Effects of blastomere biopsy on post-natal growth and behavior in mice

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STUDY QUESTION: Does blastomere biopsy (BB) of preimplantation embryos induce long-term effects on their growth and post-natal behavior?

SUMMARY ANSWER: BB induces long-term effects on body weight and behavior in male mice.

WHAT IS KNOWN ALREADY: BB is an essential technique for performing preimplantation genetic diagnosis (PGD), a screening test that can detect genetic abnormalities of embryos before their transfer in utero. There is limited understanding of the post-natal consequences and safety of BB.

STUDY DESIGN, SIZE, DURATION: Offspring who had a BB performed as embryos, as well as control offspring, were examined for body and neurological development and subjected to a screening battery of behavioral tests, designed to model symptoms of psychiatric disorders. At least 12 mice were used for each test over the course of 16 weeks.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Embryos were subjected to a single BB at the 8-cell stage and then cultured in vitro until the blastocyst stage (BB group). Two control groups were created, one consisting of embryos cultured in vitro without any manipulation (in vitro control (IVC) group) and one of embryos developed entirely in vivo (in vivo group). Embryos from in vitro groups (BB and IVC) were transferred to pseudo-pregnant female mice at the blastocyst stage. Body growth parameters and developmental landmarks of the resulting offspring were observed during their entire lifespan. Furthermore, validated behavioral tests were used to assess early communicative functions, startle reflex, and anxiety- and depression-like behaviors.

MAIN RESULTS AND THE ROLE OF CHANCE: We found that male mice derived from BB exhibited peculiar behavioral alterations and changes in body weight. BB-derived male mice showed increased body weight with respect to both controls as early as the second week of life. Adult males displayed decreased times of immobility in the tail suspension test \((P < 0.05)\) and deficits in habituation to, and pre-pulse inhibition of, the startle reflex \((P < 0.05)\). BB did not affect communicative skills and anxiety-like responses.

LIMITATIONS, REASONS FOR CAUTION: Extrapolation of these results to humans requires caution as the culture protocols used in human clinics could be better established than in mice research. Furthermore species-specific neurodevelopmental features could be a source of differences between mice and humans in the effects of BB.

WIDER IMPLICATIONS OF THE FINDINGS: Our data demonstrate that BB affects long-term programming of post-natal development and behavior in mice, suggesting that PGD procedures could be a risk factor for late-onset, neurodevelopmental and metabolic disease predisposition. Thus, in light of our observations, long-term follow-up in humans or other primates generated after BB is needed.

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Key words: blastomere biopsy / preimplantation genetic diagnosis / mouse behavior / in vitro embryo culture
Introduction

Although assisted reproductive technologies (ART) are being applied to humans at increasing rates, meaningful experimental data about the safety of ART remain controversial. In particular, little is known about the long-term effects of ART on adult well-being and disease predisposition. Studies in mice have shown that embryo culture can perturb developmental processes, leading to long-term effects on behavioral and physiological parameters (Ecker et al., 2004; Fernandez-Gonzalez et al., 2004; Watkins et al., 2007; Calle et al., 2012). Meanwhile, new ARTs are being applied in human clinics, despite the fact that the long-term consequences of embryonic manipulation on offspring health in adulthood are still unknown.

Preimplantation genetic diagnosis (PGD) is widely used in human clinical practice to screen genetic features of embryos before their transfer in utero. The procedure involves the removal of one blastomere from cleaving embryos, followed by in vitro culture until the blastocyst stage (Sermon et al., 2004). As early as the 4-cell stage, individual blastomeres 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). Thus, the removal of one cell could potentially deprive the embryo of important developmental determinants, necessary for establishing a healthy phenotype. Biopsy is also likely to have negative effects on developing embryos, interfering with cell–cell adhesion (Damsky et al., 1983; Johnson et al., 1986) and gap and tight junctions (Ducibella and Anderson, 1975; Lo and Gitula, 1979; Lee et al., 1987). So far, a limited number of reports on the medical outcome of children born after PGD have been published, but they are reassuring (Strom et al., 2000; Sermon et al., 2007; Goossens et al., 2009; Liebaers et al., 2010; Desmyttere et al., 2012). However, evidence concerning the effects of PGD on neurodevelopmental outcomes in offspring is scarce.

