IVF affects embryonic development in a sex-biased manner in mice

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Abstract

Increasing evidence indicates that IVF (IVF includes *in vitro* fertilization and culture) embryos and babies are associated with a series of health complications, and some of them show sex-dimorphic patterns. Therefore, we hypothesized that IVF procedures have sex-biased or even sex-specific effects on embryonic and fetal development. Here, we demonstrate that IVF-induced side effects show significant sexual dimorphic patterns from the pre-implantation to the prenatal stage. During the pre-implantation stage, female IVF embryos appear to be more vulnerable to IVF-induced effects, including an increased percentage of apoptosis (7.22 ± 1.94 vs 0.71 ± 0.76 , $P < 0.01$), and dysregulated expression of representative sex-dimorphic genes (*Xist*, *Hprt*, *Pgk1* and *Hsp70*). During the mid-gestation stage, IVF males had a higher survival rate than IVF females at E13.5 (male:female=1.33:1), accompanied with a female-biased pregnancy loss. In addition, while both IVF males and females had reduced placental vasculogenesis/angiogenesis, the compensatory placental overgrowth was more evident in IVF males. During the late-gestation period, IVF fetuses had a higher sex ratio (male:female=1.48:1) at E19.5, and both male and female IVF placentas showed overgrowth. After birth, IVF males grew faster than their *in vivo* (IVO) counterparts, while IVF females showed a similar growth pattern with IVO females. The present study provides a new insight into understanding IVF-induced health complications during embryonic and fetal development. By understanding and minimizing these sex-biased effects of the IVF process, the health of IVF-conceived babies may be improved in the future.

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Introduction

IVF is widely used to treat infertility (ESHRE 2014); however, a small but worrying percentage suffer from a variety of IVF-associated risks, including pregnancy loss, preterm birth, birth defects and long-term disease risks (Hansen *et al.* 2005, Ceelen *et al.* 2008, Hart & Norman 2013, Servick 2014). Interestingly, these IVF-associated health complications are at least partially dependent on the gender of IVF offspring. For example, IVF is associated with higher risks of female-biased Rett syndrome (Arnaud & Feil 2005) and male-biased Autism (Fountain *et al.* 2015), and some IVF-associated birth defects show sexually dimorphic patterns (Michalski *et al.* 2015). Furthermore, sex-specific effects of IVF on postnatal metabolism and growth of IVF-conceived offspring has been confirmed in mouse models (Scott *et al.* 2010, Donjacour *et al.* 2014, Feuer *et al.* 2014). More importantly, recent studies indicate that IVF results in a skewed sex ratio at birth (Dean *et al.* 2010, Bu *et al.* 2014, Maalouf *et al.* 2014), reflecting sex-biased or even specific disruption of embryonic development. Indeed, the suboptimal environment is known to affect the

embryonic development in a sex-specific manner as early as the pre-implantation stage, causing either a short-term sex ratio adjustment or more long-term sex-specific effects on adult health (Laguna-Barraza *et al.* 2012). Therefore, we postulated that IVF has sex-biased or sex-specific effects on embryonic or fetal development during pregnancy.

Embryonic and postnatal phenotypic variability between males and females induced by environmental factors may occur through epigenetic mechanisms (Gutierrez-Adan *et al.* 2006, Laguna-Barraza *et al.* 2012). During the IVF process, both gametes and the pre-implantation embryos are exposed to *in vitro* conditions that do not perfectly recapitulate the normal environment of the Fallopian tube and uterus (Feuer & Rinaudo 2012), in which genome-wide epigenetic reprogramming occurs (Reik 2007, Cockburn & Rossant 2010). Moreover, male and female embryos differ in their chromosomal complement, transcriptome, proteome and subsequent metabolome (Kobayashi *et al.* 2006, Bermejo-Alvarez *et al.* 2010, 2011, Gardner *et al.* 2010), and in their epigenetic processes, including

X-chromosome inactivation (XCI) (Takagi & Sasaki 1975), DNA methylation (Bermejo-Alvarez *et al.* 2008, 2010, Gebert *et al.* 2009) and gene imprinting (Kobayashi *et al.* 2006). Therefore, variation in these epigenetic mechanisms between male and female pre-implantation embryos may provide a molecular basis for the IVF-induced sex-biased or sex-specific effects.

In this study, using *in vivo* (IVO) conceived mouse embryos as controls, we investigated whether IVF has any sex-biased or sex-specific effects on embryonic or fetal development from pre-implantation to the prenatal stage. In addition, we aimed to explore the molecular basis behind these effects by monitoring the expression of related sex-dimorphic genes throughout these developmental stages in mice.

Materials and methods

Ethical approval

The protocols for the animal studies were approved by and performed in accordance with the requirements of the Institutional Animal Care and Use Committee of China Agricultural University.

Animals

We used female mice from the Institute for Cancer Research (ICR) aged 7–8 weeks and male ICR mice aged 10–12 weeks. The mice were allowed to feed *ad libitum* and housed under controlled lighting conditions (12 h light:12 h darkness photoperiod).

