REPRODUCTION
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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

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Abstract

Despite the efforts to recapitulate the follicle environment, oocytes from *in vitro* maturation (IVM) have poorer developmental potential than those matured *in vivo* and the effects on the resultant offspring are of concern. The aim of this study was to determine altered gene expression in oocytes following IVM and to evaluate the expression of the arginine rich, mutated in early stage of tumors gene (*Armet*) and mitochondrial ribosomal protein L51 (*Mrpl51*) in embryos and brains of fetal/postnatal mice and the brain development of IVM offspring. An IVM mouse model was established while oocytes matured *in vivo* were used as the controls. Suppressive subtractive hybridization (SSH) and RT-PCR/western blot were used to analyze the differential expression of genes/proteins between IVM and the control group. HE staining and water maze were used to assess the histological changes in brain tissue and cognition of the offspring. The rates of fertilization, cleavage, and live birth were significantly decreased in IVM group. Thirteen genes were upregulated in IVM oocytes compared with the control, including *Armet* and *Mrpl51*. The higher level of *Armet* in IVM oocytes was retained in brain of newborn mice, which could be related to the upregulation of activating transcription factor 6 (*Atf6*) and X-box binding protein 1 (*Xbp1*), while *Mrpl51* was expressed normally in brain of postnatal mice. No significant differences were detected in brain weight, neuronal counts, and the cognition in the offspring between the two groups. The present results suggested that IVM could affect the pregnancy outcome and the *Armet* and *Mrpl51* gene/protein expression. The change in *Armet* expression lasted while the change of *Mrpl51* disappeared after birth. However, the brain development of the offspring seemed to be unaffected by IVM.

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Introduction

In vitro maturation (IVM) of oocytes is used to avoid ovarian hyperstimulation syndromes in patients with polycystic ovaries during IVF (Chian 2004, Suikkari & Soderstrom-Anttila 2007). However, when oocytes are obtained from IVM, not all of them have the ability to be fertilized and develop into an embryo. The inability to sustain further development may be associated with incomplete maturation of the oocyte during folliculogenesis (Robert *et al*. 2001). It has become increasingly clear that genetic programs, especially the messenger RNA synthesis and storage in the oocyte, play important roles in orchestrating the multiple events required for successful folliculogenesis, fertilization, and early development (Meirelles *et al*. 2004). Although great efforts have been made to recreate *in vitro* follicular environment, the rates of blastulation and pregnancy with IVM were much lower compared with routine IVF (Lee *et al*. 2008). Some theoretic concerns regarding the safety of IVM have been raised (Fauser *et al*. 2002, Albertini *et al*. 2003, Krisher *et al*. 2007, Lee *et al*. 2008, Liu *et al*. 2010), and the potential effects of IVM on offspring are still unclear.

To identify the genes specifically expressed in IVM oocytes, we compared the mRNA profiles of mouse oocytes obtained from IVM and the control group (*in vivo* maturation) using suppressive subtractive hybridization (SSH). Thirteen specific differentially expressed genes in IVM oocytes were cloned, including two genes related to the neurology: the arginine rich, mutated in early stage of tumors gene (*Armet*) and mitochondrial ribosomal protein L51 (*Mrpl51*).

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Armet is an endoplasmic reticulum (ER) stress-responsive gene (Apostolou *et al*. 2008) required for cell protection against ER stress and is critical for specific neuronal cells to survive and is shown to play important roles in many neurodegenerative disorders (Taylor *et al*. 2002, Berke & Paulson 2003, Ciechanover & Brundin 2003, Giasson & Lee 2003). Transcription factors activating transcription factor 6 (*Atf6*) and X-box binding protein 1 (*Xbp1*) are the promoters for *Armet* transcription by recognizing the sequence ACGTGG immediately downstream of −160 bp in the *Armet* promoter when the ER stress invoked (Lee *et al*. 2003, Mizobuchi *et al*. 2007).

MRPs are encoded by nuclear genes and help in protein synthesis within the mitochondrion. MRPL51 was established as a large subunit of mammalian MRPs (Gan *et al*. 2002) and functions in the translation of genetic messages. The redistribution of mitochondria was coordinated with oocyte maturation, and lack of redistribution of mitochondria suggested incomplete cytoplasmic maturation and lower developmental competence (Krisher *et al*. 2007). Mitochondrial dysfunction (including the dysfunction of MRPL51) causes more than 50 diseases ranging from neonatal fatalities to adult-onset neurodegeneration (Lopez *et al*. 2000, DiMauro & Schon 2003, Lowell & Shulman 2005, Wallace 2005).

