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Rat BAT xenotransplantation recovers the fertility and metabolic health of PCOS mice

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Abstract

Polycystic ovarian syndrome (PCOS) is a major severe ovary disorder affecting 5–10% of reproductive women around the world. PCOS can be considered a metabolic disease because it is often accompanied by obesity and diabetes. Brown adipose tissue (BAT) contains abundant mitochondria and adipokines and has been proven to be effective for treating various metabolic diseases. Recently, allotransplanted BAT successfully recovered the ovarian function of PCOS rat. However, BAT allotransplantation could not be applied to human PCOS; the most potent BAT is from infants, so voluntary donors are almost inaccessible. We recently reported that single BAT xenotransplantation significantly prolonged the fertility of aging mice and did not cause obvious immunorejection. However, PCOS individuals have distinct physiologies from aging mice; thus, it remains essential to study whether xenotransplanted rat BAT can be used for treating PCOS mice. In this study, rat-to-mouse BAT xenotransplantation, fortunately, did not cause severe rejection reaction, and significantly recovered ovarian functions, indicated by the recovery of fertility, oocyte quality, and the levels of multiple essential genes and kinases. Besides, the blood biochemical index, glucose resistance, and insulin resistance were improved. Moreover, transcriptome analysis showed that the recovered PCOS F0 mother following BAT xenotransplantation could also benefit the F1 generation. Finally, BAT xenotransplantation corrected characteristic gene expression abnormalities found in the ovaries of human PCOS patients. These findings suggest that BAT xenotransplantation could be a novel therapeutic strategy for treating PCOS patients.

Key Words

- rat
- mouse
- xenotransplantation
- fertility
- metabolic health
- PCOS

Introduction

As a core reproductive organ, the ovary has two unique functions: producing mature oocytes for fertilization and secreting sex hormones for maintaining the normal activities of multiple organs, including itself. The normal physiology of the ovary is controlled by the hypothalamus–pituitary–ovarian axis (HPO axis). Many genetic and environmental pathological factors affect ovarian function, causing subfertility or infertility. Thus, alterations in
ovarian endocrine activity will have a systemic impact on the function of various tissues and organs. Polycystic ovarian syndrome (PCOS) is a severe and complicated ovary disorder affecting 5–10% of reproductive-age women around the world (Hendriks et al. 2007, Rosenfield et al. 2013, Walters et al. 2018). PCOS is characterized by the presence of multiple polycystic changes in the ovary, anovulation, and elevated androgen levels. PCOS patients often display additional abnormalities such as increased levels of luteinizing hormone (LH), and sometimes, decreased follicle-stimulating hormone (FSH) levels, insulin resistance (hyperinsulinemia), obesity, and hirsutism. Although the cause of PCOS is poorly defined, neuroendocrinological dysfunctions in the HPO axis, especially those in the positive and negative feedback loops, are often referred to as a major pathological mechanism (Pastor et al. 1998, Blank et al. 2006, Hendriks et al. 2007, Rosenfield et al. 2007, Walters et al. 2018). The involvement of genetic defects, abnormal gene expression, and metabolic anomalies has been extensively investigated (Escobar-Morreale et al. 2011, Azziz 2016, Streiter et al. 2016). Theoretically, intervention measures that reverse the neuroendocrinological dysfunction might have potential therapeutic value.

