Abnormal Development at Early Postimplantation Stage in Mouse Embryos After Preimplantation Genetic Diagnosis

YANG YANG,[1] YAN ZENG,[1] ZHUO LV,[2] RU WAN,[1] MAN TONG,[2] HUI ZHU,[1]*
LIU WANG,[2]* ZUOMIN ZHOU,[1] QI ZHOU,[2] AND JIAHAO SHA[1]

[1]State Key Laboratory of Reproductive Medicine, Department of Histology and Embryology, Nanjing Medical University, Nanjing, China
[2]State Key Laboratory of Reproductive Biology, Institute of Zoology, the Chinese Academy of Science, Beijing, China

ABSTRACT

Preimplantation genetic diagnosis (PGD) is an established procedure for the genetic analysis of embryos. To assess the effect of the procedure on early embryonic development, we generated a murine experimental system, including mice implanted with biopsied *in vitro* cultured embryos, control mice implanted with *in vitro* cultured embryos without biopsy, and mice with naturally conceived embryos. Embryos at the 7.5-dpc stage were isolated from all three groups and the embryo implantation rate, the survival rate of implanted embryos, and the developmental stage of surviving embryos were carefully assessed and compared among all three groups. We found the implantation rate was similar between biopsied and control group embryos (67.92% vs. 66.67%). However, the survival rate of implanted embryos in the biopsied group (49.31%) was significantly lower than that of the control (60.91%) and normal groups (96.24%) at 7.5 dpc. In addition, the survival rate of control group embryos was significant lower than that of normal group embryos. Classification of the precise developmental stages of randomly selected live implanted embryos at 7.5 dpc revealed no differences among the three groups. Our results indicate that blastomere biopsy does not adversely affect embryo implantation. The PGD procedure, in particular blastomere biopsy, increases the rate of embryo death at 4.5–7.5 dpc, but does not affect the development of surviving 7.5 dpc embryos. Anat Rec, 295:1128–1133, 2012. ©2012 Wiley Periodicals, Inc.

Key words: biopsy; blastomere; embryo; preimplantation genetic diagnosis; development; murine

INTRODUCTION

Preimplantation genetic diagnosis (PGD) has been a component of assisted reproductive technologies (ARTs) since 1990 (Handyside et al., 1990). The method is well established and is commonly applied as an effective alternative to traditional prenatal diagnosis in families with inherited disorders.

ARTs have been used widely in the treatment of human infertility. However, some processes involved in ART-mediated conception are very different from those of spontaneous conception, such as ovarian hyperstimulation and exposure of the embryo to tissue culture medium. Many investigations have indicated an increased incidence of genetic, physical, or development abnormalities

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*Correspondence to: Hui Zhu, State Key Laboratory of Reproductive Medicine, Department of Histology and Embryology, Nanjing Medical University, Hanzhong Road 140, Gulou District, Nanjing 210029, China. Fax: 86-25-86862908. E-mail: njzhuhui@njmu.edu.cn; or Liu Wang PhD, State Key Laboratory of Reproductive Biology, Institute of Zoology, CAS, 1st Beichen West Road, Chaoyang District, Beijing 100101, China. Fax: 86-10-64807299 E-mail: wangliu@ioz.ac.cn

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in ART offspring (Jackson et al., 2004; Schieve et al., 2004; McDonald et al., 2005; Wilson et al., 2011). Various factors are thought to contribute to these potential risks, including underlying parental subfertility, ARTs procedural factors, and higher maternal age. The relative contributions of these factors, especially the influence of the ARTs procedure, are still unclear (Amor and Halliday, 2008). Thus, the safety of ART procedures needs a systematically evaluation and the underlying mechanisms of those potential risks should be clarified.

Compared with other ARTs, the protocol required for PGD includes not only superovulation and embryo culture *in vitro* but also the more invasive biopsy procedure removing one or two blastomeres from the embryo. At present, research on PGD mostly focuses on making technical advancements to improve the accuracy and sensitivity of existing techniques and to develop new applications (Wilton, 2002; Wells and Levy, 2003; Hu et al., 2004, 2007). In contrast, relatively little evaluation of the influence of PGD on offspring development has been done, although the potential effects of *in vitro* fertilization (IVF) or intracytoplasmic sperm injection of eggs (ICSI) have attracted more attention. Although some clinical investigations have shown that blastomere biopsy does not produce negative effects on neonatal health outcomes (Handyside et al., 1990; Desmyttere et al., 2009; Winston and Hardy, 2009), it is difficult to convince the public that this technology will not cause adverse effects on the offspring. Therefore, a systematic and long-term assessment of PGD remains to be done.