Considering the important role of *Armet* and *Mrpl51* in nervous-related disorders, they were further analyzed in two-cell embryos and the brain tissue of fetal and postnatal mice to investigate the possible functions of the two genes in oocyte maturation and the long-term effects of IVM. In addition, the expression of *Atf6* and *Xbp1* was analyzed to reveal the possible mechanisms for the change of *Armet* induced by IVM. Meanwhile, morphology and histological analysis in the brain tissue and cognitive behavioral testing were used to show brain development of the offspring.

Results

Rates of fertilization, cleavage, and birth between IVM and the control group

The rates of fertilization and cleavage were significantly lower in the IVM group than the control group (67.9 vs 85.7% and 90.9 vs 95.7%). Similarly, the birth rate of two-cell stage transfer was significantly lower in the IVM group than the control group (15.4 vs 23.3%; Fig. 1A). No significant differences were detected in weight of newborns and the number per litter between the two groups (Fig. 1B and C).

SSH of oocytes

After sequencing, 13 differential expression genes including *Armet* and *Mrpl51* were identified as upregulated, which are listed in Table 1.

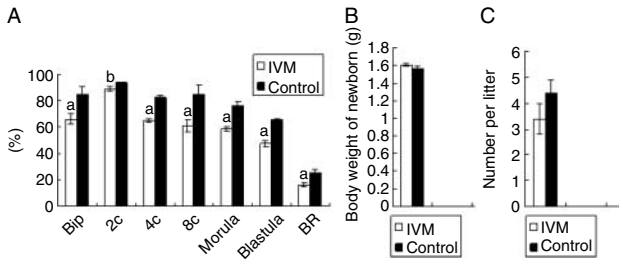

Figure 1 Effects of IVM on fertilization, preimplantation embryo development, and pregnancy outcomes. (A) The rates of fertilization and cleavage, development capability, and the birth rate (BR) of two-cell stage transfer. Bip, Bipronucleus; 2c, two-cell stage embryo; 4c, four-cell stage embryo; 8c, eight-cell stage embryo; BR, birth rate=pup number/number of two-cell embryos transferred; (B) newborn weights; (C) number per litter; a, $P<0.01$; b, $P<0.05$.

The mRNA expressions in oocytes, two-cell embryos, and brains of fetal and postnatal mice

Results of RT-PCR showed that the mRNA expression of *Armet* and *Mrpl51* in oocytes and two-cell embryos was statistically significantly higher in IVM group than the control group (Fig. 2A).

The expression of *Armet* was statistically significantly higher in the brain tissue of IVM E16.5 and newborn mice, but no differences were shown in *Mrpl51* expression (Fig. 2B and C).

Expression of *Atf6* and *Xbp1* was detected to reveal the possible pathway that might affect *Armet* mRNA expression. During development, the expression of *Atf6/Xbp1* was coincident with the change in *Armet*, higher in the brain tissue of E16.5 and newborn mice in the IVM group (Fig. 2D and E).

The protein expression of ARMET and MRPL51 in brains of fetal and postnatal mice

The results of western blotting (WB) showed that ARMET was expressed in all developmental stages of mouse brain, and it was significantly higher in E16.5 and newborn stage in the IVM group than the control group; however, no significant differences between the two groups were detected in 3 and 10 weeks stages (Fig. 3A and B). MRPL51 was expressed in all development stages of mouse brains, and no significant differences between the two groups were detected (Fig. 3C and D).

Brain development of the offspring

Morphology and histology of brain tissue

The ratio of brain to body weight, the neuronal morphology, and the neuronal counts were not significantly different between the two groups (Fig. 4A–C).

Table 1 Identification of cDNA clones found with suppressive subtractive hybridization when comparing *in vitro* maturation oocytes mRNAs with control group.

