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Persistence and intergenerational transmission of differentially expressed genes in the testes of intracytoplasmic sperm injection conceived mice*

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Abstract: Intracytoplasmic sperm injection (ICSI) is commonly used to solve male infertility problems. Previous studies showed that early environmental exposure of an embryo may influence postnatal development. To detect whether ICSI operations affect the reproductive health of a male or his offspring, we established assisted reproductive technologies (ART) conceived mouse models, and analyzed gene expression profiles in the testes of both ICSI and naturally conceived (NC) newborn F_1 mice using micro-array analysis. Among the differentially expressed genes, we focused on the expression of eight male reproduction-related genes. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was used to analyze the expression of these genes in the testes of both adult and old F_1 generation mice and adult F_2 generation mice. Our results showed that down-regulated and somatic cell-expressed genes in newborn mice retained their differential expression patterns in adult and old F_1 generation individuals, implying the persistence and fetal origin of the alteration in the expression of these genes. The intergenerational transmission of differential gene expression was observed, but most changes tended to be reduced in adult F_2 generations. Controlled ovarian hyperstimulation (COH) and in vitro fertilization (IVF) mice models were added to explore the precise factors contributing to the differences in ICSI offspring. The data demonstrated that superovulation, in vitro culture, and mechanical stimulation involved in ICSI had a cumulative effect on the differential expression of these male reproductive genes.

Key words: Intracytoplasmic sperm injection, Testis, Intergenerational transmission
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1 Introduction

Intracytoplasmic sperm injection (ICSI) is a procedure in which a single sperm is injected into an oocyte for successful fertilization. This procedure is commonly used in assisted reproduction techniques (ART) to solve male infertility problems. The pregnancy rate following ICSI is associated mostly with semen quality and ICSI treatment-induced damage, which are also crucial factors affecting the health of offspring (Lu *et al*., 2012). Besides ART-induced common birth defects (Davies *et al*., 2012; Foix-L′Hélias *et al*., 2012), it was reported that there was a higher risk of hypospadias and lower semen quality in ICSI offspring (Mau *et al*., 2004; Mau Kai

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et al., 2007). Epidemiological studies found parental subfertility probably leads to a high risk of decreased semen quality in male offspring (Ramlau-Hansen *et al.*, 2008). A father's hypospadias may not only increase the frequency of hypospadias in sons, but also decrease their semen quality. Moreover, most fathers and sons might have the same susceptible genes involved in reproductive dysfunction (Asklund *et al.*, 2007). However, a twin study indicated that genetic factors accounted for 20% of abnormal sperm density, and that environmental influence was the main factor disturbing male reproductive health (Storgaard *et al.*, 2006).

The Dutch famine near the end of World War II provided useful information to study the long-term effects of the early pregnancy environment on the development of offspring. The term 'catch-up growth' refers to the increasing body mass index found in adulthood in individuals with low birth weight. Low birth weight is also associated with many metabolic diseases in adulthood (Prader *et al.*, 1963). Poor maternal nutrition during gestation may restrict fetal growth and increase susceptibility to diseases, such as obesity, diabetes, and coronary heart disease (Roseboom *et al.*, 2006; van Abeelen *et al.*, 2012). Barker (1990) described the 'fetal origins of adult disease', and claimed that the pregnancy environment could influence fetal development. ICSI technology not only changes the intrauterine environment with gonadotropin stimulation before pregnancy, but also disturbs fertilization and the culture environment of embryos. In the entire ART process, the environment to which the embryos are exposed has a profound effect on gene expression and even on the development and subsequent health of the fetus (Rinaudo and Schultz, 2004; Lonergan *et al.*, 2006). Previous studies reported that alternations of gene expression in oocytes and embryos can be found in the postnatal brain (Wang *et al.*, 2011). Gonadotropin can regulate the expression of testicular RNA helicase, an essential post-transcriptional regulator of spermatogenesis (Tsai-Morris *et al.*, 2012). Oxidative stress may decrease semen quality and male reproductive ability (Fatemi *et al.*, 2012), and in vitro culture can increase the amount of free oxygen radicals. A latest study comparing in vitro fertilization (IVF) and ICSI mice showed that ICSI injection could increase apoptosis of spermatocytes in testes (Yu *et al.*, 2011).

In a previous study, we found no differences in semen quality or testes morphology between ICSI mice and naturally conceived (NC) mice (unpublished data). In this study, we firstly compared the gene expression profiles in the testes of ICSI-conceived newborn mice with those of NC mice to find male reproduction-related differentially expressed genes. Then, the persistence and intergenerational transmission of the differential gene expression were analyzed. Our studies provide a detailed analysis of the long-term effects of ICSI treatments on male offspring.

2 Materials and methods

2.1 ART models

All the protocols used in our investigation were approved by the Animal Care Ethics Committee of Zhejiang University. All C57BL/6J mice (6–8 weeks old) were randomly divided into four groups (controlled ovarian hyperstimulation (COH)/IVF/ICSI/NC groups) and raised in a standard environment (room temperature: (23±1) °C; humidity: (55±5)%) with a 12-h light-dark cycle.

2.1.1 COH models

Female mice were injected with 7.5 IU pregnant mare serum gonadotrophin (PMSG) (GEN's, Hangzhou, China) and, 48 h later, with 7.5 IU human chorionic gonadotrophin (hCG) (GEN's, Hangzhou, China). After superovulation, the female mice were naturally mated with male mice. COH embryos at the two-cell stage were obtained from the pregnant mice at 1.5 d (the appearance of a vaginal plug was designated as 0.5 d) and transferred to the oviducts of pseudopregnant Institute of Cancer Research (ICR) mice. COH mice were born after about three weeks.