As PCOS is commonly accompanied by abnormal metabolism, chemical-based therapies aiming at correcting metabolism are also used. Metformin significantly improved the neuroendocrinological or metabolic indexes (glucose, insulin, HOMA-IR (homeostasis model assessment of insulin resistance), triglycerides (TG), sex hormone-binding globulin (SHBG), FSH, LH, LH/FSH ratio, high-density lipoprotein cholesterol (HDL-C) levels) and restored regular menstrual cycles in some patients (Palomba et al. 2011, Morin-Papunen et al. 2012, Yang et al. 2018). The combined use of metformin and other compounds was more effective than singular use (Banaszewska et al. 2011, Tfayli et al. 2011, Bhattacharya & Jha 2012, Misso et al. 2012, Morin-Papunen et al. 2012, Ganie et al. 2013, Wu et al. 2016, Athanasiou et al. 2017, Luque-Ramirez et al. 2018, Yang et al. 2018). Moreover, BAT in PCOS patients is significantly smaller (Li et al. 2020) and less active (Oliveira et al. 2019, Shorakae et al. 2019) than that in normal control females, suggesting that BAT dysfunction might be an important cause of PCOS and that BAT supply might help to cure PCOS. In fact, rat-to-rat BAT transplantation was recently tested in a rat PCOS model. The strategy significantly improved the endocrine, metabolic, and mRNA expression profiles in the model system (Yuan et al. 2016). Moreover, the pregnancy/parturition rate was also significantly improved (Yuan et al. 2016). The primary function of BAT, which is rich in mitochondria, is to utilize glucose and lipids for thermogenesis. BAT also secretes important signaling molecules, namely the brown adipokines, or batokines, to regulate metabolism. BAT contains adipose-derived stem cells that can upregulate the expression of anti-inflammatory cytokines and specific antigens, thereby decreasing allotissue rejection and significantly promoting donor cell chimerism (Jankovič et al. 1975, González et al. 2009, Plock et al. 2017). However, while BAT is abundant in newborns, its progressive reduction with age limits its range of clinical applications.

The development of a therapeutic model should consider safety, operational simplicity, sustained efficacy, and affordability so that most patients can benefit from its application. Although BAT transplantation appears to be effective and of low toxicity, it is difficult to recruit donors from healthy young populations. From this point of view, the xenotransplantation of BAT from another species for treating PCOS is worth exploring. We recently reported that single xenotransplanted BAT significantly prolonged the fertility of aging mice by improving follicle quality (Chen et al. 2019). However, the physiology of PCOS individuals is quite different from that of young or aging individuals. For example, PCOS women have increased expression of inflammatory genes (Zhao et al. 2015, Adams et al. 2016). In addition, PCOS individuals can also have imbalanced immunity and increased autoimmunity (Petriková et al. 2010, Gong et al. 2018). Therefore, investigating whether xenotransplanted rat BAT faces immunorejection remains important. In this study, we tested rat-to-mouse (RTM) BAT transplantation in 2-month-old pubescent mice. For comparison, we performed parallel mouse-to-mouse BAT transplantation (MTM). Our aim is to assess the
potential value of BAT xenotransplantation for treating PCOS patients. Investigating gene expression patterns can aid understanding of the adaptive changes associated with cross-species BAT transplantation and aid the determination of the underlying mechanisms.

Materials and methods

Animal maintenance and experimental design

Female BALB/C (2-week-old) mice and SD (2-week-old) rats were purchased from the Animal Core Facility of Nanjing Medical University (Nanjing, Jiangsu, China). Five mice per cage were housed with a 12 h light:12 h darkness cycle in the animal facility certified by the Office of Laboratory Animal Welfare. Food and water were sufficiently provided for ad libitum intake, and the ambient temperature of animal housing is 25°C. Tomato-transgenic SD rats used for verification of the xenotransplanted BAT were introduced from the Model Animal Research Center of Nanjing University (Nanjing, China) and kept in the Animal Core Facility of Nanjing Medical University. All animal studies were conducted with the approval of the Ethics Committee of Animal Experiments at Nanjing Medical University.

Four parallel groups were included in all the experiments: Control group, 2-month-old young mice receiving normal saline injection and sham operation (the skin covering the scapula region was cut and then stitched without BAT transplantation); MTM (mouse-to-mouse) group, PCOS mice receiving allotransplanted mouse BAT; RTM (rat-to-mouse) group, PCOS mice receiving xenotransplanted rat BAT; PCOS group, 2-month-old mice accepting DHEA treatment and sham operation.

Forty-eight hour before all experiments except fertility assay and estrous cycle assay, the estrous cycle of mice were synchronized by PMSG injection.

Construction of the PCOS mouse model

DHEA used for the establishment of PCOS model was purchased from Cayman (Cat. No. 15728). DHEA (6 mg/100 g body weight, 1.2 mg/mL in sesame oil) was injected daily into the interscapular region of the recipient mice (PCOS model mice) under anesthesia. Female BALB/C mice and SD rats (2-week-old) were chosen as donors of BAT for transplantation. BAT was harvested from the interscapular region of the anesthetized donor rats or mice and placed in sterile saline. PCOS group received the same surgical operation as control group.