Gene symbol	Gene description	GenBank number
Armet	*Mus musculus* arginine rich, mutated in early stage tumors	NM_029103.3
Mrpl51	*Mus musculus* mitochondrial ribosomal protein L51	NM_025595.2
Mitd1	MIT, microtubule interacting and transport, domain containing 1	NM_026913.2
Socs3	Suppressor of cytokine signaling 3	NM_007707.2
Slc1a6	Solute carrier family 1 (high-affinity aspartate/glutamate transporter), member 6	NM_009200.2
Slc13a1	Solute carrier family 13 (sodium/sulfate symporters), member 1	NM_019481.1
Sparcl1	*Mus musculus* SPARC-like 1 (mast9 and hevin)	NM_010097.2
Srgap3	*Mus musculus* SLIT-ROBO Rho GTPase activating protein 3	NM_080448.3
Smarca1	*Mus musculus* SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily a, member 1	NM_053123.3
LOC677366	Similar to sodium- and chloride-dependent glycine transporter 2 (GlyT2) (GlyT-2) (solute carrier family 6 member 5)	XM_001004722.1 //XM_001004719.1
LOC630539	Similar to mouse RING finger 1 /// tripartite motif-containing 59	XM_904071.2
Zfp26	Zinc finger protein 26	NM_011753.2
Unknown		

Cognitive ability of the offspring

Cognition was assessed by performing a total of 24 trials with ten mice of each group over a period of six consecutive days with a Morris water maze (MWM) apparatus. The mean latency to find the platform during the first 6 days and the times of crossing the former platform in the probe trial did not differ significantly between the two groups (Fig. 4D).

Discussion

By using about 500 oocytes for each test, SSH was applied to compare the differences of whole genome transcription profiles between the oocytes matured through IVM and from *in vivo* in mice. Although it did not seem to be as sensitive as the assay with microarray in other species, the present SSH test offered much more focused information requiring relatively small sample amounts. In our research, we revealed that IVM could affect the pregnancy outcome and the expression of *Armet* and/or *Mrpl51* in mouse oocytes, embryos, and the brain of fetal mice. Although *Mrpl51* was expressed normally after birth in IVM mouse brain, the change in *Armet* lasted after the birth. The alteration in *Armet* expression in IVM was consistent with the change in *Atf6/Xbp1* expression. However, no significant differences were shown in the morphology and histological analysis of the brain and cognition in the offspring between the two groups. This is the first report of the effects of IVM on *Armet* (and *Atf6/Xbp1*) and *Mrpl51* expression in mouse oocytes, embryos, and the brains of fetal and postnatal mice.

Armet was described as a survival-promoting factor for embryonic midbrain dopaminergic neurons *in vitro* (Petrova *et al*. 2003) and protects neurons against cerebral ischemia, possibly by inhibiting cell necrosis/apoptosis in cerebral cortex (Airavaara *et al*. 2009). *Drosophila Armet*-deficient flies die as embryos, showing severe degeneration of dopaminergic axons and a drastic decrease in the level of dopamine (Lindholm *et al*. 2008). In the brain, *Armet* mRNA levels could be influenced by pathological conditions, and global forebrain ischemia could increase *Armet* expression (Lindholm *et al*. 2008). In our research, the higher *Armet* levels were not only found in IVM oocytes and embryos but also existed in brain of fetal and newborn mice conceived from IVM. Combined with the results of lower rates of fertilization, cleavage, and birth in the IVM group, it is implied that oocyte insults induced by IVM procedure lead to the specific changes in gene expression in the brains of IVM mice in the similar condition (similar newborn weight and litter size). Reports have shown that ARMET plays important roles in diseases such as ER stress (DeGracia & Montie 2004, Apostolou *et al*. 2008), type II diabetes, Parkinson's disease, and Alzheimer's disease (Lindholm *et al*. 2006, Ron & Walter 2007). Although we did not find significant differences in morphology, histological analysis, and cognitive behavioral testing in the IVM offspring, the relationship between IVM and potential diseases, especially neural disorders, needs further research with larger samples of animal or human data.

ATF6 was a specialized transcription factor for ER quality control (Adachi *et al*. 2008). ATF6 and XBP1 play key roles in the unfolded protein response by inducing ER chaperones and ER-associated protein degradation machineries (Mizobuchi *et al*. 2007). Reports showed that *Armet* gene expression in cultured cardiac myocytes could be induced by either *Atf6* or *Xbp1*(Adachi *et al*. 2008). The higher levels of *Atf6* and *Xbp1* in the IVM group proved that the change in *Armet* might be related to *Atf6/Xbp1*. IVM might upregulate the expression of *Atf6/Xbp1* and then lead to the increase in *Armet* expression.