2.1.2 IVF models

Female mice were injected with PMSG and, 48 h later, with 7.5 IU hCG. Metaphase II (MII) oocytes were obtained 14 h after the hCG injection. IVF-fertilized oocytes were obtained after 6 h of co-incubation of MII oocytes with sperm, and the fertilization procedure was performed in human tubal fluid (HTF) medium with 10% serum substitute supplement (SSS) (Irvine Scientific). IVF-fertilized

oocytes were cultured in 10% SSS G1 medium (Vitrolife). IVF embryos at the two-cell stage were transferred to the oviducts of pseudopregnant ICR mice, and IVF mice were born after about three weeks.

2.1.3 ICSI models

MII oocytes were obtained after the PMSG-hCG injection procedure. Sperm was injected into the oocytes by ICSI using an Olympus X71 inverted microscope with PIEZO (PrimeTech, Ibaraki, Japan) and micromanipulators (Narishige, Tokyo, Japan) (Wang *et al.*, 2011). The ICSI fertilization procedure was performed in warmed HEPES-buffered modified HTF (mHTF) medium (Irvine Scientific). ICSI-fertilized oocytes were cultured in 10% SSS G1 medium, and ICSI embryos at the two-cell stage were transferred to the oviducts of pseudopregnant ICR mice. ICSI mice were born after about three weeks.

Adult F_1 IVF and ICSI mice were hybridized with the mice in the same group or with the NC mice to obtain F_2 mice. Three types of IVF and ICSI F_2 mice were produced: from ICSI fathers and ICSI mothers (II), from ICSI fathers and NC mothers (IN), and from ICSI mothers and NC fathers (NI). The same terminology was applied to groups in the IVF F_2 generation.

Testes were obtained from newborns, 10-week-old and 1.5-year-old ART (F_1 and F_2) male mice and NC male mice (F_1 and F_2). All tissues were stored at −80 °C for RNA extraction. All experiments were repeated three times.

2.2 Microarray analysis

Total RNA from the testes was extracted with Trizol (Invitrogen). The gene expression profiles in the testes of ICSI newborn mice were analyzed and compared with those of NC mice, with three mice in each group (every mouse in each group had different parents). GeneChip hybridization for each sample was examined on Affymetrix 3′ IVT Expression Arrays (Mouse Genome 430 2.0 Array). The technical procedures and quality controls were performed at the CapitalBio Corporation. Hybridization assay procedures were as described in the GeneChip Expression Analysis Technical Manual (http://www.affymetrix.com).

The data were analyzed using Significant Analysis of Microarray (SAM) software to identify the differentially expressed genes. Differentially expressed transcripts were defined by a fold-change (FC) ≥2 and a q-value <5%. The functional annotation of differentially expressed genes was analyzed using Molecule Annotation System Mas 3.0 (http://bioinfo.capitalbio.com/mas3/).

2.3 Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

cDNA was synthesized from purified total RNA using an SYBR PrimeScript™ RT-PCR kit (TaKaRa). qRT-PCR was performed using an SYBR-Green I kit (TaKaRa) and an ABI 7900 thermocycler with five samples (every mouse in each group had different parents) from each group to confirm the differential expression of these genes. *Gapdh* served as an internal control for detecting the level of gene expression. The qRT-PCR primer sequences are shown in Table 1.

2.4 Statistical analysis

SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was employed for statistical evaluation. qRT-PCR data were analyzed using the $2^{-\Delta\Delta C_T}$ method ($\Delta\Delta C_T=(C_{T,\ Target}-C_{T,\ Gapdh})_{experimental\ group}-(C_{T,\ Target}-C_{T,\ Gapdh})_{control\ group}$). Statistical analysis was performed using the independent-sample t-test for comparing two different groups. One-way ANOVA (Dunnet's test) was used to analyze the differences in gene expression between the NC group and the ICSI F_2 groups. Differences were considered to be statistically significant when $P<0.05$ (two-tailed).

3 Results

3.1 Differential gene expression in the testes of ICSI mice

The array analyses showed that there were 474 (150 up-regulated and 324 down-regulated) differentially expressed genes in ICSI newborn mice compared with NC mice (Fig. 1).

3.2 Gene ontology (GO) analysis of male reproductive function

To elucidate the effect of ICSI procedures on the male reproductive system, genes that were differentially expressed in ICSI newborns were classified by GO analysis. Among 152 GO terms for biological functions, six male reproductive function-related GO

terms were related to ICSI-induced gene expression differences (Table 2). These six GO terms included eight genes: five up-regulated genes (*Dmc1*, *Prok2*, *Rec8*, *Spo11*, and *Rhox5*) and three down-regulated genes (*Adcyap1r1*, *Amh*, and *Pdgfra*). Six genes (*Adcyap1r1*, *Dmc1*, *Prok2*, *Rec8*, *Spo11*, and *Rhox5*) are involved in spermatogenesis, male meiosis I, and sperm motility. Two genes (*Amh* and *Pdgfra*) are related to gonad development.

3.3 Validation of microarray data by qRT-PCR

We amplified the number of samples to validate the microarray results (Fig. 2). qRT-PCR was used to measure the gene expression in the testes of ICSI and NC newborn mice, and the results were consistent with those from microarray analysis. Thus, the results of qRT-PCR validated the reliability of the microarray analysis.