For initial verification of the survival of the xenotransplanted rat BAT, we use global tomato-transgenic rat as previously described (Chen et al. 2019). Within the first month, we were able to easily position the transplanted rat BAT by tomato red fluorescence (Supplementary Fig. 1, see section on supplementary materials given at the end of this article). Afterwards, the rat BAT fluorescence diminished significantly (probably due to the fusion of rat BAT and mouse BAT), but we were able to detect the rat UCP1 protein and mRNA in the BAT of the xenotransplant position after six months (Chen et al. 2019). Thus the rat BAT can be at least partially active for at least 6-month, so we only did one-time transplantation.

Antibodies

Mouse monoclonal anti-α-tubulin antibodies (Cat#: F2168) were purchased from Sigma. Mouse monoclonal anti-GAPDH (Cat#: 30201ES60) was bought from YEASEN (Shanghai, China). Polyclonal antibody of RPS6 (phospho-S240, Cat#: BS4359), AMPKα1/2 (phospho-T183/172, Cat#: BS4457) and UCP1 (Cat#: BS70689) were purchased from Bioworld (Dublin, OH, USA). Rabbit polyclonal anti-HLA antibodies (Cat#: MA5-11723) was purchased from Thermo Fisher Scientific.

Glucose tolerance test and insulin tolerance test

For glucose tolerance tests (GTTs), female mice were fasted for 16 h with free access to drinking water, and injected with d-glucose (2.0 g/kg body weight, 0.4 g/mL) intraperitoneally. Blood glucose levels were measured at 0, 30, 60, 90, and 120 min with a glucose monitor significantly decreased estrus cycle and increased LH/FSH ratio according to the published criteria (Yuan et al. 2016).
(Sinocare Inc., Hunan, China). For insulin tolerance test (ITT), female mice were fasted for 16 h with free access to drinking water, and injected with insulin (0.5 U/kg body weight, 0.125 U/mL) (Yuanye Bio-Tech Co., Shanghai, China) intraperitoneally. Blood glucose levels were measured at 0, 15, 30, and 60 min. AUC (Area under the blood glucose curve) was also obtained according to the following equation: AUC = 0.5 × (0 min blood glucose + 30 min blood glucose) × 0.5 h + 0.5 × (30 min blood glucose + 60 min blood glucose) × 0.5 h + 0.5 × (60 min blood glucose + 90 min blood glucose) × 0.5 h + 0.5 × (90 min blood glucose + 120 min blood glucose) × 0.5 h.

**Oocyte collection and culture**

Mice were first anesthetized with CO₂, sacrificed by cervical dislocation, and ovaries were isolated and placed in operation medium (Hepes) containing 2.5 nM milrinone and 10% fetal bovine serum (FBS; Thermo Fisher Scientific). Oocytes were collected from the ovary by puncturing follicles with a hypodermic needle. Cumulus cells were washed off cumulus-oocyte complexes, and every 50 isolated denuded oocytes were placed in 100 mL droplets of culture medium covered by mineral oil (Sigma) in plastic dishes (BD, Brea, CA, USA). Oocytes were grown at 37°C in the culture medium (MEM; MEM with 0.01 mM EDTA, 0.23 mM Na pyruvate, 0.2 mM penicillin/streptomycin, 3 mg/mL BSA) containing 20% FBS, in the humidified atmosphere of 5% O₂, 5% CO₂. Before **in vitro** maturation, 2.5 nM milrinone was added to the culture medium to prevent the resumption of oocyte meiosis.

**Paraffin section preparation and ovarian follicle counting**

Ovaries were collected, fixed in 10% buffered formalin for 12 h, and embedded in paraffin. Serial sections were prepared at a thickness of 5 μm, and stained with hematoxylin and eosin. All follicles with a visible nucleus were counted every second section. Follicle classification was determined by Pederson’s system: oocytes surrounded by a single layer of flattened or cubical granulose cells were defined as primordial follicles; oocytes surrounded by more than one layer of cubical granulose cells with no visible antrum were counted as secondary follicles; follicles with a visible antral space and a cumulus were counted as antral. Post-ovulation follicles filled with lutein cells were determined as corpora lutea, and follicles containing degenerating oocytes, disorganized granulosa cells, pyknotic nuclei, shrunken granulosa cells, or apoptotic bodies were considered atretic.