In our previous study, we assessed the potential risk of blastomere biopsy on offspring using a mouse model. The results indicated that the development of biopsied embryos to the blastocyst stage was similar to that of control embryos following *in vitro* culture. However, when biopsied and control embryos were transferred into pseudopregnant mice, we found that the birth rate of biopsied mice was significantly lower than that of control mice (Yu et al., 2009), which suggested that biopsy manipulation may affect postimplantation development of embryos.

Mouse embryo implantation begins approximately 4.5 days postconception (4.5 dpc), at which stage the blastocyst is composed of three distinct tissue lineages: trophectoderm, primitive endoderm, and epiblast. At this time, the decidual reaction initiates and induces the formation of a spongy mass of decidual cells around a single embryo. Once implantation has been achieved, a dramatic increase in the growth rate of the embryo occurs. At about 6.5 dpc, the bilaminar embryo begins to transform into a multilayered, three-chambered conceptus in a process known as gastrulation. At 7.5 dpc, the three primary germ layers (ectoderm, mesoderm, and definitive endoderm) are formed as a result of gastrulation and the basic body plan and primordial organs are established (Nagy et al., 2003a). Therefore, the stage between 4.5 and 7.5 dpc is critical for mouse postimplantation embryo development.

In this study, we aimed to define the effect of the PGD procedure, especially biopsy manipulation, on postimplantation embryonic development by comparing three groups of murine embryos: normally conceived, biopsied, and control. Given that considerable cell division and differentiation occurs between 4.5 and 7.5 dpc, we selected the 7.5 dpc embryonic stage to observe the influence of artificial manipulations on postimplantation development. Differences between biopsied and normal group revealed potential effects due to the entire PGD procedure, and specific effects resulting from blastomere biopsy were evaluated by comparing the biopsied and control groups.

MATERIALS AND METHODS

The Mouse Model

ICR mice were used throughout this study. All experiments requiring the use of animals were approved by the ethical board of Nanjing Medical University and Institute of Zoology, Chinese Academy of Sciences. Mice were divided into three groups: biopsied, control, and normal. For the normal group, a female in estrus was placed with a male and checked the following morning for successful mating as evidenced by the presence of a vaginal copulation plug. For biopsied and control groups, female mice underwent ovarian stimulation and embryo transfer. Cleavage-stage biopsy and embryo transfer were performed as previously described (Yu et al., 2009).

Ovarian Stimulation

Female mice were given an intraperitoneal injection of 5 IU (0.1 mL) pregnant mare serum gonadotropin, followed 48 hr later by 5 IU (0.1 mL) of human chorionic gonadotropin (HCG). After the HCG injection, each female was individually placed with a male and checked the following morning for successful mating as evidenced by the presence of a vaginal copulation plug. Ensuing zygotes were dissected from the oviducts at the appropriate stage.

Cleavage-Stage Biopsy and Embryo Transfer

Groups of zygotes were transferred into a droplet of N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered CZB (Chatot, Ziomek, and Bavister) medium containing 5 mg/mL cytochalasin B. One blastomere was removed randomly from each four-cell embryo with an enucleation pipette as described for human blastomere biopsy (Sermon et al., 2004). After manipulation, embryos were transferred to CZB culture medium containing glucose and incubated for up to 2 hr at 37.5°C. Pseudopregnant ICR females were used as embryo recipients after mating with vasectomized ICR males. Biopsied three-cell embryos and four-cell control embryos were transferred into the oviducts of day 0.5 pseudopregnant ICR females.

Isolation of Embryos

We isolated 7.5 dpc embryos from each of the three groups. The morning that the vaginal plug was observed in naturally mated or pseudopregnant females was designated as 0.5 dpc.

To observe embryo morphology, we removed the Reichert's membranes from 7.5 dpc embryos after dissecting them from the deciduas. Each embryo was placed in a droplet of PBS. A detailed protocol for this procedure can be found in *Manipulating the Mouse Embryo: a Laboratory Manual* (Nagy et al., 2003b).

TABLE 1. Implantation rates of transferred embryos

Group	Embryos transferred (recipients)	Embryos/ recipient (mean ± SE)	No. of implanted embryos (%)	*P*
Biopsied	424 (23)	18.43 ± 1.0	288 (67.92)	NS
Control	330 (19)	17.37 ± 1.6	220 (66.67)	

NS = not significant, $P > 0.05$.