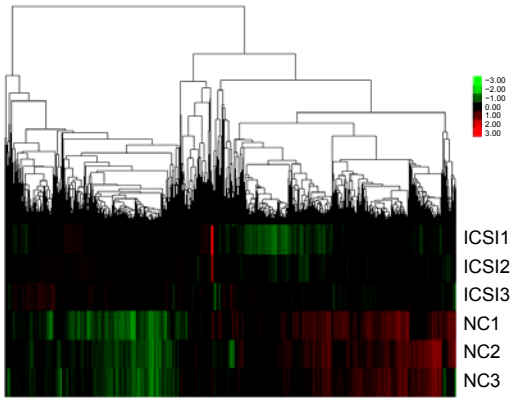

Fig. 1 Hierarchical clustering analysis of differentially expressed genes in the testes of ICSI and NC mice
The expression intensities of each gene are represented as a red-green colour spectrum: green for down-regulated genes, red for up-regulated genes and black for genes without changes in expression. According to the analysis, there were 474 (150 up-regulated and 324 down-regulated) differentially expressed genes in the ICSI group

Table 1 Primers designed for qRT-PCR

GenBank accession	Gene	Sequence (5′ to 3′)	Amplicon size (bp)
NM_008084	*Gapdh*	5′-TGTGTCCGTCGTGGATCTGA-3′	150
		5′-TTGCTGTTGAAGTCGCAGGAG-3′	
NM_007407	*Adcyap1r1*	5′-TGGCTATTGCTATGCACTCTGA-3′	104
		5′-GGAAGACTCATTTAGGCCCATCA-3′	
NM_007445	*Amh*	5′-GGCTAGGGGAGACTGGAGAA-3′	145
		5′-CCAGAGTATAGCACTAACAGGGC-3′	
NM_011202	*Pdgfra*	5′-ATGACATCGCGGAGATGGTTT-3′	113
		5′-GGGTTACTCTTACTGGGCCTT-3′	
NM_010059	*Dmc1*	5′-CCCTCTGTGTGACAGCTCAAC-3′	114
		5′-GGTCAGCAATGTCCCGAAG-3′	
NM_015768	*Prok2*	5′-GCTTGCGACAAGGACTCTCAG-3′	94
		5′-CTTGGCCCATAGGTGTGCAG-3′	
NM_020002	*Rec8*	5′-TATGTGCTGGTAAGAGTGCAAC-3′	133
		5′-TGTCTTCCACAAGGTACTGGC-3′	
NM_008818	*Rhox5*	5′-CATTTTGCAGCGCACTAATTCC-3′	106
		5′-AGCCCTCCTGATCTTAAACCA-3′	
NM_012046	*Spo11*	5′-CGTGGCCTCTAGTTCTGAGGT-3′	112
		5′-GCTCGATCTGTTGTCTATTGTGA-3′	

Table 2 Significant GO terms of biological function on male reproductive health for differentially expressed genes in the ICSI group

GO annotation	*P*-value	Protein	Gene symbol	Fold change
Spermatogenesis	4.52×10^{-16}	ADCYAP1R1; DMC1; PROK2	*Adcyap1r1*	0.35
			Dmc1	2.67
			Prok2	2.52
Male meiosis I	6.54×10^{-12}	DMC1; REC8; SPO11	*Rec8*	2.43
			Spo11	3.71
Spermatid development	3.64×10^{-11}	DMC1; REC8; SPO11		
Sperm motility	1.93×10^{-4}	RHOX5	*Rhox5*	5.31
Gonad development	8.45×10^{-4}	AMH	*Amh*	0.19
Male genitalia development	8.34×10^{-3}	PDGFRA	*Pdgfra*	0.47

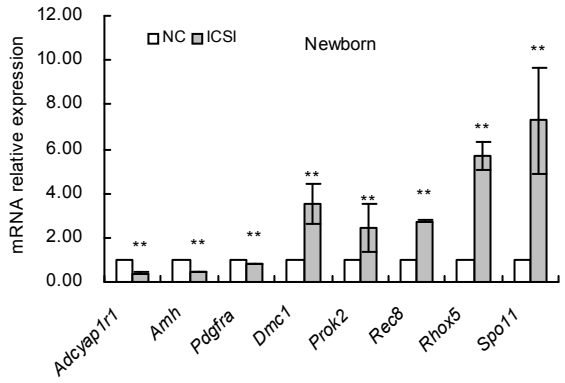

Fig. 2 qRT-PCR verification of microarray data
Values are expressed as mean±SD (n=5). Significant differences are marked by asterisks: * P<0.05, ** P<0.01

3.4 Detection of differentially expressed genes in the male reproductive systems of ART F_1 mice

Eight genes showing differential expression were detected in the adult F_1 generation. Five genes that were up-regulated in newborn ICSI mice displayed similar expression levels in ICSI and NC adult mice (Fig. 3a), while the expression levels of three genes down-regulated in newborn ICSI mice were still lower in adult ICSI mice than in adult NC mice (Fig. 3b). In adult mice, pairwise comparisons between NC and COH, COH and IVF, and IVF and ICSI groups were performed to investigate the crucial step that induced the changes in gene expression. *Adcyap1r1*, *Amh*, and *Pdgfra* were expressed at lower levels in the COH group than in the NC group (Fig. 4a). The expression of *Amh* was decreased in the IVF group compared with the COH group (Fig. 4b). The expression levels of *Adcyap1r1* and *Amh* in the ICSI group were lower than those in the IVF group (Fig. 4c). The differences in expression of the three genes among the four old F_1 generation groups were almost the same as those among the adult F_1 generation groups (Figs. 3c and 4d–4f). But *Adcyap1r1* showed no difference in expression between the NC and COH groups, and *Amh* showed no difference between the COH and IVF groups.

3.5 Detection of differentially expressed genes in the male reproductive systems of ART F_2 mice

The expressions of the three down-regulated genes were also analyzed in the F_2 generations. Except for *Pdgfra*, the genes were expressed at lower levels in the ICSI F_2 mice (Fig. 5a). Compared with the expression levels in ICSI F_1 mice, the expressions of *Adcyap1r1* and *Pdgfra* increased, while that of *Amh* decreased in the three types of ICSI F_2 mice (Fig. 5b). The differences in expression between each IVF F_2 generation group and its corresponding ICSI F_2 generation group were similar to those found in the F_1 generation (Fig. 6).