**Immunohistochemistry of paraffin section**

BATs and ovaries were fixed in 10% buffered formalin, embedded in paraffin, and sectioned at a thickness of 5 μm. After deparaffinization and rehydration, sections were processed for blocking of endogenous peroxidase activity and antigen retrieval. Immunohistochemical analyses were performed using a SPlink Detection Kits (Zhongsan Jinqiao Biotech., Beijing, China) with corresponding primary antibodies overnight at 4°C. Control sections were incubated with non-immunized IgG. DNA was stained with 1 μg/mL DAPI for 20 min. After staining, sections were observed under a confocal microscope.

**Real-time PCR**

Total RNA of ovaries was isolated with the RNAprep Pure Tissue Kit (Tiagen Biotech, Beijing, China) according to the manufacturer’s instructions. RNA was quantified with a spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific). RT of RNA (500 ng/reaction sample) was performed using a RT kit (Takara). Real-time PCR was performed with SYBRGreen PCR Kit (Yeasen Biotech., Shanghai, China) on the CFX96 Real-Time PCT Detection System (Bio-Rad). Primer sequences are shown in Supplementary Table 5. Mouse β-actin mRNA was amplified as an internal control for each sample. The mRNA levels were calculated according to the 2⁻ΔΔCt method. All the primers are in Supplementary Table 1.

**Estrous cycle analysis**

Vaginal smears were collected on glass slides with 10 μL of 0.9% NaCl at 08:00-09:00 h each morning. After air-drying, slides were stained with toluidine blue (Amresco, Solon, OH, USA) for 5 min, and washed thrice and dried. The four stages of the estrous cycle were determined by analyzing the proportion of three major cell types (epithelial cells, cornified cells, and leukocytes). Consistent cycles of proestrus, estrus, metestrus, and diestrus (4–5 days total) in mice were defined as having regular cycles.

**Detection of ROS**

ROS Assay Kit (Cat No: S0033, Beyotime Biotech., Beijing, China) was used to detect ROS levels in the oocyte. Oocytes were incubated with dichlorofluorescein diacetate
probe for 20 min at 37°C in darkness. After washing trice, the oocytes were mounted on slides, and the images were captured under an Andor Revolution spinning disk confocal workstation.

**Measurement of mitochondrial membrane potential**

Oocytes were incubated at 37°C for 20 min with JC-1 diluted at 1:200 (Cat No: 40706ES60, Yeasen Biotech., Shanghai, China), washed twice with PBS, and placed in 50 μl droplets of culture medium. The green (JC-1 as a monomer at low membrane potentials) and red (JC-1 as ‘J-aggregates’ at higher membrane potentials) fluorescent signals were monitored and captured under an Andor Revolution spinning disk confocal workstation. Mitochondrial depolarization is indicated by a decrease in the ratio of red/green fluorescence intensity.

**RNA sequencing**

Mouse ovaries were isolated and snap-frozen in liquid nitrogen and sent to BGI-Wuhan Co. Ltd. for further processing, RNA sequencing, and data collection. RNA was amplified using the SMARTer Ultra Low Input RNA Kit, the resulting cDNA was fragmented, and paired-end libraries were constructed using the Nextera XT DNA Library Preparation Kit. The libraries were sequenced on an Illumina HiSeq.

**Statistical analysis**

Statistical analysis was performed using the SPSS standard version 13.0 software. Group size for each experiment is no less than 3 or more than 3. Quantitative data was presented as mean ± S.E.M. Comparison between two groups was performed with Student’s t-test. Comparison between multiple groups (more than two) was performed with Tukey’s multiple comparisons test. \( P \leq 0.05 \) were considered statistically significant. All experiments were repeated at least three times.

**Results**

**Xenotransplanted BAT had normal function without significant immunorejection in PCOS mice**

One advantage of xenotransplantation is the easy access to BAT from the infant donor of another species. However, acute rejection is a concern. To test this, we used 2-week-old rats or mice as BAT donors, and 2-month-old B6 mice as the recipients. The experiments were carried out in four groups: control, DHEA+MTM, DHEA+RTM, and DHEA (PCOS).