Chemicals

All chemicals used were purchased from the Sigma–Aldrich Chemical Company unless otherwise specified.

Experimental design

We used a well-established experimental design (Delle Piane *et al.* 2010, Fauque *et al.* 2010, Tan *et al.* 2015) to test the sex-biased

or sex-specific effects of the IVF process from pre-implantation to the prenatal stage (Fig. 1). Briefly, all female mice were superovulated and randomly divided into two groups (IVO or IVF). After either *in vivo* fertilization and development (IVO group, control) or IVF and culture (IVF group), blastocysts in both groups were collected and transferred to pseudopregnant recipient mice. The samples of both groups were collected at blastocyst stage, E13.5 and E19.5 respectively. At blastocyst stage, well-developed late-cavitating blastocysts were collected at 96–100 h and 106–110 h post-hCG for the IVO and IVF groups respectively, according to previous descriptions about the developmental progress of IVO and IVF blastocysts (Giritharan *et al.* 2007).

We performed immunofluorescence (IF) staining to count the inner cell mass (ICM) and trophectoderm (TE) cell numbers before each blastocyst was collected and sex was determined by PCR. For gene expression analyses, each blastocyst was individually collected and their sex was determined by PCR. After that, female (IVO, $n=34$; IVF, $n=40$) and male (IVO, $n=33$; IVF, $n=40$) blastocysts were pooled respectively. Finally, qRT-PCR was performed on the pooled females and males to ascertain gene expression. At E13.5 and E19.5, the developmental rates were recorded. Fetuses and placentas were collected, and their weights and sex were determined. At E13.5, the placentas were used for morphometric and gene-expression analyses. At E19.5, the pups from the litters (of any size) were used to generate birth data; however, only animals from litters with 5–8 pups were used in the postnatal growth curve, as previously described (Donjacour *et al.* 2014).

Embryo preparation

The female mice were superovulated by intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin and 5 IU hCG after 47 hours. In the IVO group, the superovulated female mice were mated individually with male mice. The following morning, successful mating was confirmed by detecting the presence of a vaginal plug. In the IVF group, IVF was performed as previously described (Nagy *et al.* 2003). Briefly, cumulus-enclosed oocyte complexes were collected from the ampullae 14 h after the hCG injection, and sperm was obtained from the cauda epididymis and capacitated for 1 h in human tubal fluid (HTF) medium (Sage, Bedminster, NJ, USA) at 37 °C and 5% CO₂. Gametes were co-incubated in HTF

Figure 1 Overview of experimental design. Blastocysts from *in vivo* fertilization and development (IVO group) and IVF and culture (IVF group) were collected and then transferred into pseudopregnant females. Both IVO and IVF embryos/conceptuses were collected at blastocyst stage, E13.5 and E19.5 for detailed analyses.

medium for 4 h. Fertilized oocytes were washed several times in potassium simplex optimization medium containing amino acids (KSOM+AA, Millipore, Billerica, MA, USA) and transferred to 60 µl drops of KSOM+AA medium covered with paraffin oil. The embryos were cultured at 37 °C with 5% CO₂.

Blastocyst collection and embryo transfer

The criteria for collecting blastocysts for embryo transfer were based on developmental progress and morphology. IVO blastocysts were obtained from donor females by flushing the uterus with M2 medium (96–100 h post-hCG). According to previous reports, IVF embryos show delayed pre-implantation development (Giritharan *et al.* 2007, Rivera *et al.* 2008, Fernandez-Gonzalez *et al.* 2009). Therefore, as previously described (Giritharan *et al.* 2007), only well-developed late-cavitating blastocysts of similar morphology, based on the morphological landmark mentioned by Nagy *et al.* (2003), were sampled (106–110 h post-hCG) for further analysis or selected for embryo transfer in each group.

Naturally ovulating ICR female mice (aged 8 weeks), mated with vasectomized ICR males 3.5 days prior to embryo transfer, were used as recipients for both IVO and IVF embryo transfers. The morning after mating, the recipients were checked for the presence of a vaginal plug. The day of plugging was considered to be day 0.5 of the pseudopregnancy. Twelve blastocysts were surgically transferred to each recipient, with six blastocysts in each uterine horn.

Collection and morphometric analysis of post-implantation embryos

At E13.5, fetuses within their parietal and visceral yolk sac with intact placentas were dissected from their visceral yolk sac after removal of the parietal yolk sac. The sampled fetuses and placentas were weighed. Then, a proportion of placentas were placed in PBS supplemented with 4% (v/v) paraformaldehyde for 24 h and used to make paraffin sections, while the other

placentas were collected for RNA extraction and detailed analyses, as described below. At E19.5, fetuses and placentas were sampled, as described for the E13.5 collection. Placentas were weighed and then placed in PBS supplemented with 4% (v/v) paraformaldehyde. The weight of fetuses was measured weekly until week 9.