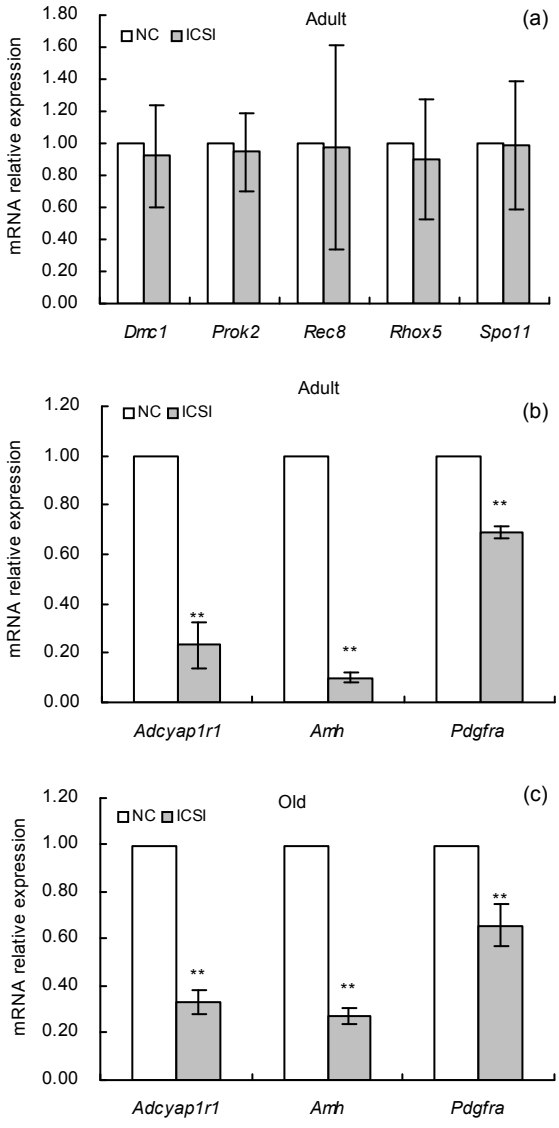

Fig. 3 Expression of the male reproductive health-related genes in adult ICSI F_1 mice
(a) Expressions of *Dmc1*, *Prok2*, *Rec8*, *Spo11*, and *Rhox5* in adult ICSI F_1 mice. (b, c) Expressions of *Adcyap1r1*, *Amh*, and *Pdgfra* in adult and old ICSI F_1 mice, respectively. Values are expressed as mean±SD (n=5). Significant differences are marked by asterisks: * P<0.05, ** P<0.01

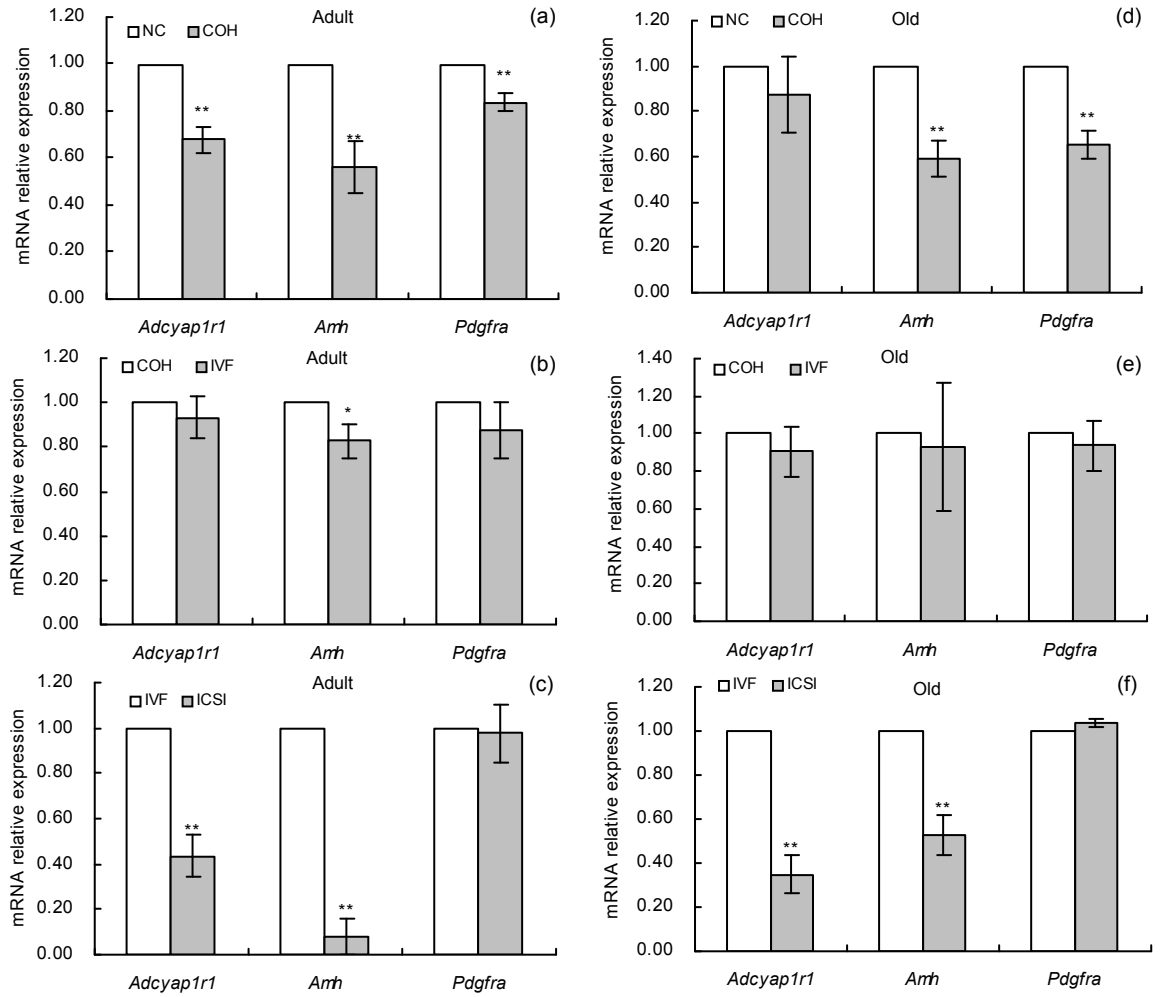

Fig. 4 Pairwise comparisons between NC and COH, COH and IVF, IVF and ICSI adult or old F_1 mice