Here, we used a mouse model to explore the long-term effects of blastomere biopsy (BB) on adult behavior. In particular, we aimed to assess the influence of the technique on the arousal of psychiatric diseases, including anxiety, depression, schizophrenia and autism. To this end, we performed a comprehensive behavioral analysis of BB, in vitro control (IVC), and naturally obtained pups and adult mice. Our results indicate that the BB procedure represents a possible risk factor for late-onset psychiatric and metabolic disorders.

Materials and Methods

Animals

Swiss albino mice, originating from the mouse colony bred in the Institute of Genetic and Animal Breeding’s Department of Experimental Embryology (Poland), were housed in 30.5 × 13 × 11 cm cages and given water and food at libitum (Labofeed H, Kcynia, Poland; metabolic energy of 13.0 MJ/kg), with 12 h light/dark alternation. Experimental procedures were conducted according to the guidelines of the European Community regulation 86/609 and conformed with the Polish Governmental Act for Animal Care.

Experimental design, embryo cultures and blastomere biopsies

Three-month-old females, naturally mated without hormonal stimulation, were used as donors. At 2.5 days post-coitum (dpc), 8-cell stage embryos were collected from the oviduct by flushing with M2 medium enriched with 0.4% bovine serum albumin. All embryos with 8 rounded blastomeres and no signs of fragmentation were incubated in Ca
+2
- and Mg
+2
- free HCBZ medium enriched with 5 mg/ml cytochalasin B. Biopsies were performed under a micromanipulator fitted on a Nikon Eclipse Ti-U inverted microscope. One blastomere per 8-cell stage embryo was removed randomly with an enucleation pipette. IVC controls (untreated) were transferred to the micromanipulation dish together with the BB embryos, but were not micromanipulated. Following the biopsy procedure, both BB and IVC embryos were transferred into 20 µl drops of KSOM medium (Millipore), covered by mineral oil, and cultured for 24–28 h in an incubator at 37°C in a humidified atmosphere of 5% CO
+2
- until they developed to the blastocyst stage. At 3.5 dpc, blastocysts (n = 6–8 per uterine horn) were synchronously transferred into pseudo-pregnant Swiss albino females who had mated with proven vasectomized males. The surrogate mothers were then transferred to the colony and allowed to deliver naturally. In addition, offspring were also produced by natural mating, as an in vivo control.

Vasectomy

Vasectomized males were produced as described previously (Nagy et al., 2003). One-month-old C57/BL males were anesthetized with intraperitoneal injections of 0.1 ml/g body weight of 12.5% solution of Vetbutal (Biovet, Pulawy, Poland) in PBS. After shaving the abdomen of each animal and cleaning it with chlorohexidine, an incision was made to access the body cavity and expose the vas deferens. The vas deferens was cut and tied off with sutures, the body cavity was sewn up and the mouse was transferred to a warm and clean cage until it recovered. Prior to being used for experiments, the sterility of vasectomized males was checked at 20 and 30 days post-surgery.

Uterine embryo transfer

Embryo transfer was performed as described previously (Nagy et al., 2003). Recipient females were anesthetized with Vetbutal (0.1 ml/g body weight of 12.5% solutions). After cleaning with chlorohexidine and shaving the back of the animal, a small incision was made and the uterus was exposed. A small hole made to the uterine wall was used to transfer embryos using a transfer pipette. The procedure was performed on both sides of the uterus, injecting seven blastocysts per side. The body cavity was sutured and the recipient female was transferred to a warm and clean cage until it recovered.