Real-time quantitative PCR analysis

Each blastocyst was collected and treated with 7 µl ice-cold Cell Lysis II Buffer (plus the Armored RNA Control). Next, 2 µl of the lysis product was digested with proteinase K at 55 °C for 2 h, and used for sex determination with TaKaRa Ex Taq Hot Start Version (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. *Rn18s* and *DYzEms3* were used for sex determination, as documented by previous studies (Bermejo-Alvarez *et al.* 2012, Rosenfeld 2012). In female samples, only one amplicon corresponding to the *Rn18S* (91 bp) gene was detected. While in male samples, an additional amplicon corresponding to the Y chromosome-specific *DyzEms3* segment (254 bp) was detected. The amplification conditions were as follows: initial denaturation for 3 min at 95 °C; 40 cycles of 10 s at 95 °C, annealing for 15 s at 52 °C, and elongation for 15 s at 72 °C; and a 5 min extension at 72 °C. The remaining 5 µl of the lysis product was used to perform RT according to the manufacturer's protocol of the Cells-to-cDNA TM II Kit (Ambion, Austin, TX, USA).

Real-time PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad) in a CFX96 real-time PCR machine (Bio-Rad). The amplification conditions were as follows: initial denaturation for 30 s at 95 °C and 40 cycles of 5 s at 95 °C, annealing and elongation for 5 s at 60 °C. Negative controls were either reactions without reverse transcriptase or with the RNA sample replaced by diethylpyrocarbonate treated MilliQ water (to test for any RNA or DNA contamination). *Actb* and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) were used as the normalization control. Primer information is provided in Table 1. A threshold was set where the

Table 1 Primer sequences.

Gene name	Forward (5'–3')	Reverse (5'–3')
<i>Actb</i>	AGGTCATCACTATTGGCAAC	ACTCATCGTACTCTGCTTG
<i>Dlk1</i>	GCAAGAAGAAGAACCTCCT	CACAGCAACACAAGACAATA
<i>Dnmt3a</i>	TCACTCTTCTCTTCTCTCC	CACATATCTACACTCCATCCA
<i>Dnmt3b</i>	GAGATGATGAAGTAGATGATGG	GTGCTTGCTGAAGATGATG
<i>DYzEms3</i>	TAGGATGGTAAGCCCAATGC	TGGTTGGTTAATTGTTTGGG
<i>G6pdx</i>	ACCAGACTTCCCAGAGGCTA	AGGCAGCATGAGGTCTCTTC
<i>Gapdh</i>	TGCCCCCATGTTTGTGATG	TGTGGTCATGAGCCCTTCC
<i>H19</i>	GTGATGGAGAGGACAGAAG	GAGAGCAGCAGAGAAGTG
<i>Hprt1</i>	CAGCGTCGTGATTAGCG	GCCTCCCATCTCCTTCAT
<i>Hsp70</i>	GGCACCGATTACTGTCAA	GGCTACTGGAACACTGATAT
<i>Igf2</i>	GTGGAAAGAATCAGGAGGAG	GTGGAGAGCAGGACATTG
<i>Kcnq1ot1</i>	ATGTTGATGCTGCTCTTCT	CCTGATGGTCTCTGGATTC
<i>Pgk1</i>	CAGTTGCTGCTGAACCTCAAATC	CCCTCCCTTCTTCTCTACAT
<i>Rn18s</i>	AGAAACGGCTACCACATCCAA	CCTGTATTGTTATTTTCGTACTACCT
<i>Slc2a1</i>	ATTCAGATAAGCAGCAGGTT	CTCAGAGTTCCGTTATTAGTGT
<i>Slc2a3</i>	AGGAGATGAAGGATGAGAGT	CTGGAGGACAATGGAGATG
<i>Slc38a4</i>	GAATGACGGTTGCCAGTA	AAGAGTGAGAACGGAAGAC
<i>Snrpn</i>	AATGGTCAGCAAGGCAAT	GGAGCAAGTTGGAGATGG
<i>Xist</i>	GGCGGTGCAAACTAAACTC	CAGTAGGCTTAGAGAACCCG

amplification was close to the reaction's maximum rate, which was not achieved in the negative controls. The threshold cycle (C_t) was used to calculate the relative quantity of the genes of interest in the cDNA sample. A plot of $2^{-(\text{normalized } \Delta C_t)}$ is shown.

At E13.5, five placentas (including spongiotrophoblast and labyrinth) from five litters of each group, with the wet weights closest to the mean weight of the litter, were pooled for total RNA extraction. The total RNA extraction from placentas of E13.5 embryos was performed using TRIzol (Invitrogen) according to the manufacturer's instructions. Before RT, RNA samples were digested with DNase I (Fermentas, Hanover, MD, USA) to remove the contaminating genomic DNA. The concentration and the quality of the extracted total RNA were assessed by the A260 nm/A280 nm and A260 nm/A230 nm ratios determined using a DS-11 spectrophotometer (Denovix, Wilmington, NC, USA). RT was accomplished by using the commercially available first strand cDNA synthesis kit (iScript cDNA synthesis kit, Bio-Rad Laboratories). The real-time quantitative PCR was performed as described above.