(a), (b), and (c) show the gene expression in adult mice; (d), (e), and (f) show the gene expression in old mice. The comparison between the NC and COH groups is presented in (a) and (d). The comparison between the COH and IVF groups is presented in (b) and (e). (c) and (f) show the comparison between the IVF and ICSI groups. Values are expressed as mean±SD (n=5). Significant differences are marked by asterisks: $^{*}P<0.05$, $^{**}P<0.01$

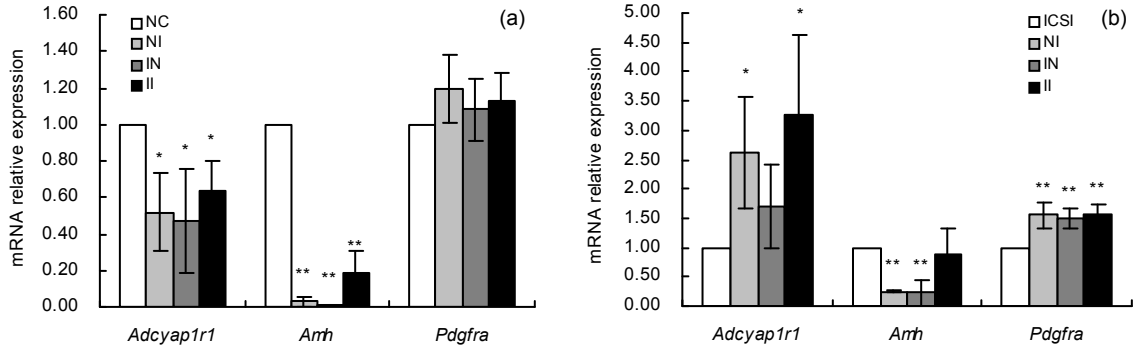

Fig. 5 Expressions of *Adcyap1r1*, *Amh*, and *Pdgfra* in adults of F_2 generations

The comparisons between NC mice and three types of ICSI F_2 mice are shown in (a), while the comparison between ICSI F_1 and F_2 generations are presented in (b). Values are expressed as mean±SD (n=5). Significant differences are marked by asterisks: $^{*}P<0.05$, $^{**}P<0.01$

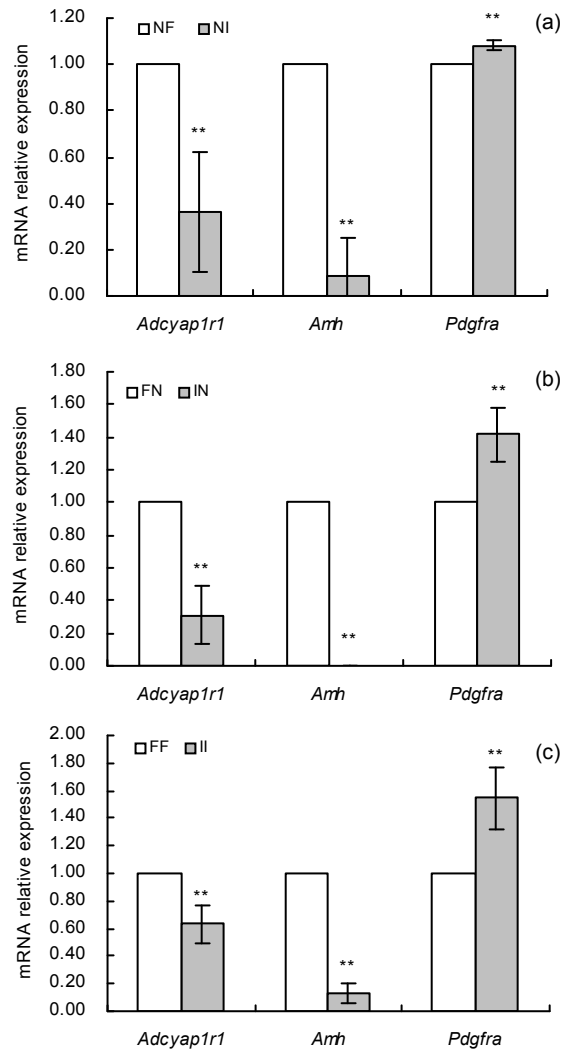

Fig. 6 Comparison of the expressions of *Adcyap1r1*, *Amh*, and *Pdgfra* between IVF and ICSI F_2 generations
IVF and ICSI F_2 mice: NF (NC father+IVF mother), NI (NC father+ICSI mother), FN (IVF father+NC mother), IN (ICSI father+NC mother), FF (IVF father+IVF mother), II (ICSI father+ICSI mother). (a) NF vs. NI; (b) FN vs. IN; (c) FF vs. II. Values are expressed as mean±SD (n=5). Significant differences are marked by asterisks: * P<0.05, ** P<0.01

4 Discussion

The use of mouse models successfully avoided the interference of individual differences in genetic and many environmental factors. The testis was used to study the effect of ART methods on the male reproductive system. Microarray analysis showed that there were a large number of genes that were differentially expressed between the ICSI and NC mice. We analyzed differentially expressed genes related to male reproductive function using GO analysis. In total, eight differentially expressed genes were found in newborn mice of the ICSI group, among which *Amh* and *Rhox5* are expressed in Sertoli cells (Hu *et al.*, 2007; Goulis *et al.*, 2008), *Adcy1p1r1* and *Pdgfra* are expressed in Leydig cells (Koh and Won, 2006; Basciani *et al.*, 2010), while the other four genes are mainly expressed in spermatocytes (Lee *et al*., 2002; Abreu *et al*., 2010; Bellani *et al*., 2010; Puverel *et al*., 2011). There are two essential stages for testicle development: embryonic and pubertal age. Sex determination and phenotype development are completed in the embryonic phase, while spermatogenesis starts well before the pubertal phase, and male meiosis is initiated at pubertal stage. Therefore, we should also detect the expression level of these differently expressed genes in adult mice.