Assessment of post-natal development and general health

The litter size and birthweights were recorded at delivery. Pups were then weighed every 2 days from post-natal Day 2 (P2) to P20. Physical landmarks, including eyes/ear opening, incisor eruption and fur development, were also scored during this period. Postural reflex was evaluated using the righting reflex test, performed on P6 and P10. Each animal was turned on its back (ventral side up) on a flat surface and given a maximum of 60 s to return to the four paws position. Time of success was recorded for three consecutive trials for each pup. After weaning, mice were weighed and observed for general health and the presence of visible tumors weekly. At 3 months of age, reproductive fitness was evaluated by the ability to obtain progeny. Subsequently, animals were sacrificed and selected organs (liver, lungs, heart, spleen and kidneys) were collected, weighed and examined for the presence of morphological abnormalities, and then frozen in liquid nitrogen for future analysis.

Behavioral screening

All tests were performed during the end of the light phase in an experimental room isolated from the colony. Mice were introduced into the testing room.
1 h before the test to permit environmental habituation, and returned to their home cage at the end of each test. Each apparatus was washed with 70% ethanol and rinsed with water between subjects to avoid olfactory cueing behaviors. All behavioral tests were made blind to experimental conditions and videotaped for further detailed analysis. First, the isolation-induced ultrasound vocalization (USV) test was performed in pups. In adults, each behavioral test was conducted starting at P75, in the following order: open field (OF), elevated plus maze (EPM), tail suspension (TS) and startle reflex. There was at least 1 week interval between tests performed on the same animal. Each group consisted of 25–30 animals (half males and half females).

Isolation-induced ultrasound vocalization

Testing for isolation-induced USV was done on P4, P8 and P12, as described previously (Hofer et al., 2002), with slight modifications. Briefly, pups (on average 30 per group; 15 males and 15 females) were isolated one by one from their mother and littermates and placed in a clean plastic container into a sound-attenuating chamber, under controlled temperature (28°C). To record vocalizations, an ultrasound sensitive microphone, a bat detector (US Mini-2 bat detector, Summit, Birmingham) tuned in the range of 60–80 kHz, was suspended above the isolated pup and USVs were recorded for 5 min using the Audacity software. For analysis, recordings were imported into Avisoft SASLab software. Background noise was removed, and call detection was provided by an automatic threshold-based algorithm. An experienced human observer checked the accuracy of call detection, and obtained a 100% concordance between automated and observational detection. Parameters analyzed included the total number and sound pressure amplitude of calls. No differences in patterns of calling were detected in a comparison between male and female pups; therefore data were collapsed across sex. Subgroups of mice (n = 15 per group) were isolated on P4 in the presence of nest bedding material, in order to analyze the effects of a familiar environment cue on USV activity (Oswalt and Meier, 1975; Lyons and Banks, 1982).

Open field

OF was used to assess anxiety-related behavior and general locomotor activity. As we previously described (Swiergiel and Dunn, 2007), the apparatus consisted of a square arena (60 × 60 cm) divided into 16 squares and enclosed by continuous 30-cm-high walls. The 12 squares adjacent to the wall represented a protected field, while the 4 central squares represented an exposed field. The test was initiated by placing a single mouse in the middle of the arena (in one of the four central squares) and letting it move freely for 6 min. A number of conventional parameters were collected during the session: (i) time to leave the center: time spent by the mouse to leave the central square; (ii) locomotor activity: the number of lines crossed by the mouse during the entire test; (iii) central square entries: the number of entries into the central area; and (iv) central square duration: the cumulative time spent in the central area.

Elevated plus maze

EPM was used to assess exploration activity and anxiety-like behavior. The apparatus consisted of two open arms (30 × 5 cm) and two closed arms (30 × 5 cm), enclosed by 15 cm-high walls, with the two pairs of identical arms emerging oppositely from a central platform (5 × 5 cm). The apparatus was elevated 45 cm above the floor. The test was initiated by placing the mouse on the central platform, facing one of the closed arms and letting it move freely for 6 min. A number of conventional parameters were collected during the session: (i) total arms entries: the number of total entries into both the open and closed arms; (ii) open arms entries: the number of entries into the open arms with all four paws; (iii) open arms exploration: the number of times the mouse entered the open arms with only its forward paws (stretch—attend posture); and (iv) time spent in the open arms: the cumulative time spent in the open arms (Swiergiel and Dunn, 2007).