To determine if the xenotransplanted BAT was alive and exerted normal function, we used the tomato transgenic rat as the donor. BAT cells from these rats can be easily detected based on their fluorescence signals. We found that, 3 weeks after BAT xenotransplantation, the xenotransplanted rat BAT fused well with the innate mouse BAT and expressed rat-specific BAT marker genes, for example, AdipoQ (Supplementary Fig. 1A and B), indicating that the xenotransplanted rat BAT survived and adopted well. UCP1 is an essential protein marker for heat generation in BAT. The function of BAT in the RTM group was supported by the UCP1 immunohistochemistry (Fig. 1A) and immunoblotting (Fig. 1B and C) results, which showed significantly stronger UCP1 signals in the RTM group than in the PCOS group.

We compared the protein levels of major histocompatibility complex class I A (HLA-A) in various tissues among the four groups. All the examined tissues, including BAT, had similar HLA-A levels, indicating that no severe acute rejection occurred (Fig. 1D, E and F).

Finally, we examined the mRNA levels of several adipose marker genes (Ucp1, Ppar1a, Pgc1a, Pcg1b, Dio2, Mcad, Adipoq) and found that, while the expression levels of these genes in the PCOS group were significantly decreased compared to the control group, RTM xenotransplantation successfully restored their expression to levels closer to, or even higher than, that of the control group (Supplementary Fig. 2A, B, C, D, E, F and G). Overall, the results suggest that RTM BAT xenotransplantation did not cause global rejection and that the xenotransplanted rat BAT survived and functioned well.

**RTM BAT transplantation recovered PCOS mouse fertility effectively**

We set up four mating cages for each group and started a 10-month long fertility comparison. The PCOS mice had significantly decreased overall fertility, as manifested by a reduction in the number of total pups (Fig. 2A and B). In addition, the average number of pups per litter (Fig. 2C) and the total number of litters (Fig. 2D) tended to decrease (although not significantly); and the PCOS mice displayed elongated time from mating to the first birth (although not significantly) (Fig. 2E). However, all these indices in the RTM group were recovered to levels comparable to
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rt that of the control group (Fig. 2A, B, C, D and E). These results indicate that rat BAT xenotransplantation can significantly improve the fertility of PCOS mice.

RTM BAT transplantation recovered the overall health and partially corrected the metabolic abnormality of the PCOS mice

The measurement of over 20 common biochemical blood indices showed that, although the average levels of most indices were not significantly different among the four groups, the distribution range of values for several indices, including glutamic pyruvic aminotransferase (ALT), glutamic oxaloacetic aminopherase (AST), HDL-C, and blood urea nitrogen (BUN), was much broader in the PCOS group than in the control group, and rat BAT xenotransplantation led to narrower distribution, a pattern similar to that of the control group (Supplementary Fig. 3A, B, C and D). These results indicate that rat BAT xenotransplantation can improve the overall health conditions of the PCOS mice.

PCOS leads to increased risk of metabolic diseases such as diabetes, which is characterized by abnormality in glucose tolerance and insulin tolerance. In this aspect, our experiments showed that, in the intraperitoneal glucose tolerance test (IPGTT), the PCOS group had significantly higher blood glucose levels than the control group within 1.5 h of glucose administration (Fig. 2F and G). For the intraperitoneal insulin tolerance test (IPITT), upon insulin injection, the PCOS group had higher glucose levels (although not significantly) at each test point than the control group (Fig. 2H and I). Importantly, rat BAT xenotransplantation successfully rescued the glucose levels back to that of the control group in both tests (Fig. 2F, G, H and I). These results indicate that the PCOS model mice had a metabolic abnormality, and that rat BAT xenotransplantation largely corrected this abnormality.

Rat BAT transplantation recovered PCOS mouse ovarian performance

The ovary is the core organ of female fertility. Our examination of the ovary structure showed that the PCOS mice had more (although not significantly) total and primordial follicles, and significantly fewer primary and antral follicles (Fig. 3A, B, C and D). Moreover, the
atretic follicles were increased almost 10-fold in the PCOS mice compared to the control group (Fig. 3F). BAT xenotransplantation significantly increased the number of antral follicles (Fig. 3D) while decreasing the numbers of atretic follicles (Fig. 3E and F). Besides, the 3-week long estrus cycle analysis showed that the PCOS group had significantly fewer estrus cycles than the control group, whereas the rat BAT xenotransplantation recovered the cycles close to the normal levels in the control group (Supplementary Fig. 4). These results indicate that rat BAT xenotransplantation effectively improved the ovarian performance of the PCOS mice.