In adult males, the five up-regulated genes returned to normal levels, while the three down-regulated genes were expressed at lower levels. PDGFRA is essential for early embryonic development of Leydig cells (Basciani *et al.*, 2010). Testosterone is produced by the Leydig cells. PDGFRA deficiency can delay the production of testosterone (Schmahl *et al.*, 2008). The decrease in AMH and ADCYAP1R1 in newborn males reflects the maturation status of Sertoli cells (Rey *et al.*, 1993; Koh and Won, 2006; Boukari *et al.*, 2009). Another study reported that the loss of AMH was associated with non-obstructive azoospermia (Fenichel *et al.*, 1999). RHOX5 is an androgen-dependent protein, which regulates spermatogenesis (Domanskyi *et al.*, 2007; Hu *et al.*, 2007). Sertoli cells mediate spermatogenesis by nourishing the development of sperm cells, thus the expression levels of AMH, RHOX5, ADCYAP1R1, and PDGFRA reflect the functions of Sertoli cells (Fenichel *et al.*, 1999; Jamen *et al.*, 2000; Basciani *et al.*, 2002; Denolet *et al.*, 2006). However, if ICSI affects spermatogenesis, why was the down-regulation of *Pdgfra* followed by the increased expression of *Rhox5*? Luteinizing hormone (LH) in male rats may compensate for the dysfunction of Leydig cells as it activates cAMP for testosterone production (Koh and Won, 2006; Nurmio *et al.*, 2008). Therefore, we re-analyzed the results from GeneChip, and found that expression of the LH receptor

(LHCGR) in ICSI mice was 1.61-fold higher than that in NC mice. The increased expression of *Lhcgr* induced by ART might make up for its effect on Leydig cells, and accelerate spermatogenesis in early infancy leading to the observed up-regulation of *Prok2*, *Dmc1*, *Rec8*, and *Spor11*.

In adult mice, the continuing down-regulation of *Amh* and *Adcyap1r1* indicated that their differential expression in newborn mice may not be associated with testosterone secretion, but with the decreased function of Sertoli cells. Moreover, we detected these three down-regulated genes in old mice, and again found decreased expression in the ICSI group. These results suggest that ICSI treatments had a persistent effect on the three genes and that the changes were of fetal origin. To find the precise factors that induced the differences in ICSI mice, we included data from COH and IVF models of adult and old mice. Comparisons between the NC and COH, COH and IVF, and IVF and ICSI groups were made to analyze the effects of superovulation, in vitro culture and ICSI mechanical stimulation. Our results showed that superovulation was the only factor that induced the down-regulation of *Pdgfra*, while the decreases in *Adcyap1r1* and *Amh* resulted mainly from both hormonal and ICSI mechanical stimulation. These three factors had a cumulative effect on the changes in *Amh*.

Intergenerational transmission of differential gene expression has been demonstrated in many studies (Jimenez-Chillaron *et al.*, 2009; Hoile *et al.*, 2011). We built three types of ICSI F_2 models by hybridizing ICSI and NC F_1 mice to simulate the phenomenon in human beings and observed the effects of the parents. *Amh* and *Adcyap1r1* were still down-regulated in ICSI F_2 mice compared with the control NC group, but *Pdgfra* showed no difference. Compared with ICSI F_1 mice, *Adcyap1r1* and *Pdgfra* showed higher expression, while *Amh* showed lower expression in ICSI F_2 mice, which implies that most of the differences induced by ART treatments tended to be reduced during intergenerational transmission. In F_2 offspring, we also compared the expression of *Amh*, *Adcyap1r1*, and *Pdgfra* between each pair of groups of IVF and ICSI F_2 mice. The results were similar to those of F_1 offspring, except that significant differences in the expression of *Pdgfra* were found in F_2 mice, indicating that ICSI mechanical stimulation can also induce intergenerational transmission of differential gene expression.

5 Conclusions

In summary, we found that the differential expression of genes in ICSI adult mice might originate from the newborn period or even earlier. Superovulation, in vitro culture and mechanical stimulation had a cumulative effect on the differential expression of male reproductive genes in the ICSI mice. ICSI treatments, or the mechanical stimulation involved in ICSI, can induce the intergenerational transmission of differential gene expression in the F_1 generation. The effect of the F_1 generation groups that induced the differential gene expression tended to be reduced during intergenerational transmission. However, differences in *Amh* expression, which were maintained from ICSI newborn to old age, showed a more significant difference in F_2 than in F_1 mice. The potential effect of its decreased expression in ICSI testes on male reproductive health needs further investigation. Our studies provide a detailed analysis of the effect of ICSI procedures on the expression of male reproductive genes, which will be a useful reference for future studies on ART methods.

Compliance with ethics guidelines

Li-ya WANG, Ning WANG, Fang LE, Lei LI, Le-jun LI, Xiao-zhen LIU, Ying-ming ZHENG, Hang-ying LOU, Xiang-rong XU, Xiao-ming ZHU, Yi-min ZHU, He-feng HUANG, and Fan JIN declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

References

Abreu, A.P., Kaiser, U.B., Latronico, A.C., 2010. The role of prokineticins in the pathogenesis of hypogonadotropic hypogonadism. *Neuroendocrinology*, **91**(4):283-290. [doi:10.1159/000308880]

Asklund, C., Jorgensen, N., Skakkebaek, N.E., Jensen, T.K., 2007. Increased frequency of reproductive health problems among fathers of boys with hypospadias. *Hum. Reprod.*, **22**(10):2639-2646. [doi:10.1093/humrep/dem217]