Tail suspension

This test was performed as we described previously (Juszczak et al., 2006; Swiergiel et al., 2008). Briefly, a short piece of paper adhesive tape (~6 cm) was attached along half the length of the tail. The free end of the tape was attached to a 30 cm long rigid tape, which hung from a horizontal bar clamped to a heavy laboratory support stand. Suspended animals were surrounded by a black wooden enclosure (45 cm high, 40 cm wide, 40 cm deep) such that the mouse’s head was ~20 cm above the floor. For testing, each mouse was suspended by its tail and observed for 6 min. An observer scored the total duration of a passive, ‘dead weight’ hanging (immobility), between the periods of wriggling of the animal to avoid the aversive situation.

Acoustic startle reflex and pre-pulse inhibition

The animals were tested for acoustic startle reflex (ASR) and pre-pulse inhibition (PPI) using the Startle Box (Med. Associates, Inc., USA). The mice were placed in a Plexiglas cylinder within a sound-attenuating chamber, upon a piezoelectric transducer, which allowed movements to be quantified and displayed on a computer. Calibration of acoustic stimuli was confirmed with an external digital sound level meter (Med. Associates, Inc., USA) before each day of testing. Each test consisted of 3 blocks of trials, for a total of 40 trials, presented after 5 min of acclimation under 60 decibels (dB) of ambient white noise. The first and the last blocks consisted of five trials in which 120 dB white noise stimulus was presented alone without prepulse (ASR-simple). The central block consisted of a total of 30 stimuli, presented as 120 dB white noise signals with or without PPI. Prepulses (at 70, 75, 80 and 85 dB) were presented in pseudo-random order 100 ms prior to the 120 dB main stimulus (ASR-pp). Inter-trial intervals varied randomly from 10 to 20 s. The ASR was defined as the movement amplitude after the 120 dB stimulus, resulting in a peak value. Mean PPI for each pre-pulse intensity was calculated as a percentage reduction in the mean of ASR-pp compared with ASR-simple, according to the formula (ASR-pp/ASR-simple) × 100. Habituation was calculated as the difference in mean response of the first block compared with the last block of ASR-simple stimuli, according to the formula ((ASR block3 — ASR block 1)/ASR block 1) × 100 (Haave et al., 2011).

Statistical analyses

Data were analyzed using STATview (version 5.0.1). Comparisons of body weight and behavioral measurements were firstly made among BB, IVC and in vivo-derived offspring using one-way ANOVA (nonparametric Kruskal–Wallis test + Dunn’s multiple comparison test). Two-way repeated measures ANOVA was used to analyze differences in body weight between the groups during the P2–20. In multiparous species, litter size is a factor that can potentially influence phenotypic features. Because we found differences in litter size between the groups, a further multiple regression analysis was performed including litter size as a random effect. For each data set, litter size was dichotomized around its average value, and its effect on all weight and behavioral measurements was estimated calculating odd ratios (OR), confidence intervals (CI) and P-values. For neonatal tests, data were collapsed across sexes. Differences were considered statistically significant when P-values < 0.05. Success of development to term was analyzed using χ² test, by the percentage of delivered pups on the number of embryos transferred to the individual recipient. All variances displayed in figures, tables and within the text are expressed as mean value and standard error of the mean (SEM), except for litter size and birthweight, which are expressed using standard deviation (SD).
Results