Apoptosis analysis

At the blastocyst stage, the embryos were washed three times with 0.1% polyvinyl alcohol (PVA)/PBS and then transferred to PBS supplemented with 4% (v/v) paraformaldehyde and 0.5% Triton X-100 for simultaneous fixation and permeabilization at 37 °C for 45 min. Apoptotic nuclei were detected by TUNEL assay using *In Situ* Cell Death Detection Kit (Roche). According to the manufacturer's instructions, fixed embryos were incubated in the TUNEL reaction medium for 1 h at 37 °C in the dark. After the reaction was stopped, the embryos were washed, transferred into a 2 µg/ml DAPI (4', 6-diamidino-2'-penyindole dihydrochloride, Vector Laboratories Inc., Burlingame, CA, USA) solution and mounted on slides. The number of apoptotic nuclei and the total number of nuclei were determined under a confocal laser scanning microscope (Digital Eclipse C1, Nikon, Tokyo, Japan).

IF staining

IF staining was performed as previously described (Inoue *et al.* 2010), with minor modifications. Briefly, embryos were fixed with 4% paraformaldehyde in PBS (pH 7.4) at 4 °C for 1 h and then permeabilized with PBS/0.5% Triton X-100/0.1% PVA (PBST/PBS/0.5% Triton X-100)-PVA) at room temperature for 1 h. After three washes with PBS/0.1% PVA at 37 °C for 10 min, the embryos were blocked in PBS/1% BSA at 4 °C for 6 h. Fixed embryos were then incubated with primary anti-CDX2 antibodies (1:200, Santa Cruz Biotechnology) overnight at 4 °C. Following three washes with PBST-PVA, the embryos were incubated with secondary antibodies conjugated with Alexa Fluor 488 (anti-mouse, Invitrogen) for 1 h at room temperature. Finally, the samples were counterstained with DAPI. The fluorescence signals were determined under a confocal laser scanning microscope (Digital Eclipse C1). The sex of each embryo was determined by PCR according to the method described above.

Histological and morphometric analysis of the placentas

At E13.5, at least one placenta, with the weight closest to the mean weight of the litter, was selected from each litter. These samples were fixed and then embedded in paraffin wax, and 6 µm cross-sections were obtained and stained with hematoxylin and eosin. The resulting sections were observed and photographed under an epifluorescence microscope (BX51, Olympus). The border between the labyrinth and the spongiotrophoblast was identified visually and outlined using Photoshop software (Adobe). The border between maternal and fetal components of the placenta was identified by the presence of trophoblast giant cells; a continuous line connecting the trophoblast giant cells was created likewise using Photoshop. The total cross-sectional areas of fetal and labyrinthine zones were measured and calculated using the DP72 plug-ins Image Processing Toolkit (Olympus).

For endothelial staining of fetal blood vessels, a subgroup of placentas was stained for laminin (Sigma). Briefly, 6 µm paraffin sections were deparaffinized and rehydrated in PBS. The sections were treated with blocking solution (1% BSA/PBS), and incubated with primary anti-laminin antibodies overnight at 4 °C. Following three washes with PBST-PVA, the embryos were incubated with secondary antibodies conjugated with Alexa Fluor 594 (anti-rabbit, Invitrogen) for 1 h at room temperature. Finally, the samples were counterstained with DAPI. The fluorescence signals were determined under an epifluorescence microscope (BX51, Olympus). As described previously (Rosenstein *et al.* 1998), four regions (1 mm²) within each image were sampled by using a per area ratio measurement, and the data were analyzed by Mann-Whitney *U*-test to determine significant differences between IVO and IVF placentas.

Statistical analysis

One-way ANOVA was used to compare the differences among the groups, followed by *post-hoc* comparisons using IBM SPSS statistics software version 20. Experimental replicate was incorporated as a covariate. Relative gene expression data was first subjected to arcsine transformation. The developmental rate of embryos to the blastocyst stage was assessed by binary logistic regression analysis. The sex ratio was calculated as the proportion of males (number of males/total number of embryos or fetuses) and compared with the naturally expected ratio (50%) using a corrected χ^2 procedure (Kimura *et al.* 2005).

Results

The effects of IVF on pre-implantation embryonic development

At the blastocyst stage, the sex ratio (male/female) was not significantly changed in the IVF group compared with the IVO group (1.02 vs 1.05, Table 2). However, male IVF blastocysts had a higher total cell number compared with their female counterparts (54.22 ± 1.02

Table 2 The developmental rate of IVO and IVF preimplantation embryos.

Group	No. of zygotes	Sex	No. of blastocysts	Zygote to blastocyst (%)	Male/female
IVO			176		1.02
		Male	89		
		Female	87		
IVF	309		238	77.02±4.31	1.05
		Male	122	39.48±2.27	
		Female	116	37.54±2.14	

Data represent mean ± s.d.

vs 46.59 ± 1.02 , $P < 0.05$, Table 3). In addition, male IVF blastocysts, but not females, had a significantly increased ratio of TE/ICM cells compared with IVO blastocysts (males: 2.64 ± 0.11 vs 2.25 ± 0.09 , $P < 0.05$; females: 2.43 ± 0.11 vs 2.22 ± 0.10 , $P > 0.05$, Table 3). Moreover, while the apoptosis rates of IVF male and female blastocysts was significantly higher than that of their IVO counterparts ($P < 0.05$, Fig. 2A), the increased rate of apoptosis in female IVF blastocysts was more pronounced than that in IVF males.

