proportion of non-intact zona embryos transfected was after digestion for 3 to 4 min. However, in previous reports, the time of pronase removing ZP had greater difference, in which 5 to 10 min might remove the ZP in some reports (Hiroshi et al., 1995), whereas other reports showed that 3 to 5 s might remove the ZP (Ji and Bavister, 2000).

Although, there was higher transfection efficiency for zona-free embryos, it did not match non-intact zona embryo on the cultured methods and development rate. All zona-free embryos in this study were transfected but the transfection rate of zona-free embryos was less than 100% in some reports (Hiroshi et al., 1995; Gordon, 2002). Perhaps in these studies, it was mistakenly believed that the time of pronase exposure was adequate but some embryos ZP were not digested completely, so these embryos could not be all transfected, and resulted to reduction of real rates of transfection. Another reason, perhaps is that, nonviable embryos were not excluded, and this resulted to low real rates of transfection. In the present study, zona-free embryos were all transfected as long as the embryo survived after digestion process. Although, all zona-free embryos could be transfected, embryonic development was significantly lower than non-intact zona embryo (Tables 3 and 4). Vajta et al. (2000) established well of the well (WOW) methods which could improve development of the zona-free embryos, but the operating procedures of this method were more complicated than that of droplet method. In addition, the embryo adhesion does not occur even if non-intact embryos were cultured in a droplet but zona-free embryos cultured in a droplet produce embryos adhesion. Our laboratory data indicated that if the zona-free and non-intact zona embryo were all cultured in the same WOW, the development rate of non-intact zona embryo was still significantly higher than that of zona-free embryos (unpublished). Therefore, we thought that zona-free embryos could not match non- intact zona embryo with the cultured methods and development rate.

In the present experiment, the morulas (or blastocysts) rates of zona-intact and non-intact zona embryos were both significantly higher than that of the zona-free embryos. Similarly, in previous studies, the rate of blastocyst formation of zona-free embryos was lower than that of the zona-intact embryos (Tan et al., 2007; Suzuki et al., 1995). It was reported that the ZP might prevent blastomeres from separating (Bronson and McLaren, 1970). Furthermore, cells of zona-intact embryos were more tightly packed than that of zona-free



(A)



(B)

Figure 5. A. The morula or blastocysts of non-intact zona embryos. The embryos were observed under natural light and blue light (490 nm) under epifluorescence microscope (Nikon 120, Tokyo, Japan). “a” Indicates a blastocyst of non-intact zona embryos; “b” indicates a zona-free embryo; “c” indicates an undeveloped embryo of non-intact zona embryos. The arrows indicate non-intact zona pellucida (40×10). When the natural light and blue light of microscope were simultaneously turned on, other embryo could be observed as green fluorescence (b), another embryo was observed as faint green fluorescence (a and c). Moreover, zona pellucida was clearly observed on embryo, (a and c; indicate with arrows). However, when the natural light was turned off (only blue was turn on), the embryos (b and c) were observed as a strong green fluorescence. B. The non-intact zona embryos were observed under only blue light (490 nm) with epifluorescence microscope (Nikon 120, Tokyo, Japan) (40×10). Embryo gleams green fluorescence (Figure 5B).

embryos (Suzuki et al., 1995). In mice, developmental rates were lower for zona-free embryos than zona-intact embryos (Suzuki et al., 1987), although the ZP was apparently unnecessary for hamster embryo development *in vitro* from the zygotic embryo stage to blastocyst (Ji and Bavister, 2000). Moreover, the birth rate of zona-free embryos after transplantation was significantly lower than non-intact zona embryos (our laboratory, unpublished).

Table 3. Comparison of non-intact zona embryos with zona-free embryos after pAd-GFP transfection.

Item	Cleavage (%)	Morulas (or blastocysts) (%)	Abnormal embryos (%)
Entire zona embryo	85.2±2.5	78.2±2.1[a]	0
Transfected non-intact zona embryo	82.8±2.1	69.2±3.4[b]	0
Zona-free embryo	81.5±2.7	46.5±4.5[c]	11.3±2.5
Non-transfected, non-intact zona embryo	82.7±1.9	75.9±1.2[a]	0

Each mean is from four times of replication and each used ~80 embryos. The data are mean ± SEM except for abnormal embryos, which are mean ±SD. [a-c]Within a column, values without a common superscript differ (P< 0.05). Cleavage (%) = no. of 2-cell embryos / no. of embryos.