No effects of BB on offspring rate, pre-weaning development nor fertility

We did not find any effects of BB on the ability of embryos to develop to the blastocyst stage, nor on the incidence of development to term. For the BB group, 267 embryos were transferred, while 323 were transferred for the IVC group, resulting in 12 and 18 litters, respectively. The total number of embryos that developed to term was 56 (21.0%) and 64 (19.8%) for BB and IVC, respectively. None of these differences were deemed significant by $\chi^2$ analysis. Differences in litter size were not significant between the BB and IVC groups; nevertheless in vivo litters were more numerous respect to the in vitro groups (Table I). We did not find any effect of BB when the offspring were subjected to a battery of pre-weaning developmental assays that included the righting reflex ability and observed for developmental landmarks, such as incisor appearance, eye opening, fur appearance and ear-pinnæ detachment, which all developed normally in all groups. BB had no effect on the fertility of BB-derived males and females. All F1 mice produced offspring at similar rates (litter sizes: $9.9 \pm 1.2$ (BB); $10.1 \pm 1.4$ (IVC); $9.8 \pm 0.8$ (in vivo)).

Increased body weight but not organomegaly in biopsy-derived offspring

Although birthweight was increased in IVC compared with BB and in vivo pups ($P = 0.002$) (Table I), BB pups were significantly heavier than IVC pups from P10 till weaning (Fig. 1a). Table II shows the average weights from each litter, at birth and weaning. Adult male BB offspring showed an increase in body weight compared with the IVC controls (the difference was significant at Weeks 6, 10, 12–16) (Fig. 1b), but for adult females, the weight differences were not significant (Fig. 1c). Further regression analysis revealed that litter size was associated with body weight at birth ($OR = 1.27; CI = 0.62–2.61; P = 0.046$). However, no effect of litter size was detectable at P20, when BB pups had a higher risk of having an increased body weight ($OR = 5.46; CI = 1.98–15.09; P = 0.001$), even after including litter size as a random variable ($OR = 2.49; CI = 1–6.23; P = 0.051$). Likewise, regression analysis did not reveal any effect of litter size on adult body weight. Postmortem examination of selected organs (liver, lungs, heart, spleen and kidneys) did not reveal any morphological abnormalities nor weight differences (Table III).

Normal USV activity, anxiety-like behaviors and locomotor activity in BB-derived offspring

USVs are emitted by rodent pups, to elicit nursing and care-giving behavior in the mother. Quantitative and qualitative data on USV emission profiles provide valuable information on the pup’s neurodevelopment and communicative skills (Branchi et al., 2001). Analysis of USV activity in pups socially isolated from their mothers revealed no differences between the groups in the rate nor in the sound amplitude of calls (Table IV). This was true for all developmental stages and conditions. Anxiety-related behaviors were assessed with the OF and the EPM tests, which exploit the natural aversion of mice to exposed spaces. The anxiety level is considered high when the number of entries into, and the length of time spent in, the aversive area is low and short (Carola et al., 2002). We did not find any effect of BB on anxiety-like behavior. No differences in locomotor activity, number of entries into, and time spent in open spaces were observed between the groups (Table IV).

Decreased depression-like behaviors in BB-derived male offspring

The duration of immobility in the tail suspension test (TST) reflects the response and the motivation of the animal to avoid acute stress (Cryan et al., 2005). The biopsy exerted significant effect on immobility duration in the TST. Male mice derived from the biopsied embryos displayed a significantly lower duration of immobility compared with both IVC and in vivo control mice. In contrast, no significant effects of the biopsy procedure on the duration of immobility were observed in female mice (Fig. 2).

Altered habituation to the startle reflex and PPI in BB-derived male offspring

The startle reflex is a fast motor response to sudden, intense acoustic stimuli, which can be modulated by a variety of pathological conditions and experimental manipulations. Thus, it is used to model neuropathological dysfunctions of sensorimotor information processing (Koch, 1999). All mice responded to the 120 dB acoustic stimuli (Fig. 3a). No differences were observed among groups in the amplitude of the motor reflex exhibited when 120 dB acoustic stimuli were presented without PPI ($P > 0.05$). All in vivo-produced mice showed a clear habituation to the acoustic stimulus throughout the testing sessions, with a 44.8% decrease in the startle response between the first and the last block of the test. Conversely, male mice derived from biopsied embryos had decreased habituation to the acoustic stimulus compared with the in vivo-derived mice, with stable levels of startle response during all testing periods, and only a 9.3% decrease in amplitude between the beginning and end of the test (Fig. 3b). Normal habituation and PPI responses were observed in females of the BB group (Fig. 3b and d). No differences in habituation were observed between IVC and either BB or in vivo controls. PPI expression was significantly lower in male BB-derived mice compared with control mice. This difference was observed consistently across all pre-pulse dB intensities, except $+

Table I  Effects of blastomere biopsy on embryo development.