TABLE 2. Survival rate of implanted embryos at 7.5 dpc

Group	No. of implanted embryos (recipients)	Implanted embryos/ recipient (mean ± SE)	No. of surviving embryos (%)
Biopsied	288(23)	12.52 ± 2.62	142 (49.31)[a,b]
Control	220(19)	11.58 ± 0.76	134 (60.91)[a]
Normal	186(16)	11.63 ± 0.99	179 (96.24)

[a]$P < 0.01$ *versus* normal.
[b]$P < 0.01$ *versus* control.

Fig. 1. Embryos and surrounding deciduas at 7.5 dpc. The scale bar is 100 μm. 1, a surviving 7.5 dpc embryo inside the decidua; 2, 3, and 4, decidua containing a dead 7.5 dpc embryo. The circle indicates the position of the embryo inside the decidua.

Developmental Classification of Surviving 7.5 dpc Embryos

We defined the developmental stage of selected surviving 7.5 dpc embryos according to the morphological criteria for mouse embryo development defined by Downs and Davies (1993). These criteria have been accepted by http://www.emouseatlas.org/emap/home.html to supplement Theiler's criteria, which is the most used general criteria for describing mouse embryo development.

Statistical Analysis

Differences between groups were evaluated using the χ^2 test. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) software version 18.0 for Windows (SPSS, Inc., USA).

RESULTS

Embryo Implantation Rates are Similar for Biopsied and Control Groups

To assess early embryo development, biopsied (N = 424) and control (N = 330) embryos were transferred into recipient mothers, and the number of embryos transferred into each recipient were similar to the number of embryos normally present under natural conception conditions. At 7.5 dpc, recipient mice were dissected and the embryos were carefully observed. Embryos that were encapsulated by a spongy mass of decidual cells were defined as imbedded embryos. There were 288 imbedded embryos in the biopsied group and 220 imbedded embryos in the control group, corresponding to an implantation rate of 67.92% for the biopsied group and 66.67% for the control group (Table 1). These results indicate that blastomere biopsy had no influence on either the *in vivo* preimplantation development or the implantation ability of these embryos.

In addition, the average number of implanted embryos in a single recipient mother was compared among the biopsied, control, and normal groups. We found no difference in this index among the three groups (Table 2), which further suggests that PGD procedures cause no adverse effects on embryo development before implantation.

Reduced Survival Rates of Implanted Embryo at 7.5 dpc in Biopsied and Control Groups

Careful dissection of deciduas revealed that not all deciduas from mice in the biopsied and control groups contained a living embryo (Fig. 1). Only 49.31% of implanted embryos in the biopsied group survived to 7.5 dpc. The 7.5 dpc survival rate of implanted embryos was slightly higher (60.91%) in the control group and highest (96.24%) in the normal group (Table 2).

Statistical analysis indicated that the survival rate of embryos in the biopsied group was significant lower than that in the normal group ($P < 0.01$, Table. 2), which suggests that the PGD procedure increases the likelihood of embryo death occurring between 4.5 and 7.5 dpc. Given that the survival rate in the control group was also significant lower than that in the normal group ($P < 0.01$, Table 2), it is likely that the manipulations of superovulation, embryo culture *in vitro*, and embryo transfer also contribute to embryo death between 4.5 and 7.5 dpc. In addition, the survival rate in the biopsied group was significant lower than that of the control group ($P < 0.01$, Table. 2), which suggests that blastomere biopsy is a risk factor for embryonic death at this developmental stage.

The Developmental Stage of Surviving Embryos at 7.5 dpc is Similar in all Groups

Embryos of the same gestational age may differ in their developmental stage. Downs and Davis (1993) defined a set of morphological criteria for classifying the gastrulation stage of mouse embryos, from just before formation of the primitive streak (approximately 6.75 dpc) until the beginning of organogenesis at

TABLE 3. Developmental stages of surviving 7.5 dpc embryos

Developmental stage(range)	Biopsied (%)	Control (%)	Normal (%)
MS stage	7 (15.91)	0	6 (12.00)
MS–LS stage	1 (2.27)	0	0
LS stage	7 (15.91)	4 (8.16)	7 (14.00)
OB stage	1 (2.27)	9 (18.37)	7 (14.00)
EB stage	11 (25.00)	7 (14.29)	6 (12.00)
LB stage	6 (13.64)	6 (12.24)	5 (10.00)
EHF stage	5 (11.36)	12 (24.49)	12 (24.00)
EHF–LHF stage	1 (2.27)	1 (2.04)	2 (4.00)
LHF stage	4 (9.09)	9 (18.37)	4 (8.00)
Abnormal	1 (2.27)	1 (2.04)	1 (2.00)
No. of 7.5 dpc embryos	**38 (86.36)**[a,b]	**38 (77.55)**[a]	**43 (86.00)**
Total	44	49	50