Table 4. The effect of ZP removal and pAd-GFP expression on embryonic development (two-factor crossover test).

Item	Cleavage (%)	Morulas (or blastocysts) (%)
Non-transfected non-intact zona embryo	84.3±2.3	74.3±2.4[a]
Transfected non-intact zona embryo	82.6±1.3	69.2±3.3[b]
Non-transfected zona-free embryo	81.3±1.9	54.5±4.3[c]
Transfected zona-free embryo	82.3±1.7	46.7±5.5[d]
Non-intact zona embryo	83.5±1.5	71.8±1.6[w]
Zona-free embryo	81.8±1.3	50.6±2.2[x]
Transfected embryo	82.5±1.4	56.0±2.1[y]
Non-transfected embryo	82.8±1.3	64.4±1.9[z]

Each mean is from four times of replication, in which each used ~90 to 110 embryos; [a-d; w-z] Within a column, values without a common superscript differ (P< 0.05). Morulas (or blastocysts) (%) = Morulas + blastocysts / embryos of the group.

The ZP could augment the tight junctions between blastomeres during compaction (Wassarman et al., 1999; Breed et al., 2002), protecting the early embryo from potential immunological rejection (Ozgur et al., 1998), and inhibiting their adhesion or fusion to the oviductal wall (Modilinski, 1970) or to other cells (Mintz, 1962). Further, ZP might act to protect against infiltration of leukocytes (Modilinski, 1970) and infection by bacterial or fungal agents in the genital tract (Singh, 1987). Zona-free mouse embryos transferred to recipient mice at the one-, two- and four-cell stages did not implant (Modilinski, 1970), and developmental rates into fetuses that were carried to term were lower for zona-free than zona-intact embryos (Suzuki et al., 1987). In the present study, the blastocyst rate of non-intact zona embryos was lower than that of the zona-intact embryos. The micro-environment of embryos (e.g. the ZP and perivitelline space) might affect normal preimplantation development (Suzuki et al., 1995); consequently, exposure to pronase could destroy this microenvironment, resulting in suppressed development of non-intact zona embryos.

The expression of pAd-GFP and the removal of ZP reduced developmental potential of embryos in the current study (Table 4). In some previous reports, the toxicity of the adenovirus vector inhibited development of murine embryos (Kubisch et al., 1997; Gordon, 2002). Although, adenovirus vector toxicity was evident in the previous study (Gordon, 2002), the developmental rate of TNZE was significantly higher than that of TZFE in the present study. Thus, the presence of the ZP, even if it was not intact, seemed to mitigate the effect of the adenovirus vector on embryonic development, and non-intact ZP presence seemed to overcome adenovirus vector toxicity. However, ZP-digestion was more severe than pAd-GFP expression in suppressing developmental potential of embryos. For example, the developmental rate of TNZE was not significantly different with <3.5 min of digestion time, but developmental rate of TZFE appeared to decrease with digestion time increase. For zona-free, the pronase would further damage the plasmalemma of the zygote after ZP removal. The glycoprotein on the plasmalemma of the zygote, as an informational molecule, could be lysised or its function lost, resulting in abnormalities of signal transduction, material transport and intercellular interactions, thereby suppressing embryos developmental potential. Although, either expression of pAd-GFP and removal of the ZP could reduce developmental potential, we concluded that the main factor of harming developmental potential was removal of the ZP but not toxicity of the adenovirus vector.

We only discussed two problems: non-intact zona embryos could be transfected by pAd-GFP, and the advantage of non-intact embryos was more than that of the zona-free embryos in the present study. However, other problems (integrate of adenovirus-vector, multiple

insertions, constraints with size of transgene, choice of animal, transgenic animals and so on) did not belong to the scope of the present study. In fact, our laboratory had transfected non-intact zona embryos from mice, rabbits and pigs, and we obtained a similar rule (our laboratory, unpublished). In addition, we used other viral vectors transfecting mouse non-intact zona embryo; we also got a similar rule. These data would be published separately in succession. We again emphasized that this finding might be used to generate transgenic animals in future, but this paper was not a study on transgenic animals.

In summary, non-intact zona murine embryos were readily transfected by the pAd-GFP, and had much greater development potential than zona-free embryos. Although, the susceptibility of the ZP to digestion by pronase varied among embryos, on average, approximately 3.5 to 4.0 min of digestion resulted in partial removal of the ZP and promoted both transfection and satisfactory embryonic development. It is expected that this method could be used to increase the efficiency of generating transgenic animals.

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