As differences in gene expression regulation between male and female pre-implantation embryos may explain the variable susceptibility to IVF, we detected several representative X-linked (*Xist*, *Hprt*, *G6pdx* and *Pgk1*) and autosomal (*Slc2a3*, *Hsp70*, *Dnmt3a* and *Dnmt3b*) genes that are differentially expressed between genders (Bermejo-Alvarez et al. 2011). Compared with their IVO counterparts, IVF males showed significant higher expression levels of *G6pdx* and *Hsp70*; while IVF females showed significantly higher the expression levels of *Hprt*, *Pgk1*, *Hsp70* and significantly lower the expression level of *Xist* (Fig. 2B).

The effects of IVF on mid-gestation placental and fetal development

At E13.5, we found that the implantation rate of IVF embryos was comparable with that of IVO embryos (Table 4). However, IVF embryos showed a significantly higher abortion rate compared with IVO embryos (23.64% vs 11.63%, $P < 0.05$). Furthermore, the sex ratio (male/female) of IVF fetuses at E13.5 was significantly higher than the expected natural sex ratio (1.33 vs 1, $P < 0.05$) (Table 4). Moreover, IVF fetal weight at E13.5 was significantly lower than that in the IVO group (112.84 ± 7.87 mg vs 126.40 ± 2.52 mg, $P < 0.05$); but

placental weight was similar between the two groups. We also observed a significantly higher ratio of placental/fetal weight for IVF males compared with the IVO group (0.68 ± 0.03 vs 0.59 ± 0.01 , $P < 0.05$), but not for IVF females (Table 5).

Next, we analyzed the histological characteristics of the placenta at E13.5. Although the total placental area and labyrinth area in both male and female IVO placentas were comparable to their IVF counterparts, the ratio of labyrinth area to total placental area was significantly lower in IVF males and females compared with their IVO counterparts (males: 0.60 ± 0.03 vs 0.67 ± 0.02 , $P < 0.05$; females: 0.59 ± 0.01 vs 0.66 ± 0.01 , $P < 0.05$, Fig. 3A). As the labyrinth is crucial for the exchange of nutrients between maternal and fetal blood (Desforges & Sibley 2010), we examined vasculogenesis in this area using laminin to stain blood vessels (Fig. 3B). By quantifying the blood vessels per area, we showed that IVF fetuses had reduced placental vasculogenesis in the labyrinth layer compared with IVO fetuses (Fig. 3B).

Based on the aberrant placental morphology, we attempted to determine whether IVF affects the expression of genes involved in placental development and nutrient transport. Previous studies have demonstrated that IVF can dysregulate the expression of genes involved in placental nutrient transport (*Slc2a1*, *Slc2a3* and *Slc38a4*) (Chen et al. 2015), as well as of imprinted genes that play an important role in placental development and function (*Dlk1*, *H19*, *Igf2*, *Kcnq1ot1* and *Snrpn*) (Constancia et al. 2005, Angiolini et al. 2006). We found that compared with their IVO counterparts, IVF males showed significantly lower expression of *Dlk1*, *Kcnq1ot1*, *Slc2a1*, *Slc2a3* and *Slc38a4*; while IVF females showed significantly lower expression of *Dlk1* and *Slc2a3* (Fig. 3C).

The effects of IVF on late-gestation fetal and placental development and postnatal growth

At E19.5, the litter size in the IVF group (5.47 ± 1.31 , $n = 15$ litters) was significantly smaller than that in IVO group (6.75 ± 1.42 , $n = 12$ litters, $P < 0.05$). The sex ratio (male/female) of IVF fetuses was significantly higher when compared with the expected natural ratio (1.48 vs 1, $P < 0.05$, Table 6). Furthermore, both male and female IVF fetuses showed comparable weight to their IVO counterparts; while both male and female IVF placentas showed significantly higher weight than their IVO

Table 3 Cell numbers of IVO and IVF blastocysts.

Sex	Group	No. of blastocysts	No. of total cells	No. of TE cells	No. of ICM cells	Percentage of ICM	Ratio TE/ICM
Male	IVO	38	49.63 ± 0.92^b	33.39 ± 0.85^b	16.24 ± 0.74	32.61 ± 0.95	2.25 ± 0.09^b
	IVF	37	54.22 ± 1.02^a	38.38 ± 0.96^a	15.84 ± 0.72	29.71 ± 0.99	2.64 ± 0.11^a
Female	IVO	36	47.78 ± 1.26^{bc}	32.03 ± 1.10^b	15.75 ± 0.77	33.12 ± 1.05	2.22 ± 0.10^b
	IVF	34	46.59 ± 1.02^c	32.29 ± 0.79^b	14.29 ± 0.62	30.67 ± 0.79	2.43 ± 0.11^{ab}

Data represent the mean ± s.e.m. Different superscript letters if $P < 0.05$.