Bold values are total amount of MS-EHF embryos.
[a]$P > 0.05$ *versus* normal.
[b]$P > 0.05$ *versus* control.

approximately 8.0 dpc (Downs and Davies, 1993). Based on these criteria, nine developmental stages were defined: prestreak (PS, 6.75–7.25 dpc), midstreak (MS, 7.0–7.75 dpc), late streak (LS, 7.25–7.75 dpc), no allantoic bud (OB, 7.25–7.75 dpc), early allantoic bud (EB, 7.5–8.0 dpc), late allantoic bud (LB, 7.5–8.0 dpc), early head fold (EHF, 7.5–8.0 dpc), and late head fold (LHF, 8.0 dpc). According to this classification, MS and EHF stage embryos correspond to the developmental stage expected for 7.5 dpc.

To further evaluate the effect of PGD on embryo development, we randomly selected 44, 49, and 50 surviving 7.5 dpc embryos from biopsied, control, and normal group, respectively, and classified their developmental stages according to the Downs and Davis criteria. Of these, one embryo in each group was found to be morphologically abnormal and could not be classified, and all others could be defined in the range from MS to LHF (Table 3, Fig. 2a). Of these, 38 of the 44 biopsied embryos (86.36%), 38 of the 49 control embryos (77.55%), and 43 of the 50 normal embryos (86.00%) were identified as MS–EHF stages. Thus, the proportion of embryos reaching the developmental stage corresponding to 7.5 dpc was similar among all three groups, indicating that there are no obvious differences in the development of surviving embryos during the 4.5–7.5 dpc period among the groups (Table 3, Fig. 2b).

DISCUSSION

PGD is an established procedure for the genetic analysis of embryos. It allows couples carrying genetic diseases to have an unaffected child, without facing invasive prenatal diagnosis and termination of the pregnancy. The first 1,000 PGD births were reached by 2004, and perhaps close to 10,000 additional PGD babies have been born since 2004 till 2010 (Verlinsky et al., 2004; Simpson, 2010). As the technology is being so widely applied, a systematic evaluation of its safety needs to be done. Here, we evaluated the influence of the PGD procedure on early embryonic development using a mouse model. We found that rates of embryo implantation are similar in biopsy and control groups, which indicated that blastomere biopsy does not impair either embryo development at the preimplantation stage or embryo implantation.

However, a proportion of imbedded embryos in all groups were found to be dead at 7.5 dpc. The ratio of surviving 7.5 dpc embryos to implanted embryos in the biopsied group was significant lower than that of normal group (49.31% vs. 96.24%, $P < 0.01$). Therefore, the PGD procedure was found to impair embryo postimplantation development, resulting in increased embryonic mortality between 4.5 and 7.5 dpc. Except for blastomere biopsy, all other PGD manipulations (superovulation, *in vitro* culture, and embryo transfer) are common to other ARTs protocols, such as IVF and ICSI. Coincidently, Delle Piane et al. (2010) reported that mice that had conceived using IVF had higher abortion rates than mice that had conceived naturally. Moreover, ovarian stimulation itself has also been reported to negatively affect murine embryonic development (Ertzeid and Storeng, 2001; Van der Auwera and D'Hooghe, 2001). According to these publications and our results, we suggest that superovulation, *in vitro* culture, and embryo transfer might contribute to the negative effect of PGD on postimplantation embryo development. This is supported by the observation that the survival rate in the control group (60.91%) was significantly lower than that in normal group (96.24%; $P < 0.01$). However, the relative contribution of each manipulation to the overall effect remains to be evaluated.

We found that blastomere biopsy (which is unique to PGD) was associated with an increased rate of embryo death, since the percentage of surviving 7.5 dpc embryos in the biopsied group (49.31%) was significant lower than that of the control group (60.91%; $P < 0.05$). This result confirmed that blastomere biopsy enhances the damage to embryo development during 4.5–7.5 dpc caused by the other manipulations, such as superovulation, *in vitro* embryo culture and embryo transfer. In conclusion, the PGD procedure led to increased embryo mortality between 4.5 and 7.5 dpc and blastomere biopsy contributed to embryo death as a single risk factor.