Barker, D.J., 1990. The fetal and infant origins of adult disease. *BMJ*, **301**(6761):1111. [doi:10.1136/bmj.301.6761.1111]

Basciani, S., Mariani, S., Arizzi, M., Ulisse, S., Rucci, N., Jannini, E.A., Della Rocca, C., Manicone, A., Carani, C., Spera, G., *et al.*, 2002. Expression of platelet-derived growth factor-A (PDGF-A), PDGF-B, and PDGF receptor-α and -β during human testicular development

and disease. *J. Clin. Endocrinol. Metab.*, **87**(5):2310-2319. [doi:10.1210/jc.87.5.2310]

Basciani, S., Mariani, S., Spera, G., Gnessi, L., 2010. Role of platelet-derived growth factors in the testis. *Endocr. Rev.*, **31**(6):916-939. [doi:10.1210/er.2010-0004]

Bellani, M.A., Boateng, K.A., McLeod, D., Camerini-Otero, R.D., 2010. The expression profile of the major mouse SPO11 isoforms indicates that SPO11β introduces double strand breaks and suggests that SPO11α has an additional role in prophase in both spermatocytes and oocytes. *Mol. Cell. Biol.*, **30**(18):4391-4403. [doi:10.1128/MCB.00002-10]

Boukari, K., Meduri, G., Brailly-Tabard, S., Guibourdenche, J., Ciampi, M.L., Massin, N., Martinerie, L., Picard, J.Y., Rey, R., Lombes, M., *et al.*, 2009. Lack of androgen receptor expression in Sertoli cells accounts for the absence of anti-Mullerian hormone repression during early human testis development. *J. Clin. Endocrinol. Metab.*, **94**(5):1818-1825. [doi:10.1210/jc.2008-1909]

Davies, M.J., Moore, V.M., Willson, K.J., van Essen, P., Priest, K., Scott, H., Haan, E.A., Chan, A., 2012. Reproductive technologies and the risk of birth defects. *N. Engl. J. Med.*, **366**(19):1803-1813. [doi:10.1056/NEJMoa1008095]

Denolet, E., de Gendt, K., Allemeersch, J., Engelen, K., Marchal, K., van Hummelen, P., Tan, K.A., Sharpe, R.M., Saunders, P.T., Swinnen, J.V., *et al.*, 2006. The effect of a sertoli cell-selective knockout of the androgen receptor on testicular gene expression in prepubertal mice. *Mol. Endocrinol.*, **20**(2):321-334. [doi:10.1210/me.2005-0113]

Domanskyi, A., Zhang, F.P., Nurmio, M., Palvimo, J.J., Toppari, J., Janne, O.A., 2007. Expression and localization of androgen receptor-interacting protein-4 in the testis. *Am. J. Physiol. Endocrinol. Metab.*, **292**(2):E513-E522. [doi:10.1152/ajpendo.00287.2006]

Fatemi, N., Sanati, M.H., Zavarehei, M.J., Ayat, H., Esmaeili, V., Golkar-Narenji, A., Zarabi, M., Gourabi, H., 2012. Effect of tertiary-butyl hydroperoxide (TBHP)-induced oxidative stress on mice sperm quality and testis histopathology. *Andrologia*, in press. [doi:10.1111/j.1439-0272.2012.01335.x]

Fenichel, P., Rey, R., Poggioli, S., Donzeau, M., Chevallier, D., Pointis, G., 1999. Anti-Mullerian hormone as a seminal marker for spermatogenesis in non-obstructive azoospermia. *Hum. Reprod.*, **14**(8):2020-2024. [doi:10.1093/humrep/14.8.2020]

Foix-L′Hélias, L., Aerts, I., Marchand, L., Lumbroso-le Rouic, L., Gauthier-Villars, M., Labrune, P., Bouyer, J., Doz, F., Kaminski, M., 2012. Are children born after infertility treatment at increased risk of retinoblastoma? *Hum. Reprod.*, **27**(7):2186-2192. [doi:10.1093/humrep/des149]

Goulis, D.G., Iliadou, P.K., Tsametis, C., Gerou, S., Tarlatzis, B.C., Bontis, I.N., Papadimas, I., 2008. Serum anti-Mullerian hormone levels differentiate control from subfertile men but not men with different causes of subfertility. *Gynecol. Endocrinol.*, **24**(3):158-160. [doi:10.1080/09513590701672314]

Hoile, S.P., Lillycrop, K.A., Thomas, N.A., Hanson, M.A., Burdge, G.C., 2011. Dietary protein restriction during F_0 pregnancy in rats induces transgenerational changes in the hepatic transcriptome in female offspring. *PLoS One*, **6**(7):e21668. [doi:10.1371/journal.pone.0021668]

Hu, Z., MacLean, J.A., Bhardwaj, A., Wilkinson, M.F., 2007. Regulation and function of the *Rhox5* homeobox gene. *Ann. N. Y. Acad. Sci.*, **1120**(1):72-83. [doi:10.1196/annals.1411.011]

Jamen, F., Rodriguez-Henche, N., Pralong, F., Jegou, B., Gaillard, R., Bockaert, J., Brabet, P., 2000. PAC_1 null females display decreased fertility. *Ann. N. Y. Acad. Sci.*, **921**(1):400-404. [doi:10.1111/j.1749-6632.2000.tb07004.x]

Jimenez-Chillaron, J.C., Isganaitis, E., Charalambous, M., Gesta, S., Pentinat-Pelegrin, T., Faucette, R.R., Otis, J.P., Chow, A., Diaz, R., Ferguson-Smith, A., *et al.*, 2009. Intergenerational transmission of glucose intolerance and obesity by in utero undernutrition in mice. *Diabetes*, **58**(2):460-468. [doi:10.2337/db08-0490]

Koh, P.O., Won, C.K., 2006. Decrease of pituitary adenylate cyclase activating polypeptide and its type I receptor mRNAs in rat testes by ethanol exposure. *J. Vet. Med. Sci.*, **68**(6):537-541. [doi:10.1292/jvms.68.537]