Figure 2 Apoptosis analysis and gene detection in blastocysts. (A) Apoptosis analysis of male and female blastocysts from the IVO and IVF groups. The apoptosis rate (%) in male and female blastocysts in each group is shown. The number of blastocysts in each group is indicated. Data represent mean $\pm$ s.d. * $P < 0.05$, ** $P < 0.01$. (B) Expression levels of representative X-linked (*Xist*, *Hprt*, *G6pdx* and *Pgk1*) and autosomal (*Slc2a3*, *Hsp70*, *Dnmt3a* and *Dnmt3b*) genes in male and female blastocysts from the IVO and IVF groups. Five independent experiments were performed. Values are expressed as means $\pm$ s.d. Values with different letters (a, b and c) are significantly different ($P < 0.05$).

counterparts (Table 7 and Supplementary Table S1, see section on supplementary data given at the end of this article). Correspondingly, the ratio of placental/fetal weight in the IVF group was higher than that of the IVO group for both males and females (Table 7). We also detected the postnatal growth of males and females from birth until week 9. From birth to weaning (week 3), both IVF male and female mice had comparable weights to their IVO counterparts; however, after weaning, IVF males showed a significantly higher body weight gain, which was not observed in IVF females (Fig. 4 and Supplementary Table S2).

Discussion

This study highlights the sex-biased effects of the IVF process on embryonic and placental development, and postnatal growth. We found that the IVF manipulation during the pre-implantation stage impacts embryonic and placental development, and that these IVF-induced effects can persist from the pre-implantation stage through to the postnatal period. Our results support the idea that the pre-implantation period constitutes a window of development sensitive to reprogramming

(Lee & Bartolomei 2013, Donjacour *et al.* 2014), and provide evidence that many of these IVF-induced effects are sex-biased.

During the pre-implantation stage, the developmental potential of both female and male embryos was compromised by IVF; however, female embryos appear more vulnerable. Indeed, we showed that male pre-implantation embryos cleaved faster than females under IVF conditions. As faster cleavage during pre-implantation stage can result in higher pregnancy and implantation rates (Lundin *et al.* 2001), our results suggest that male IVF blastocysts may have a higher developmental potential. Moreover, while male and female IVF blastocysts had an increased apoptosis rate, as shown in previous studies (Hardy 1997, Lonergan *et al.* 2006), the increased rate of apoptosis in female IVF blastocysts was more pronounced than that in IVF males. This sex-biased effect during the pre-implantation stage was supported by the expression pattern of genes that are crucial for early development. In particular, X-linked genes, *Xist*, *Hprt* and *Pgk1*, were significantly dysregulated in IVF female blastocysts, implying that XCI, a female-specific epigenetic event to balance the X-linked gene dosage between males and females during early

Table 4 Implantation rate in mice at E13.5.

Group	No. of transferred blastocysts (recipients)	Average of implantation sites	Average of abortive sites	Abortion rate (%)	No. of fetuses (litters)	Range of litter size	Male/female [†]
IVO	60 (5)	8.60 $\pm$ 1.02	1.00 $\pm$ 0.63	11.63 ^b	38 (5)	6–9	1.11
IVF	84 (7)	7.86 $\pm$ 1.55	1.86 $\pm$ 0.76	23.66 ^a	42 (7)	5–8	1.33 ^a

Data represent mean $\pm$ s.d.. Different superscript letters if $P < 0.05$.

[†]The sex ratio was compared with the expected sex ratio (1.00) using a corrected χ^2 procedure (* $P < 0.05$).

Table 5 Fetal and placental weight and placenta/fetus ratio in mice at E13.5.

Sex	Group	No. of fetuses (litters)	Fetal weight (mg)	Placenta weight (mg)	Ratio placenta/fetus
Male	IVO	20 (5)	131.62 ± 4.60 ^a	76.93 ± 3.39 ^a	0.59 ± 0.01 ^b
	IVF	24 (7)	119.57 ± 4.38 ^b	80.99 ± 2.63 ^a	0.68 ± 0.04 ^a
Female	IVO	18 (5)	121.17 ± 0.96 ^{ab}	68.57 ± 4.11 ^b	0.57 ± 0.03 ^b
	IVF	18 (7)	106.12 ± 5.95 ^c	67.68 ± 4.72 ^b	0.64 ± 0.04 ^{ab}

Data represent mean ± s.d. Different superscript letters if $P < 0.05$.

development, may be impaired. However, the long-term effect of this IVF-induced female-specific epigenetic error should be further investigated.