Mrpl51 functions in the translation of genetic messages, and the redistribution of mitochondria was coordinated with oocyte maturation competence (Krisher *et al*. 2007). Liu *et al*. (2010) reported that the

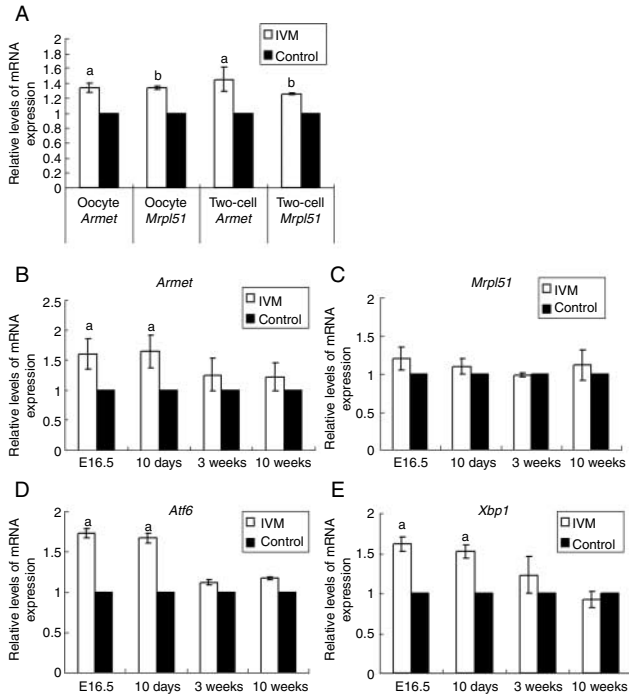

Figure 2 The mRNA expression of oocytes, two-cell embryos, and mouse brains. The comparison of mRNA expression was made between IVM and control group. Summary data show the relative expression levels in oocytes, embryos, and brains of fetal and postnatal mice after real-time PCR analysis. The relative mRNA levels represent the amount of mRNA expression normalized to *Gapdh*. (A) *Armet* and *Mrpl51* mRNA expression in oocytes and two-cell embryos; (B) *Armet* mRNA expression in mouse brains (E16.5, 10 days, 3 weeks, and 10 weeks); (C) *Mrpl51* mRNA expression in mouse brains (E16.5, 10 days, 3 weeks, and 10 weeks); (D) *Atf6* mRNA expression in mouse brains (E16.5, 10 days, 3 weeks, and 10 weeks); (E) *Xbp1* mRNA expression in mouse brains (E16.5, 10 days, 3 weeks, and 10 weeks). a, $P<0.01$; b, $P<0.05$.

distribution of mitochondria in human oocytes was changed after maturation, which may partially explain the reduced developmental potential of oocytes from IVM compared with the control. The increased level of *Mrpl51* in the IVM group might be a way to increase the maternal reserve to ensure subsequent normal development; however, whether or how much the mitochondrial function could be compensated is unknown. Disorders of MRPs are related to mitochondrial diseases (Seyda *et al*. 2001). The gene abnormality can decrease the efficiency of oxidative phosphorylation and lead to the disorder of energy metabolism. In order to reveal whether the abnormal expression of *Mrpl51* in IVM oocytes and embryos would be a potential risk of some mitochondrial diseases for the offspring, we detected the mRNA and protein expression during the brain development from the fetus to adulthood. No statistical differences were shown between the IVM and control groups, which indicated that the influence of IVM on *Mrpl51* expression was limited to the preimplantation stage, or that embryos with abnormal *Mrpl51* expression failed to go through post-implantational development.

Genes related to neuron development were found to be abnormally expressed in the IVM group; however, along with the growth and development, most abnormal expression levels disappeared in the same condition. Meanwhile, no differences were found due to IVM in postnatal brain histology and cognition compared with those of control animals, which suggests that mice born following IVM could correct some abnormal gene expression profile and develop normally, and the technique is comparatively safe if a healthy baby is delivered.

In conclusion, upregulation of the ER stress pathway as indicated by increased *Armet, Xbp1*, and *Atf6* expression following IVM may, to some degree, be beneficial for oocyte health and subsequent development by restoring protein folding and cell function leading to normal postnatal brain development as observed in the current study. However, upregulation of the ER stress pathway is not sufficient to restore cell function to *in vivo* levels as demonstrated by decreased fertilization rate, preimplantation embryo development, and decreased number of viable pups following transfer of embryos derived from IVM oocytes. Meanwhile, this study was performed with mice of normal fertility and does not exclude effects of the etiology of the underlying fertility within clinical populations. Additional studies are required to determine IVM effects in the patients under other more complex circumstances.

