Embryo biopsy and development: the known and the unknown

Federica Zacchini¹, Roberta Arena¹, Adam Abramik¹ and Grazyna E Ptak^{1,2,3}

¹*Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Jastrzebiec, Poland*, ²*Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy* and ³*Malopolska Center of Biotechnology, Jagiellonian University, Kraków, Poland*

Correspondence should be addressed to F Zacchini; Email: f.zacchini@ighz.pl

Abstract

Preimplantation genetic diagnosis (PGD) has been introduced in clinical practice as a tool for selecting 'healthy' embryos before their transfer in utero. PGD protocols include biopsy of cleaving embryos (blastomere biopsy (BB)) or blastocysts (trophectoderm biopsy (TB)), followed by genetic analysis to select 'healthy' embryos for transfer in utero. Currently, TB is replacing the use of BB in the clinical practice. However, based on the European Society of Human Reproduction and Embryology Preimplantation Genetic Diagnosis Consortium reports, BB has been used in >87% of PGD cycles for more than 10 years. An exhaustive evaluation of embryo biopsy (both BB and TB) risks and safety is still missing. The few epidemiological studies available are quite controversial and/or are limited to normalcy at birth or early childhood. On the other hand, studies on animals have shown that BB can be a risk factor for impaired development, during both pre- and postnatal life, while little is known on TB. Thus, there is an urgent need of focused researches on BB, as it has contributed to give birth to children for more than 10 years, and on TB, as its application is significantly growing in clinical practice. In this context, the aim of this review is to provide a complete overview of the current knowledge on the short-, medium- and long-term effects of embryo biopsy in the mouse model.

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General overview

Since the 90s, PGD has been offered to couples undergoing assisted reproduction. In PGD, embryos produced by *in vitro* fertilization or intracytoplasmic sperm injection are screened for genetic defects, allowing only 'healthy' embryos to be selected for transfer *in utero*. One component of PGD protocol is embryo biopsy to obtain one or few cells to be genetically screened. Embryo biopsy can be performed on cleaving embryos (blastomere biopsy (BB), consisting in the removal of one or two blastomere from 8-cell embryos) or on blastocyst stage embryos (trophectoderm biopsy – TB, consisting of the removal of few cells from trophectoderm). Based on the ESHRE Preimplantation Genetic Diagnosis Consortium reports (Harper *et al.* 2012, Montou *et al.* 2014, De Rycke *et al.* 2015), BB has been used in the majority of PGD cycles (BB > 87% vs TB < 1%) for over 10 years. However, in the last years, the use of TB is replacing that of BB in the clinical practice.

Since genetic testing of a single cell obtained by early embryo biopsy was shown to be possible (Monk *et al.* 1987), much attention has been devoted to optimizing the technique to improve embryo survival. To this aim, Takeuchi *et al.* (1992) compared three different protocols – enucleation, aspiration and extrusion – for the removal of one blastomere from a developing embryo, showing

that the aspiration method was the less harmful protocol for BB when the procedure was performed on both 4- and 8-cell stage embryos. Pierce and coworkers reported that 8-cell stage embryos were less sensitive to the procedure than 4-cell ones (Pierce *et al.* 1997). Concerning TB, Carson and coworkers showed that the excision of herniating trophectoderm after hatching was the safest way to perform blastocyst biopsy compared to the aspiration method (Carson *et al.* 1993).

First reports in 90s, concerning development to blastocyst stage and the rate of cell division after BB (Wilton & Trounson 1989, Takeuchi *et al.* 1992, Pierce *et al.* 1997) as well as embryo survival following TB (Carson *et al.* 1993), were quite reassuring. More recently, time-lapse analysis revealed impaired developmental dynamic in both human and mouse BB-embryos, in terms of timing of blastulation, expansion/contraction movements and hatching (Duncan *et al.* 2009, Terada *et al.* 2009, Ugajin *et al.* 2010, Bar-el *et al.* 2016, Cimadomo *et al.* 2016, Yao *et al.* 2016). No information on developmental dynamic following TB has been published so far.