During the mid-gestation stage, E13.5 was selected for sampling based on developmental considerations, as the placental architecture and function is well established and spongiotrophoblast and labyrinthine areas can be clearly distinguished at this time (Delle Piane *et al.* 2010). Although the implantation ability of IVF embryos

was comparable with that of IVO embryos, we found that the abortion rate of IVF embryos is significantly higher than that of IVO embryos. Further analysis showed that this phenomenon is mainly attributed to IVF females, indicating that IVF embryos may have a female-biased developmental defect before the mid-gestation stage, leading to a female-biased pregnancy loss. This agrees with the above observation that female blastocysts are more vulnerable to the IVF process. Therefore, a

Figure 3 Morphological analysis and gene detection in placentas at E13.5. (A) Representative images of cross-sections of the entire male and female placentas from the IVO and IVF groups. The areas of the labyrinth (L) and spongiotrophoblast (S) layers were measured. Data represent mean ± s.d. D, decidua. (B) Representative images of laminin-labeled placental vasculogenesis in male and female placentas from the IVO and IVF groups. Nuclei (blue) are stained with DAPI. The blood vessel per area (1 mm²) was quantified. Values are expressed as means ± s.d. (C) Expression levels of representative genes involved in placental development (*Dlk1*, *H19*, *Igf2*, *Kcnq1ot1* and *Snrpn*) and nutrient transport (*Slc2a1*, *Slc2a3* and *Slc38a4*) in male and female placentas from the IVO and IVF groups. Five independent experiments were performed. Values are expressed as means ± s.d. Values with different letters (a, b or c) are significantly different ($P < 0.05$).

Table 6 Litter characteristics of IVO and IVF fetuses at E19.5.

Group	No. of pups (litters)	Litter size	Range of litter size	Live pups (%) [†]	Male/female [‡]
IVO	81 (12)	6.75 ± 1.30 ^a	5–9	56.25 ± 11.83 ^a	1.08
IVF	82 (15)	5.47 ± 1.31 ^b	3–8	45.58 ± 10.92 ^b	1.48*

Data represent the mean ± s.d. Different superscript letters if $P < 0.05$.

[†]Number of live pups/number of embryos transferred. [‡]The sex ratio was compared with the expected ratio (1.00) using a corrected χ^2 procedure (* $P < 0.05$).

disrupted female-specific epigenetic event, such as XCI, may also play a role in IVF-induced effects during the mid-gestation period. Indeed, previous studies show that impaired XCI by *Xist* mutation can result in significant female-specific lethality during the peri-implantation period (Hoki *et al.* 2011, Senner *et al.* 2011). Another possible explanation for the female-biased growth defects is the gender-specific disruption of other epigenetic events, such as gene imprinting and DNA methylation (Kobayashi *et al.* 2006, Bermejo-Alvarez *et al.* 2008, 2010, Gebert *et al.* 2009).

We found both IVF males and females have an increased ratio of placental/fetal weight, which is considered as a marker of intrauterine stress (Barker *et al.* 1990). The increased ratio of placental/fetal weight observed in this study is similar to that described in human IVF pregnancies, where IVF offspring were associated with hypertension later in life (Barker *et al.* 1990, Moore *et al.* 1996, Koudstaal *et al.* 2000). The reasons for the increased ratio of placental/fetal weight are likely complicated; one possible explanation involves a compensatory process for the depressed fetal growth or the impaired function of the placenta itself (Feuer *et al.* 2014, Chen *et al.* 2015). The placental/fetal weight ratio was higher for IVF males than IVF females. In part, this may be due to the higher TE/ICM ratio observed in IVF male blastocysts. These results suggest that although the growths of both male and female fetuses were depressed after the IVF process, male placentas may have a stronger compensatory potential.

Correspondingly, we examined some representative genes that are essential for placental development and nutrient transport, and found that *Dlk1*, *Kcnq1ot1*, *Slc2a1*, *Slc2a3* and *Slc38a4* were dysregulated in IVF males, while *Dlk1* and *Slc2a3* were dysregulated in IVF females. This observation implies that the mechanisms responsible for the placental overgrowth may vary between males and females. *Dlk1* is a paternally expressed imprinted gene, which is a marker for a subpopulation of glycogen cells in the placenta, and is

essential for nutrient metabolism (Yevtodiynenko & Schmidt 2006, Charalambous *et al.* 2014). On the other hand, *Kcnq1ot1* is a maternally imprinted gene that organizes a lineage-specific nuclear domain for epigenetic gene silencing (Redrup *et al.* 2009), and its decreased expression is accompanied with placental overgrowth (Chen *et al.* 2015). *Slc2a1*, *Slc2a3* and *Slc38a4* are also well-documented genes involved in placental nutrient transport (Chen *et al.* 2015). Our results suggest that both males and females have an impaired placental development and function; however, the placental compensatory effect is more evident in males. This observation also suggests that the sex-biased effects of IVF on pre-implantation embryos can persist after implantation.