Lee, J., Yokota, T., Yamashita, M., 2002. Analyses of mRNA expression patterns of cohesin subunits Rad21 and Rec8 in mice: germ cell-specific expression of Rec8 mRNA in both male and female mice. *Zoolog. Sci.*, **19**(5):539-544. [doi:10.2108/zsj.19.539]

Lonergan, P., Fair, T., Corcoran, D., Evans, A.C., 2006. Effect of culture environment on gene expression and developmental characteristics in IVF-derived embryos. *Theriogenology*, **65**(1):137-152. [doi:10.1016/j.theriogenology.2005.09.028]

Lu, Y.H., Gao, H.J., Li, B.J., Zheng, Y.M., Ye, Y.H., Qian, Y.L., Xu, C.M., Huang, H.F., Jin, F., 2012. Different sperm sources and parameters can influence intracytoplasmic sperm injection outcomes before embryo implantation. *J. Zhejiang Univ.-Sci. B (Biomed. & Biotechnol.)*, **13**(1):1-10. [doi:10.1631/jzus.B1100216]

Mau, C., Juul, A., Main, K.M., Loft, A., 2004. Children conceived after intracytoplasmic sperm injection (ICSI): is there a role for the paediatrician? *Acta Paediatr.*, **93**(9):1238-1244. [doi:10.1111/j.1651-2227.2004.tb02756.x]

Mau Kai, C., Main, K.M., Andersen, A.N., Loft, A., Skakkebaek, N.E., Juul, A., 2007. Reduced serum testosterone levels in infant boys conceived by intracytoplasmic sperm injection. *J. Clin. Endocrinol. Metab.*, **92**(7):2598-2603. [doi:10.1210/jc.2007-0095]

Nurmio, M., Kallio, J., Toppari, J., Jahnukainen, K., 2008. Adult reproductive functions after early postnatal inhibition by imatinib of the two receptor tyrosine kinases, c-kit and PDGFR, in the rat testis. *Reprod. Toxicol.*, **25**(4):442-446. [doi:10.1016/j.reprotox.2008.03.004]

Prader, A., Tanner, J.M., von Harnack, G.A., 1963. Catch-up growth following illness or starvation: an example of developmental canalization in man. *J. Pediatr.*, **62**(5):646-659. [doi:10.1016/S0022-3476(63)80035-9]

Puverel, S., Barrick, C., Dolci, S., Coppola, V., Tessarollo, L., 2011. RanBPM is essential for mouse spermatogenesis

and oogenesis. *Development*, **138**(12):2511-2521. [doi:10.1242/dev.062505]

Ramlau-Hansen, C.H., Thulstrup, A.M., Olsen, J., Bonde, J.P., 2008. Parental subfecundity and risk of decreased semen quality in the male offspring: a follow-up study. *Am. J. Epidemiol*., **167**(12):1458-1464. [doi:10.1093/aje/kwn076]

Rey, R., Lordereau-Richard, I., Carel, J.C., Barbet, P., Cate, R.L., Roger, M., Chaussain, J.L., Josso, N., 1993. Anti-mullerian hormone and testosterone serum levels are inversely during normal and precocious pubertal development. *J. Clin. Endocrinol. Metab*., **77**(5):1220-1226. [doi:10.1210/jc.77.5.1220]

Rinaudo, P., Schultz, R.M., 2004. Effects of embryo culture on global pattern of gene expression in preimplantation mouse embryos. *Reproduction*, **128**(3):301-311. [doi:10.1530/rep.1.00297]

Roseboom, T., de Rooij, S., Painter, R., 2006. The Dutch famine and its long-term consequences for adult health. *Early Hum. Dev*., **82**(8):485-491. [doi:10.1016/j.earlhumdev.2006.07.001]

Schmahl, J., Rizzolo, K., Soriano, P., 2008. The PDGF signaling pathway controls multiple steroid-producing lineages. *Genes Dev.*, **22**(23):3255-3267. [doi:10.1101/gad.1723908]

Storgaard, L., Bonde, J.P., Ernst, E., Andersen, C.Y., Spano, M., Christensen, K., Petersen, H.C., Olsen, J., 2006. Genetic and environmental correlates of semen quality: a twin study. *Epidemiology*, **17**(6):674-681. [doi:10.1097/01.ede.0000239730.47963.4e]

Tsai-Morris, C.H., Sato, H., Gutti, R., Dufau, M.L., 2012. Role of gonadotropin regulated testicular RNA helicase (GRTH/Ddx25) on polysomal associated mRNAs in mouse testis. *PLoS One*, **7**(3):e32470. [doi:10.1371/journal.pone.0032470]

van Abeelen, A.F., Elias, S.G., Bossuyt, P.M., Grobbee, D.E., van der Schouw, Y.T., Roseboom, T.J., Uiterwaal, C.S., 2012. Famine exposure in the young and the risk of type 2 diabetes in adulthood. *Diabetes*, **61**(9):2255-2260. [doi:10.2337/db11-1559]

Wang, N., Wang, L., Le, F., Zhan, Q., Zheng, Y., Ding, G., Chen, X., Sheng, J., Dong, M., Huang, H., *et al.*, 2011. Altered expression of Armet and Mrlp51 in the oocyte, preimplantation embryo, and brain of mice following oocyte in vitro maturation but postnatal brain development and cognitive function are normal. *Reproduction*, **142**(3):401-408. [doi:10.1530/REP-11-0152]

Yu, Y., Zhao, C., Lv, Z., Chen, W., Tong, M., Guo, X., Wang, L., Liu, J., Zhou, Z., Zhu, H., *et al.*, 2011. Microinjection manipulation resulted in the increased apoptosis of spermatocytes in testes from intracytoplasmic sperm injection (ICSI) derived mice. *PLoS One*, **6**(7):e22172. [doi:10.1371/journal.pone.0022172]