Molecular analyses on mouse embryos developed after BB, but not TB, have been carried out by few researchers (Wu *et al.* 2014, Zacchini *et al.* unpublished). These studies showed deregulated expression of genes involved in DNA methylation, chromatin remodeling,

cell cycle and differentiation, oxidative stress, metabolism (Wu *et al.* 2014) and of three imprinted genes *UBE3A*, *SNRPN* and *PEG1* (S Sampino, F Zacchini, GE Ptak, unpublished observation) in 4.5 dpc blastocysts. Epigenetic analysis of 7.5 dpc embryos, following BB, revealed a reduced global methylation profile vs that of control embryos. In particular, 20 differentially methylated loci were identified in the BB embryo group, 11 of which involved in important developmental events (i.e. cell proliferation and differentiation) or the onset of developmentally related diseases (i.e. cardiovascular disease and infertility) (Wu *et al.* 2014).

Regarding the effects of BB and TB on the offspring's further development, epidemiological studies are quite controversial and/or are limited to normalcy at birth or early childhood (Banerjee *et al.* 2008, Middelburg *et al.* 2011, Desmyttere *et al.* 2012, Thomaidis *et al.* 2012, Winter *et al.* 2015, Cimadomo *et al.* 2016, Sunkara *et al.* 2016). Though the majority of studies did not show an increased incidence of pregnancy complications following embryo biopsy, some reported that BB is associated with reduced implantation rates, increased risks of low birth weight or preterm birth (Banerjee *et al.* 2008, Thomaidis *et al.* 2012, Cimadomo *et al.* 2016) and, in early childhood, with impaired cognitive and/or motor development (Banerjee *et al.* 2008, Middelburg *et al.* 2011, Thomaidis *et al.* 2012, Cimadomo *et al.* 2016), while no indication on TB pregnancy/babies are available. Research on mouse model has shown that BB is not only associated with increased pregnancy loss and reduced/delayed fetal development, but also with impaired placental functions and epigenetic defects in both fetuses and placentae (Sugawara *et al.* 2012, Yang *et al.* 2012, Sato *et al.* 2014, Wu *et al.* 2014, Yao *et al.* 2016). Regarding TB, research on mouse model is very limited so far. Gentry and Critser (1995) reported neither pregnancy complication nor reduced birthweight after TB. More recently, Taskin and coworkers described that, even if blastocyst viability was not affected, TB was associated with reduced implantation rate and development to term (Taskin *et al.* 2016). Additional studies are necessary to confirm whether (or not) TB may affect post implantation development.

Concerning the postnatal development of mice obtained following BB and TB, the first reports were again quite reassuring, as they did not show any adverse effects of both procedures on the offspring at birth (i.e. congenital defects, developmental delays or reduced body weight) or general health in adulthood (i.e. body weight, blood parameters or gross morphology of major internal organs) (BB: Cui *et al.* 1993, 1994; TB: Gentry & Critser 1995). However, in the last decade, different research groups have described increased body weight/size and impaired behavior in BB mice and have suggested reduced fertility in adult females (Yu *et al.* 2009, Zeng *et al.* 2013, Zhao *et al.* 2013, Sampino *et al.* 2014, Wu *et al.* 2014).

Studies to assess epigenetic, behavioral, reproductive and/or metabolic health in the pre- or postnatal life following TB have not been published so far, while few research groups have performed studies to assess BB risk(s) and safety (Yu *et al.* 2009, 2013, Sugawara *et al.* 2012, Yang *et al.* 2012, Zeng *et al.* 2013, Zhao *et al.* 2013, Sampino *et al.* 2014, Sato *et al.* 2014, Wu *et al.* 2014). Thus, in the following sections, we will focus our attention on BB, rather than TB.