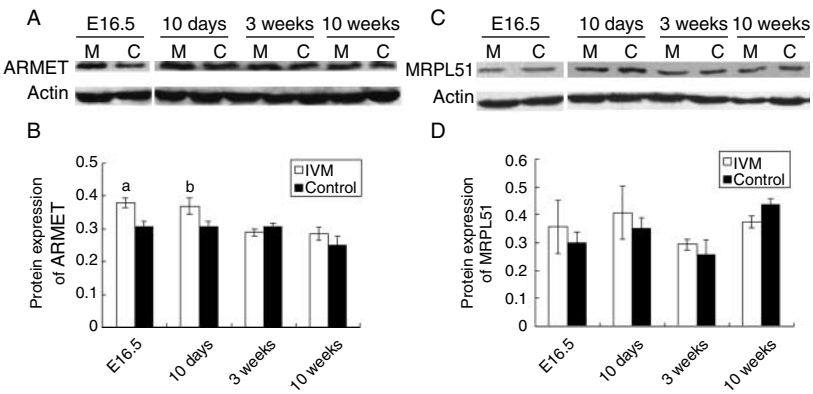

Figure 3 Protein expression in mouse brains. Bands of western blotting (WB) were captured and analyzed by Quantity One Software from three replicate experiments. Protein loads of 50 μg were applied. (A) Bands of ARMET and actin from the two groups; (B) the ratio of ARMET/actin in E16.5, 10 days, 3 weeks, and 10 weeks between IVM and the control group; (C) bands of MRPL51 and actin from the two groups; (D) the ratio of MRPL51/actin in E16.5, 10 days, 3 weeks, and 10 weeks between IVM and the control group. a, $P<0.01$; b, $P<0.05$.

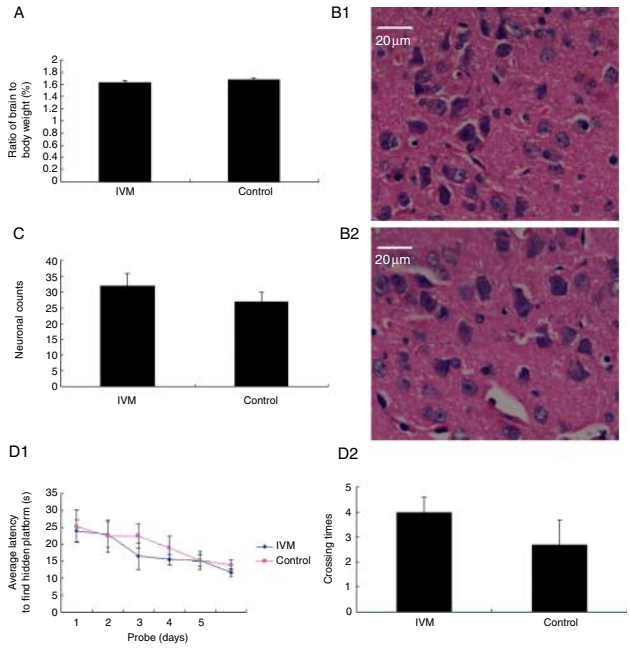

Figure 4 Brain development of the offspring. (A) The ratio of brain weight to body weight between the two groups ($P > 0.05$); (B) morphology of the neuronal cell. (B1) IVM, (B2) control; bar = 20 μm; (C) neuronal counts between the two groups ($P > 0.05$); (D) results from the Morris water task ($n = 10$). (D1) average latency to find the hidden platform (acquisition trials); (D2) crossing times ($P > 0.05$).

Materials and Methods

Establishment of the IVM mouse model

Experimental animals

Animal care and procedures were carried out following Institutional Guidance of the Laboratory Animal of the Animal Care of Usage Committee (ACUC) of Zhejiang University and the protocol was approved by the ACUC of Zhejiang University School of Medicine. Female C57BL/6J and ICR mice (6–7 weeks old) and male mice (8–12 weeks old) were housed in 12 h light:12 h darkness cycle at 25 ± 0.5 °C and 50–60% humidity. The mice were fed with a standard pellet diet and water.