At birth, there were no significant differences in fetal weight between IVO and IVF males and females. This phenomenon, that the differences in fetal weight observed at E13.5 disappear 6 days later (at E19.5), is in accordance with that reported by previous study (Bloise *et al.* 2012), in which the relative IVF fetal weight to IVO controls is lowest at the mid-gestation stage. After that, differences in fetal weight between IVF and IVO embryos reduce gradually until they disappear. One plausible explanation is the compensatory effect due to the larger placentas in IVF groups compared with that in control groups (Bloise *et al.* 2012). Indeed, we found evident placental overgrowth in IVF males and females. This overgrowth may be due to the dysregulated expression of some key genes responsible for placental development and function, as described above. Among the most striking effects following IVF was an alteration in the growth curve in male IVF mice; IVF males grew faster than their IVO counterparts and showed a significantly higher body weight after weaning, which cannot be observed in females. This suggests that the IVF has long-term sex-biased effects that can persist to postnatal period. Similarly, a previous study demonstrated that male IVF mice tend to have aberrant glucose tolerance and pancreatic defects, which may be

Table 7 Fetal and placental weight and placenta/fetus ratio in mice at E19.5.

Sex	Group	No. of pups (litters)	Range of litter size	Fetal weight (g)	Placenta weight (g)	Ratio placenta/fetus
Male	IVO	37 (11)	5–8	1.45 ± 0.15	0.12 ± 0.01 ^b	0.087 ± 0.01 ^b
	IVF	38 (11)	5–8	1.45 ± 0.15	0.15 ± 0.03 ^a	0.10 ± 0.01 ^a
Female	IVO	35 (11)	5–8	1.41 ± 0.19	0.11 ± 0.01 ^b	0.084 ± 0.02 ^b
	IVF	29 (11)	5–8	1.39 ± 0.14	0.14 ± 0.01 ^a	0.10 ± 0.01 ^a

As the litter size may contribute considerably to the fetal and placental weight, litters with smaller or larger numbers of pups were excluded for analysis of fetal and placental weight. Data represent mean ± s.d. Different superscript letters if $P < 0.05$.

Figure 4 Comparisons of postnatal growth curves of IVF and IVO conceived offspring. Male (A) and female (B) IVF mice were compared separately with their IVO counterparts. The IVF males (A) were significantly larger in weight (*) than their IVO counterparts from 4 to 9 weeks. Values are expressed as means $\pm$ s.d.

associated with the observed postnatal overgrowth (Donjacour *et al.* 2014). However, the postnatal growth of male IVF mice appeared normal after *in vitro* culture using KSOM+AA medium under 5% O₂ (Donjacour *et al.* 2014). Therefore, the use of atmospheric O₂ (20%), as was used in this study, may be a suboptimal culture condition which contributes to the IVF-induced aberrations (Rinaudo *et al.* 2006). Future studies should be designed to define which particular constituent(s) in the culture medium and/or what oxygen concentration is responsible for IVF-induced sex-biased phenotypes.

Our present study showed that the sex ratio at birth was skewed in IVF offspring. Indeed, this phenomenon has been repeatedly reported in IVF embryos of domestic animals, especially in bovines (Avery *et al.* 1991, Gutierrez-Adan *et al.* 2001, Wrenzycki *et al.* 2002, Torner *et al.* 2014). However, in humans, a recent study on assisted reproductive technologies (ART) indicated that the sex ratio among abnormal embryos is male-biased during the pre-implantation stage, but is female-biased among normal embryos (Orzack *et al.* 2015). In the study by Orzack *et al.* (2015), ART included both IVF and ICSI, and the sex ratio was determined for the combined IVF- and ICSI-produced embryos. In most previous studies, the sex ratios of IVF- or ICSI-produced embryos/offspring were analyzed separately, and

IVF was shown to result in a male-biased sex ratio (Avery *et al.* 1991, Gutierrez-Adan *et al.* 2001, Wrenzycki *et al.* 2002, Dean *et al.* 2010, Maalouf *et al.* 2014, Torner *et al.* 2014), while ICSI resulted in a female-biased sex ratio (Bu *et al.* 2014, Maalouf *et al.* 2014, Tarin *et al.* 2014). Therefore, ICSI and IVF may affect sex ratios via different mechanisms. Moreover, two recent epidemiologic analyses based on very large clinical populations confirmed the IVF-induced higher male birth rate (Dean *et al.* 2010, Maalouf *et al.* 2014), which is in agreement with our results in mice. Therefore, the different strategy of analysis (i.e. analyzing IVF and ICSI together or separately) may explain the inconsistencies among these studies.

To the best of our knowledge, the skewed sex ratio observed in this study had not been reported in mice before. One possible explanation for this is that the sex skewing in IVF mouse embryos occurs during the post-implantation stage, but not at the blastocyst stage. This makes it difficult to perform a population analysis of the sex ratio phenomenon based on a large sample sizes. Indeed, a skewed sex ratio induced by maternal dietary nutrition was observed in mice at birth (Rosenfeld *et al.* 2003), suggesting that a long-term analysis (i.e. at the embryo stage or at birth) is needed to confirm sex skewing in IVF-produced offspring.

In summary, we have found that the effects of the IVF process, including IVF and culture, on embryonic or fetal development showed significant and long-term sexual dimorphic patterns. Future studies should be directed at understanding the molecular mechanisms underlying the described findings, and preventing these sex-biased side effects by optimizing the IVF system. By specifically targeting these sex-biased side effects, we hope to reduce IVF-associated health risks and improve the health of IVF-conceived babies.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-15-0588>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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