<table>
<thead>
<tr>
<th>Blastocysts transferred</th>
<th>Litter</th>
<th>Litter size</th>
<th>Offspring</th>
<th>Birthweight (g)</th>
<th>male/female ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB</td>
<td>267</td>
<td>12</td>
<td>4.6 ± 0.56</td>
<td>56</td>
<td>1.16 ± 0.04</td>
</tr>
<tr>
<td>IVC</td>
<td>323</td>
<td>18</td>
<td>3.8 ± 0.64</td>
<td>64</td>
<td>1.22 ± 0.01</td>
</tr>
<tr>
<td>in vivo</td>
<td>/</td>
<td>10</td>
<td>9.8 ± 0.36</td>
<td>97</td>
<td>1.13 ± 0.01</td>
</tr>
</tbody>
</table>

*In vivo-derived litters had significantly increased numbers of pups. $P < 0.0001$ with one-way ANOVA, Dunn’s Multiple Comparison Test. Variances are expressed as mean ± SD.
the lower 70 dB pre-pulse. While in IVC and in vivo mice (of both sexes), the percentage of PPI generally increased as a function of pre-pulse intensity, male BB-derived mice displayed a stable 42% decrease in all pre-pulse conditions (Fig. 3c).

Discussion

Nowadays, several clinical interventions include the use of BB to screen genetic abnormalities of human embryos prior to implantation (Harper...
Although widely applied for the diagnosis of many hereditary disorders, the magnitude of post-natal consequences of BB procedures has not been studied extensively. Here we report that BB induced significant changes in body weight and behavior in male offspring. This sexual dimorphism has been observed in psychiatric diseases known to be characterized by a male gender bias: indeed, males are more susceptible to psychiatric disorders such as schizophrenia, addiction and autism (Constantino and Todd, 2003; Kirkbride et al., 2006). It is likely that endocrinological differences are involved in the sexual dimorphism of psychiatric and metabolic disorders, though the specific molecular mechanisms at play have not been elucidated yet. Recent studies have demonstrated the existence of a female protective factor able to preserve females from the effects of heritable and de novo autism risk factors (Robinson et al., 2013).

In our study, male mice derived from biopsied embryos gained more weight throughout their lifespan than IVC controls. Such differences were already detectable during pre-weaning development, with BB mice gaining more weight than controls starting at P10. Since BB did not affect organ weight, it is possible that the increased body weight observed in BB males was due to increased fat deposition, indicating that fat metabolism could be altered as a consequence of BB. Increased body weight in mice derived from BB was observed previously (Yu et al., 2009). Thus, the effects of BB on fat metabolism and energy balance systems should be further explored to better clarify the possible risk of obesity in children born after BB. Moreover an increased body weight in mice derived from the IVC was observed. Body weight at birth was also affected by litter size. While litters from the in vitro groups (BB and IVC) had reduced numbers of littermates respect to in vivo ones, differences in body weight between IVC and in vivo-derived animals have to be seen in light of this limitation.

The behavioral data obtained in the TS and in the startle reflex tests suggest that mice produced with BB develop an antidepressant-like phenotype and deficits in PPI of, and habituation to, the startle reflex. PPI refers to the attenuation of the startle response to an intense pulse stimulus when it is shortly preceded by a weaker prepulse stimulus. PPI is a measure of sensory-motor gating, and its impairment, together with habituation deficits, has been detected in several neuropsychiatric diseases, including schizophrenia (Braff and Geyer, 1990; Braff et al., 1992).