Collection of oocytes

For IVM experiments, the cumulus-enclosed oocytes at germinal vesicle (GV) stage were selected from ovaries 46–48 h after injected (i.p.) with 7.5 IU pregnant mare serum gonadotropin (PMSG; Pregnyl, Organon, Oss, The Netherlands). Oocytes were matured to metaphase II (MII) stage as described previously (Junk *et al.* 2003, Nishi *et al.* 2003). Briefly, GV stage oocytes were cultured in human tubal fluid medium (HTF; Irvine Scientific, Santa Ana, CA, USA) containing 10% serum substitute supplement (SSS, Irvine Scientific) with 0.1 IU/ml FSH (Gonal F, Serono) and 0.5 IU/ml human chorionic gonadotropin (hCG, Pregnyl, Organon) for 16–18 h at 37 °C in a humidified atmosphere of 5% CO_2. For the control group, all mice received an i.p. injection of 7.5 IU hCG 46–48 h after the administration of 7.5 IU PMSG. Mice were killed by cervical dislocation 12–14 h after hCG injection and the oviducts were excised and cumulus masses with oocytes matured *in vivo* were obtained (Ruan *et al.* 2006).

The collected cumulus masses from the two groups were digested with hyaluronidase (80 IU/ml, Sigma) to remove granulosa cells. The naked oocytes were either cultured in pre-equilibrated 10% SSS HTF medium at 37 °C in humidified 5% CO_2 for fertilization or washed three times with PBS for mRNA extraction.

ICSI and embryo transfer

ICSI was performed immediately with fresh sperm after collection from epididymis using PIEZO (PrimeTech, Ibaraki, Japan) assistance on an Olympus X71 inverted microscope with Narishige micromanipulators (Narishige, Tokyo, Japan) at 37 °C warm plate as described previously (Yoshida & Perry 2007). Injected oocytes were cultured in 10% SSS HTF medium at 37 °C in humidified 5% CO_2 overnight. Some of the two-cell embryos were used for transfer; the others were washed three times with PBS for mRNA extraction.

Embryos at two-cell stage were transferred into the oviducts (15 per oviduct) of 0.5 d pseudopregnant female mice (Lacham-Kaplan *et al.* 2003). Some pregnant recipients were killed at E16.5 and the uteri were excised to obtain fetal mice. The rest were allowed to go to term and give birth to live offspring for analysis and subsequent breeding.

Brain tissues obtained from mice at E16.5, 10 days, 3 weeks, and 10 weeks stages of IVM and the control group were snap frozen in liquid nitrogen just after collection for half an hour and finally stored at −80 °C for further RNA and protein extraction.

Suppressive subtractive hybridization

RNA was extracted from 500 oocytes using Trizol reagent (Takara, Tokyo, Japan) according to the manufacturer's protocol. SMART cDNA synthesis kit (Clontech Laboratories) was used for the synthesis of double-stranded cDNA. SSH was performed with the PCR-Select cDNA Subtraction Kit (Clontech Laboratories) according to the manufacturer's instructions.

The subtracted PCR products were cloned into the PMD-19 vector (Takara). After transformation by heat shock in 200 μl competent DH5a *Escherichia coli* cells, colonies were then isolated and grown. The insertion of each colony was amplified using PCR. After electrophoresis on the 2% agarose gel, positive and single clones were sent to a sequencing service, and their sequences were submitted for a Blast analysis in GenBank for identification.

Quantitative real-time RT-PCR

Total RNA was extracted from oocytes (500), embryos (500), and brain tissues (about 100 mg; including several developmental stages E16.5, 10 days, 3 weeks, and 10 weeks) using Trizol reagent according to the manufacturer's protocol. Differential gene expressions were detected using the SYBR PrimeScript RT-PCR Kit (Takara). For RT, 4 μl 5×PrimeScript

Buffer PCR buffer, 1 μl PrimeScript RT enzyme mix I, 1 μl Oligo dT primer (50 μM), 1 μl random 6 mers (100 μM), and 10 μl RNA sample for oocytes (or 1 ng for brain tissues) were added into the 20 μl reaction system. The mixture was incubated at 37 °C for 15 min, and the reaction was inactivated at 85 °C for 5 s. RT products were amplified by real-time PCR with SYBR-Green I (Takara, Foster City, CA, USA) on ABI 7900 real-time PCR system (Applied Biosystems, Tokyo, Japan) according to the manufacturer's protocol and using *Gapdh* as an internal control. Primers used for the experiments were designed by the Primer Express 3.0 program. Primer sequences for the following genes are as follows:

Armet: (f) TGCTGCCACCAAGATCATCAA, (r) AGGTCCACTGTGCTCAGGTCAA; *Mrpl51*: (f) TTGGCCTTCGTAAGGCTCACTC, (r) CGGACACAACGCTGCAATTC; *Atf6*: (f) AGTCCCAAGTCCAAAGCGAAGA, (r) CTGATTGGCAGGGCTCACACTA; *Xbp1*: (f) AGTTAAGAACACGCTTGGGAATGG, (r) CTGCTGCAGAGGTGCACATAGTC; *Gapdh*: (f) TGACGTGCCGCCTGGAGAAA, (r) AGTGTAGCCCAAGATGCCCTTCAG.