Effects of blastomere biopsy on feto-placental development

A gross morphological evaluation of developing mouse fetuses obtained following BB did not show delayed development in early pregnancy (7.5 dpc, Yang *et al.* 2012), while reduced fetal, but not placental, weight was observed starting from 12.5 dpc (Sato *et al.* 2014, Yao *et al.* 2016). Of relevance, a low fetal/placental weight ratio was observed in BB conceptuses, suggesting increased risk of cardiovascular diseases later in life (Thornburg *et al.* 2010). Although placental gross morphology and weight seem not to be affected by BB, impaired placental functions have recently been described (Sugawara *et al.* 2012, Sato *et al.* 2014, Yao *et al.* 2016). Sugawara and coworkers showed alterations in steroid metabolism, such as increased progesterone levels and impaired steroid clearance/elimination, in placentae and fetal livers (Sugawara *et al.* 2012). Compromised steroid metabolism has been associated with (i) defective placental vasculogenesis, which may affect nutrient supply to the fetus; (ii) defective signaling for the induction of labor and (iii) delayed fetal development, resulting in low birth weight and indicative of a higher predisposition to cardiovascular disease later in life. Other studies have reported deregulation of STAT, SOCS and MMP signaling pathways, alteration of oxidative stress, inflammation and impaired expression of imprinted genes (*Igf2p0*, *H19* and *Igf2r*) in full-term mouse placentae (Sato *et al.* 2014, Yao *et al.* 2016). Altogether, these data suggest that BB may be a risk factor for defective placental vascular development and function, as well as intrauterine growth restriction and obstetric complications (i.e. premature membrane rupture and dysfunctional labor).

Reproductive fitness in mice conceived following blastomere biopsy

No effect of BB was described with regard to pregnancy rate and litter size when comparing BB mice vs control(s) at reproductive age (Sampino *et al.* 2014, Yu *et al.* 2013). However, Yu and coworkers showed that aged (i.e. 40-week-old) females, born following BB, had reduced pregnancy rates, numbers of developing follicles and ovarian weights, in addition to altered steroid levels (Yu *et al.* 2013). To investigate

the underlying mechanism(s) responsible for reduced ovarian function, proteomic analyses of ovarian tissues from pubertal (10-week-old) and aged (40-week-old) females were performed. These analyses showed that ovarian tissues from both BB-pubertal and BB-aged females were characterized by impaired expression of three proteins (HSPA4, PSMB8 and ALDH1A1) associated with ovarian development, follicle formation and could indicate a higher risk of ovarian cancer. Further analyses are necessary to confirm this study and to evaluate whether reproductive function could also be impaired in male BB subjects.

Neurodevelopmental disorders induced by blastomere biopsy

In the last decade, a few research groups have investigated whether BB could be associated with an increased risk of neurodevelopmental disorders (Yu *et al.* 2009, Zeng *et al.* 2013, Zhao *et al.* 2013, Sampino *et al.* 2014, Wu *et al.* 2014). Behavioral tests showed that mice born following BB had impaired memory and motor coordination, increased anxiety and altered responses to threatening or stress stimuli. Of relevance, these studies showed an increased predisposition to behavioral anomalies in male subjects, but not in females. To clarify the mechanism behind the impaired behavior and stress response, the brain and adrenal glands (peripheral organs) were selected as target organs. Histological and ultrastructural examination of the brain revealed structural abnormalities in the corpora striatum and hippocampus, such as hypomyelination, axonal swelling, neuronal regression and mitochondrial degeneration (Yu *et al.* 2009, Wu *et al.* 2014). Proteomic analysis of brain tissues from BB mice identified 36 differentially expressed proteins, 17 of which are associated with neurodegenerative diseases, including Alzheimer's and Parkinson's disease (Yu *et al.* 2009). Epigenetic analysis of brain tissues showed reduced methylation and increased hydroxymethylation. In particular, methylated DNA immunoprecipitation (MeDIP) analysis identified 11 differentially methylated loci, six of which are associated with neural disorders (Wu *et al.* 2014). Furthermore, Zhao and coworkers showed that impaired behavior in BB mice was associated with epigenetic defects in brain tissues, while the same association was not observed in control mice (Zhao *et al.* 2013). Regarding the adrenal glands, Zeng and coworkers described an altered response to cold stress in BB mice, which was associated with impaired structure and function of this peripheral organ, and not with anomalies in the hypothalamus (Zeng *et al.* 2013). Altogether, these studies suggest that BB may be responsible for a higher incidence of altered behavior in offspring and could predispose them to neurodevelopmental and psychiatric diseases.