The decreased time of immobility observed in the BB group in the TST also occurs in mice following the injection of antidepressant drugs prior TS test sessions (Cryan et al., 2005). This phenotype could also reflect an altered coping response to a threatening inescapable situation, or could be related to general hyperactivity.

In all stages of the experiments, careful control strategies were employed to ensure that the differences reported here could only be attributed to the blastomere ablation and not to other experimental conditions. In each replicate, embryos were collected from different donors, pooled together and split into two groups to generate either BB or IVC

**Table II** Average weight measurements at birth and at weaning.

<table>
<thead>
<tr>
<th>Birth</th>
<th>Weaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB</td>
<td>IVC</td>
</tr>
<tr>
<td><strong>in vivo</strong></td>
<td><strong>in vivo</strong></td>
</tr>
<tr>
<td>BB</td>
<td>IVC</td>
</tr>
<tr>
<td>1470</td>
<td>1385</td>
</tr>
<tr>
<td>1263</td>
<td>1613</td>
</tr>
<tr>
<td>1140</td>
<td>2040</td>
</tr>
<tr>
<td>1486</td>
<td>1144</td>
</tr>
<tr>
<td>1248</td>
<td>1070</td>
</tr>
<tr>
<td>1518</td>
<td>1162</td>
</tr>
<tr>
<td>0.802</td>
<td>1307</td>
</tr>
<tr>
<td>1140</td>
<td>1450</td>
</tr>
<tr>
<td>0.796</td>
<td>1348</td>
</tr>
<tr>
<td>1065</td>
<td>1220</td>
</tr>
<tr>
<td>0.967</td>
<td>1085</td>
</tr>
<tr>
<td>1015</td>
<td>1280</td>
</tr>
<tr>
<td>1283</td>
<td></td>
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<tr>
<td>1113</td>
<td></td>
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<tr>
<td>1423</td>
<td></td>
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<tr>
<td>1310</td>
<td></td>
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<tr>
<td>1115</td>
<td></td>
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<tr>
<td>1183</td>
<td></td>
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<tr>
<td></td>
<td>Average</td>
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<tr>
<td></td>
<td>1225</td>
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<td></td>
<td>11992</td>
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<tr>
<td></td>
<td>10374</td>
</tr>
</tbody>
</table>

Each value represents the average weight, in milligrams, among the pups from the same litter.

**Table III** Mean organ weight (g) of male and female offspring (n = 10–18 per treatment).

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Lung</th>
<th>Heart</th>
<th>Left kidney</th>
<th>Right kidney</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BB</td>
<td>2.167 ± 0.222</td>
<td>0.436 ± 0.084</td>
<td>0.261 ± 0.034</td>
<td>0.400 ± 0.037</td>
<td>0.435 ± 0.034</td>
<td>0.229 ± 0.047</td>
</tr>
<tr>
<td>IVC</td>
<td>2.126 ± 0.151</td>
<td>0.401 ± 0.070</td>
<td>0.265 ± 0.026</td>
<td>0.395 ± 0.039</td>
<td>0.405 ± 0.042</td>
<td>0.221 ± 0.057</td>
</tr>
<tr>
<td><strong>in vivo</strong></td>
<td>2.095 ± 0.137</td>
<td>0.380 ± 0.028</td>
<td>0.246 ± 0.041</td>
<td>0.365 ± 0.040</td>
<td>0.347 ± 0.061</td>
<td>0.212 ± 0.018</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BB</td>
<td>1.962 ± 0.413</td>
<td>0.376 ± 0.079</td>
<td>0.193 ± 0.026</td>
<td>0.264 ± 0.016</td>
<td>0.284 ± 0.034</td>
<td>0.295 ± 0.094</td>
</tr>
<tr>
<td>IVC</td>
<td>1.657 ± 0.268</td>
<td>0.374 ± 0.068</td>
<td>0.208 ± 0.030</td>
<td>0.265 ± 0.038</td>
<td>0.268 ± 0.051</td>
<td>0.245 ± 0.061</td>
</tr>
<tr>
<td><strong>in vivo</strong></td>
<td>2.025 ± 0.272</td>
<td>0.365 ± 0.056</td>
<td>0.194 ± 0.020</td>
<td>0.269 ± 0.016</td>
<td>0.271 ± 0.012</td>
<td>0.288 ± 0.043</td>
</tr>
</tbody>
</table>

*Variances are expressed as mean ± SEM.*
Post-natal consequences of blastomere biopsy

offspring. Moreover, blastocysts from the different groups were transferred to female recipients originating from the same litter. This strategy minimized genetic differences between groups in the source of embryos and in the maternal environment.