Our experimental design did not allow us to determine whether the all of the surviving 7.5 dpc embryos would be viable. However, a clinical study of 581 PGD/preimplantation genetic screening (PGS)-conceived children and 2,889 ICSI children (Liebaers et al., 2010) found a higher rate of stillbirth in children born after PGD/PGS (3.09%) than in ICSI-conceived children (1.70%). In this study, stillbirth was defined as an intrauterine death of a child at a gestation of $\geq$20 weeks or an intrapartum death of a child with a birthweight of $\geq$500 g. Therefore, it is possible that in our experiments some surviving 7.5 dpc biopsied embryos would die before birth from the effects of blastomere biopsy. However, more studies are required to prove whether blastomere biopsy affects the intrauterine development of embryos throughout gestation.

To further elucidate the influence of the PGD procedure on the early development of postimplantation embryos, we classified some surviving embryos from each groups based on morphological criteria. We randomly selected 44 surviving biopsied embryos, and 49 and 50 surviving embryos from the control and normal groups, respectively, to be classified. In general, the developmental stage of all embryos was consistent with a gestational age of 7.5 dpc. Moreover, there was no

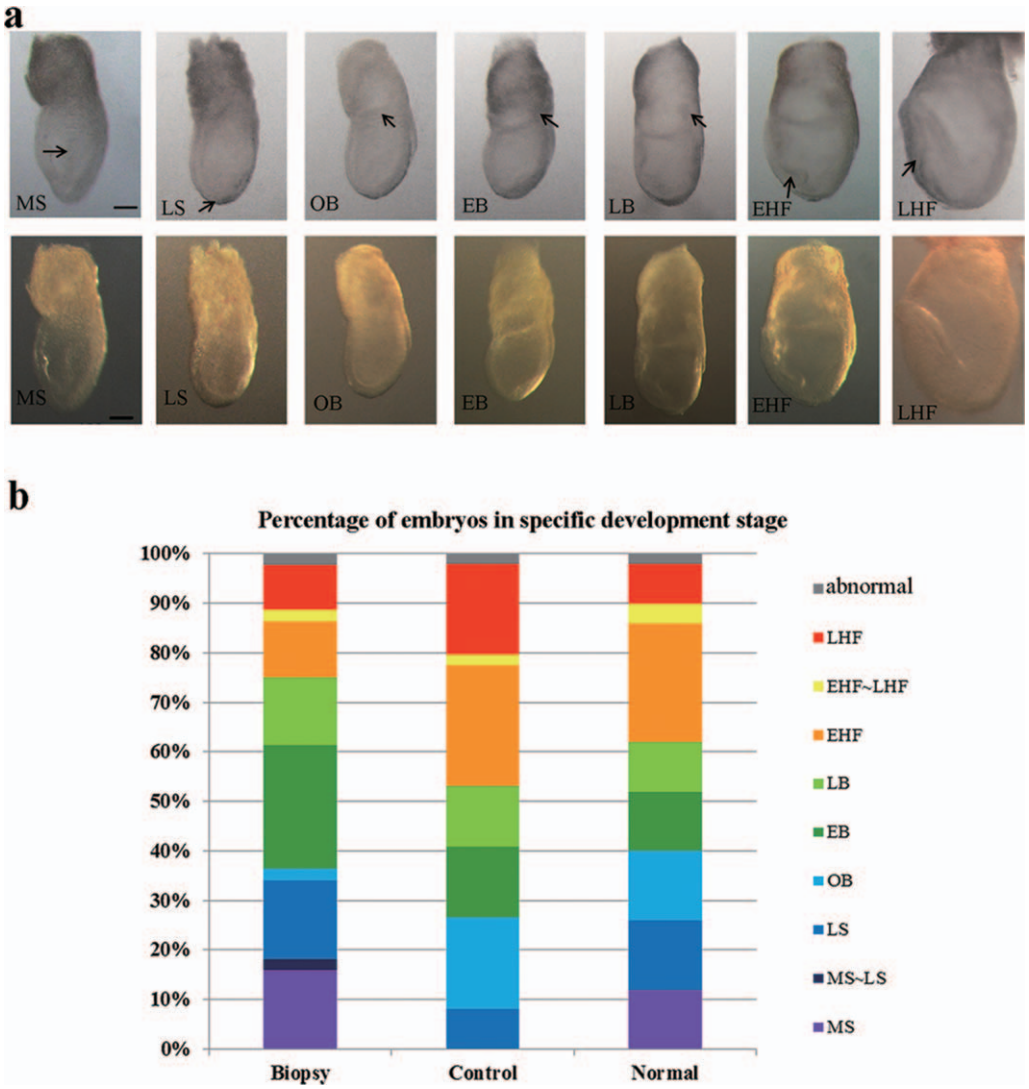

Fig. 2. The developmental stage of live embryos at 7.5 dpc was defined according to the detailed criteria of Downs and Davis. **a**. Morphological characteristics of gastrulation-stage mouse embryos (from MS to LHF). All embryos are shown on the same scale with posterior on the right side. The scale bar in MS is 100 μm. Mid-streak stage (MS): mesoderm extending between 51% and 100% of the posterior side (indicated by black arrow); Late streak stage (LS): the node is visible at distal tip embryo (indicated by black arrow); posterior amniotic fold not yet fused to anterior proamniotic fold to form the amnion; No bud stage (OB): the full closure of the amnion in the OB stage embryo (indicated by black arrow) and expansion of the exocoelomic cavity as compared with the LS stage embryo. Early bud stage (EB): a small allantoic bud (indicated by black arrow) could be seen at the posterior junction of the embryonic and extraembryonic portions of the egg cylinder; Late bud stage (LB): larger allantois, projecting into the exocoelomic cavity (indicated by black arrow), anterior neurectoderm thickening but not yet forming headfolds; Early head fold stage (EHF): anterior ectoderm thickening to form headfolds (indicated by black arrow), no foregut is yet visible; Late head fold stage (LHF): the anterior neurectoderm has thickened to form distinct headfolds and a foregut pocket has become visible (indicated by black arrow). **b**. Most embryos were defined as having reached the developmental stage corresponding to 7.5 dpc (from MS to MHF) in biopsied, control, and normal groups. The proportion of embryos at each developmental stage was similar among the three groups.