Real-time PCR was carried out in a 20 μl reaction system containing 10 μl SYBR Premix Ex Taq, 0.4 μl PCR forward primer (10 μM), 0.4 μl PCR reverse primer (10 μM), 0.4 μl ROX reference dye, and 2 μl cDNA sample. PCR was performed with 95 °C for 10 s and 40 cycles at 95 °C for 5 s, 60 °C for 30 s. The mRNA levels of target genes were standardized with those of *Gapdh* reference gene and the average fold values were calculated. Data (three biological repeats and three technical repeats at each time point) were analyzed by the comparative threshold cycle (C_T) method and the standard formula (Schefe *et al.* 2006). Each reaction was performed in triplicate. We choose three pools of oocytes/embryos from 15 mice and three brains from three mice every time, and the same experiment was repeated three times.

Western blotting

The brain proteins were extracted using about 100 mg tissues in a radioimmunoprecipitation buffer (Takara) according to the manufacturer's protocol. Protein samples were separated by SDS–PAGE (6% gel; 50 μg per lane) and then transferred to a polyvinylpyrrolidone membrane. The membrane was blocked and then incubated overnight with the primary antibody (goat polyclonal antibody, MRPL51, 1:500; Santa Cruz Biotechnology, Inc. (Paso Robles, CA, USA); goat polyclonal antibody, ARMET, 1:500; Santa Cruz Biotechnology, Inc.; rabbit anti-β-actin, 1:1000; Sigma). After washing three times with tris-buffered saline with 0.1% Tween-20, the membrane was incubated with secondary antibody (HRP-conjugated rabbit anti-goat IgG (H+L) for MRPL51 and ARMET and HRP-conjugated goat anti-rabbit IgG (H+L), 1:5000; Sigma) conjugated with HRP for 1 h at RT. The signal was detected with the ECL Advance WB detection reagents (Amersham). Five mice of each group were used each time, and the experiment was repeated three times. Data were analyzed by the Quantity One software (Bio-Rad).

Brain development of the offspring

In order to detect whether the changes in the gene/protein expression could affect the brain development of the postnatal mice, we chose morphology, histological analysis, and cognitive behavioral testing as the targets.

Morphology and histology of brain tissue

Ten-week-old mice were weighed, and the brains were removed from the body and weighed after the mice were killed. The ratio of organs to body weight was calculated. The brains were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin section (5 mm) was stained by hematoxylin and eosin (H&E). The slides were observed under the microscope, and five to eight regions within the H&E section of cortex were examined and scored at 400× magnification. Morphology of the brain was observed and neuronal counts in the cerebral cortex were measured (Ratai *et al.* 2011).

Cognition of the offspring

The MWM test was performed using a tracking system (PolyTrak; San Diego Instruments, San Diego, CA, USA) as described previously (Ecker *et al.* 2004, Vorhees & Williams 2006). Spatial training occurred at 8 weeks of age over a 6-day period in a MWM using ten male mice from each group. Each mouse was given 60 s to find the hidden platform, and the latency to find the platform was recorded. The animals were placed in four different quadrants for four trials every day. After completing the trials, each animal performed one probe trial, in which the platform was removed from the pool. The probe trial was performed to verify the animal's understanding of the platform location and observe the strategy that the animal follows when it discovered the platform was not there. The number of times each animal crossed the former location of the platform was recorded during 30 s. The data were analyzed by the tracking system software to evaluate the animals' learning and memory.

Statistical analysis

Data represent mean (s.d.). For comparison of the rates of fertilization, cleavage, and development capability in the preimplantation embryos and the rates of birth in the offspring, the χ^2 test was used. The other data were analyzed by independent *t*-test for significant differences among different groups. (Statistical Package for the Social Science (SPSS) 16.0 (SPSS, Inc., Chicago, IL, USA) was used and differences were considered significant at P value <0.05.

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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