How the removal of a blastomere influences brain development is not known. It has been observed that mice derived from biopsied embryos present an altered steroid metabolism in placental tissues (Sugawara et al., 2012; Sugawara and Ward, 2013). Therefore, it is possible that BB exerts negative effects on neurodevelopment indirectly by interfering with placental functions. Moreover, the lack of developmental determinants and/or adaptative metabolic changes that are consequences of BB could irreversibly alter embryonic programs and in the maternal environment.

In conclusion, our data demonstrate that BB could affect long-term programming of post-natal development and behavior in mice, and suggest that PGD procedures could be risk factors for late-onset disease predisposition. However, modeling ARTs in mice must be viewed in light of its limitations. Culture protocols used in human clinical practice could be better established than in mouse research, and specific neurodevelopmental features could also be the source of differences between mice and humans in the effects of BB. Therefore, it is needed to understand the molecular and physiological consequences of BB on placental physiology and on the developing and adult brain. BB-derived embryos present an altered steroid metabolism in placental tissues, and direct effects of BB could irreversibly alter embryonic programs and contribute to altered brain development in the offspring. Moreover, blastocysts from the different groups were transferred to female recipients originating from the same litter. The strategy minimized genetic differences between groups in the source of embryos and in the maternal environment.

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Table IV Behavioral results from ultrasound vocalization (USV), open field and elevated plus maze tests. Variances are expressed as mean ± SD.

<table>
<thead>
<tr>
<th></th>
<th>Number of USV</th>
<th>Open field</th>
<th>Elevated plus maze</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of entries</td>
<td>Time spent</td>
</tr>
<tr>
<td>BB P4</td>
<td>98.09 ± 10.54</td>
<td>12.93 ± 1.24</td>
<td>28.75 ± 2.83</td>
</tr>
<tr>
<td>BB P8</td>
<td>119.7 ± 19.11</td>
<td>10.58 ± 1.38</td>
<td>25.66 ± 1.97</td>
</tr>
<tr>
<td>BB P12</td>
<td>112.87 ± 13.76</td>
<td>11.79 ± 1.62</td>
<td>25.11 ± 2.06</td>
</tr>
<tr>
<td>IVC P4</td>
<td>119.7 ± 19.11</td>
<td>10.58 ± 1.38</td>
<td>25.66 ± 1.97</td>
</tr>
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</tr>
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<td>In vivo PA</td>
<td>112.87 ± 13.76</td>
<td>11.79 ± 1.62</td>
<td>25.11 ± 2.06</td>
</tr>
</tbody>
</table>

Figure 2 Decreased time of immobility in BB-derived male mice (n = 12 mice/group/sex). Male derived from biopsied embryos showed significantly less time of immobility in the tail suspension test compared with IVC and in vivo mice (P < 0.05). Variances are expressed as mean ± SEM.
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Authors’ roles
S.S. conceived the trial and performed the behavioral tests and data analysis and drafted the manuscript; F.Z. performed the embryo manipulation, in vitro cultures and embryo transfers; A.H.S. and A.J.M. substantially contributed to the acquisition and interpretation of data; P.L. revised the manuscript for intellectual content; and G.E.P. supervised the trial and prepared the final manuscript.

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Conflict of interest
None declared.

References
Carola V, D’Olimpo F, Brunamonti E, Mangia F, Renzi P. Evaluation of the elevated plus-maze and open-field tests for the assessment of