How embryo biopsy may affect embryo development

The preimplantation period is a short window of mammalian development characterized by a well-defined sequence of programming events that will decide the future of the developing embryo. In this period, epigenetic and/or metabolic changes (i.e. adaptations), occurring to promote survival in an inadequate environment (e.g. a suboptimal culture system, maternal undernutrition), may cause permanent changes to developmental programming, thus leading to increased risk of developmental defects and/or metabolic disorders (Barker 2007).

With regard to blastomere biopsy, no research has been carried out to investigate whether or how the removal of one cell from a developing embryo could trigger adaptations that persist further in development (both pre- and post-natally). However, based on the current knowledge in the field of developmental and reproductive biology, we can speculate that:

- The removal of one blastomere deprives the embryo of important developmental determinants necessary to produce a healthy individual. This speculation is based on different studies showing that embryos at early stages of development (including at the 4-cell stage) are characterized by individual blastomeres that differ in their developmental fate and potency (Piotrowska-Nitsche *et al.* 2005, Tarkowski *et al.* 2010), as well as in their transcriptomic profile and epigenetic features (Torres-Padilla *et al.* 2007, Tang *et al.* 2011). In this context, BB may be responsible for the embryo's adaptations that occurred as a consequence of blastomere/determinant removal.
- BB acts as a 'stress stimulus' that interferes with the proper sequence of events necessary for successful development. As widely described in human and animal models, a suboptimal preimplantation environment (such as *in vitro* culture) can perturb proper epigenetic remodeling or critical transcriptional events during early development (Thompson *et al.* 2006, Lucas 2013), resulting in impaired fetoplacental and postnatal development. With respect to BB, there is limited knowledge of epigenetic or transcriptional perturbation in early embryos (Wu *et al.* 2014); however, due to defects observed in BB conceptuses and offspring (Sugawara *et al.* 2012, Zhao *et al.* 2013, Wu *et al.* 2014), it is plausible that the removal of one blastomere from the developing embryo perturbs the proper sequence of developmental programming events.

With regard to trophectoderm biopsy, there is no extensive knowledge concerning detrimental effects (if any) on both pre- and postnatal life. However, as the procedure is carried out at the end of preimplantation

window, it could be speculated that the removal of few cells for trophoblast could affect proper placental development and function. If so, impaired placentation

could also perturb both fetal and offspring development. Much effort must be done to assess potential risk (if any) of TB.

Figure 1 Effects of blastomere biopsy in the short, medium and long term. Schematic overview of the current state of the art in the mouse model.

Conclusion

The present review summarizes the current knowledge of the short-, medium- and long-term effects of BB in the mouse model (Fig. 1) and provide the limited information available on TB. What emerges is that, even from the animal model, we still have a fragmentary picture of the real risks (or safety) of BB, and there is no information on the potential side effect (if any) of TB. Thus, much research is still necessary to provide a complete and satisfactory picture of the potential embryo biopsy-induced risk(s) and of the mechanisms behind the onset of developmental defects. Researches focused on BB are still important, as they will provide indication on the potential long-term risk that could affect the already born children in adulthood and/or their progeny, while studies focused on TB are necessary to provide useful information on an invasive procedure whose application is growing nowadays. Through focused investigations, it will be possible to improve not only our scientific knowledge in the field of developmental/reproductive biology, but also current practices in human assisted reproductive technology, to ultimately prevent and manage the onset of diseases in modern society.

Declaration of interest

The authors declare no conflicts of interest. The contents of this publication are the sole responsibility of the Institute of Genetics and Animal Breeding of the Polish Academy of Sciences and do not necessarily reflect the opinion of the European Union.

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Author contribution statement

F Z conceived and prepared the manuscript; R A contributed to the manuscript and figure preparation; A A contribute to bibliography research and G E P contributed to the intellectual content and edited the final version of the manuscript.

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