significant difference in the percentage of 7.5 dpc embryos classified as corresponding to stages from MS to EHF in the three groups. This suggests that no developmental retardation of live embryos occurred in biopsied and control groups compared to normal embryos at 7.5 dpc.

Although postimplantation embryo mortality was increased by the PGD procedure, the underlying mechanism was not elucidated. One possibility is that epigenetic changes occurring as a consequence of our manipulations might contribute to embryonic mortality. Epigenetic alterations do not change the DNA sequence, but change gene expression by the direct modification of nucleotides that is inherited during cell division. Genomic imprinting is an example of epigenetic inheritance in eutherian mammals, in which differences in gene expression depend on the parental origin of the allele. Many population-based cohort studies have reported a higher risk of imprinting defects in ARTs-conceived babies (DeBaun et al., 2003; Gicquel et al., 2003;

Maher et al., 2003). However, this finding needs to be confirmed by further epidemiologic studies.

It is known that an important epigenetic event, that is, whole genomic demethylation initiated immediately after fertilization, does not affect methylation of imprinted genes. Thus, parental imprints are preserved. Genome hypomethylation has been observed until the blastocyst stage (Santos et al., 2002). Recent research indicates that DNA methylation begins to re-establish during or after implantation at 4.5 dpc (Borgel et al., 2010), and that this remethylation (defined as *de novo* methylation) is carried out by Dnmat3a and 3b. By 6.5 dpc, remethylation of almost all genes has been completed and corresponds to the methylation pattern characteristic of adult somatic tissues. ARTs-related manipulations, such as *in vitro* embryo culture, blastomere biopsy, and embryo transfer, are all done during the demethylation stage. As DNA methylation and demethylation are both sensitive to environmental factors, it is possible that ARTs-related manipulations can disturb demethylation and may even obstruct remethylation postimplantation. Disruption of these processes may explain our finding that embryo mortality was significantly higher in both biopsied and control groups.

In conclusion, we have used a mouse model to assess the influence of PGD on early embryonic development. We found that increased embryo death during 4.5–7.5 dpc might be associated with ovarian stimulation, *in vitro* embryo culture, and transfer, which are general manipulations for ARTs, such as IVF, ICSI, and PGD. Moreover, the manipulation unique to the PGD procedure, that is, blastomere biopsy, increases embryo mortality during 4.5–7.5 dpc. However, more studies are required to elucidate the underlying mechanism, for example, to investigate the possible influence of epigenetic factors.

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