**Rat BAT transplantation significantly improved the follicle and oocyte quality in PCOS mice**

To determine on the molecular level if rat BAT xenotransplantation could recover PCOS mouse fertility, we performed western blotting analysis of p-AKT, p-RPS6, and p-AMPK, the components of the mTOR signaling pathway essential for the survival or activation of the primordial follicles. Low p-AKT and p-RPS6 levels and high p-AMPK levels usually suggest that more primordial follicles are inactivated, a hallmark of PCOS. Here, p-AKT and p-RPS6 levels were significantly decreased in the PCOS group than in the control group, while p-AMPK levels were significantly increased in the PCOS group than in the control group, suggesting inactivation of mTOR signaling in the PCOS mouse ovaries. Rat BAT xenotransplantation led to significant recovery of p-AKT, p-AMPK and p-RPS6 close to the levels of the control group (Fig. 4A, C, D and E). These findings demonstrate that mTOR activity was abnormally lower in the PCOS group and that rat BAT xenotransplantation improved it.

BAT secretes important signaling molecules, namely the adipokines, or batokines, to modulate the metabolism (Villarroya et al., 2013, 2017, Wang et al., 2015). BAT contains adipose-derived stem cells that can upregulate the expression of anti-inflammatory cytokine and specific antigens, thereby inhibiting allotissue rejection and promoting donor cell chimerism (30–32). Here, we examined several adipokines and found that NRG4 was significantly reduced in the PCOS group. However, a significant increase in NRG4 levels was observed in the rat BAT xenotransplantation group (Fig. 4F and G).

BMP15 and GDF9 are two important oocyte-derived paracrine factors (ODPFs) essential for follicle maturation. Here, real-time PCR showed that while Bmp15 and Gdf9 mRNA levels were markedly decreased in the PCOS group, they were largely recovered in the RTM group (Fig. 4H).
and Supplementary Fig. 5A). GDF9 immunofluorescence experiments yielded consistent results for the protein levels (Fig. 4I and J). Moreover, real-time PCR showed that while the mRNA levels of five granular cell–expressed, steroidogenesis-related genes were significantly lower (Amhr and Aromatase, Supplementary Fig. 5B and C) or higher (Cyp17a, Cyp19a, and Fshr, Supplementary Fig. 5D, E and F) in the PCOS group, they were largely recovered in the RTM group as compared to the control group. Cyp19a immunohistochemistry of the


Figure 4
Rat BAT transplantation significantly improved the follicle and oocyte quality in PCOS mice. (A) Western blotting analysis. (B, C and D) Densitometry analysis of (A). p-RPS6 and p-Akt levels were significantly lower in the PCOS group than in the control group, whereas p-AMPK levels were significantly higher than that in the control group. Rat BAT xenotransplantation restored the expression levels of these three genes in the RTM group. (E) Immunofluorescence in the ovaries showed that p-RPS6 levels were significantly lower in the PCOS group than in the control group, while rat BAT xenotransplantation increased it close to that of the control group. (F) Western blotting analysis of NRG4 expression. (G) Densitometry analysis of (F). NRG4 protein levels were significantly reduced in the PCOS group, while rat BAT xenotransplantation increased NRG4 levels. (H) The results of real-time PCR showed that GDF9 mRNA levels were decreased in the PCOS group, whereas rat BAT xenotransplantation restored them. (I) Immunofluorescence in ovaries show that GDF9 levels were significantly lower in the PCOS group than in the control group, while rat BAT xenotransplantation recovered it close to that of the control group. (J) Fluorescence intensity analysis of (I). Different lowercase alphabets above the columns in the graphs indicate a significant difference between groups.

Ovaries demonstrated its restoration at protein level (Supplementary Fig. 5G).

Reduced mitochondrial membrane potential and increased ROS level are indicative of impaired follicle or oocyte quality. Here, the mitochondrial membrane potentials, as indicated by the JC-1 fluorescence ratio, were significantly lower in the MII oocytes of the PCOS mice than in the control mice (Fig. 5A and B). ROS levels
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in the MII oocytes of the PCOS mice were significantly higher than that in the control mice (Fig. 5C and D). Again, rat BAT xenotransplantation successfully recovered both ROS levels and mitochondrial membrane potentials close to that of the control group (Fig. 5A, B, C and D). These results suggested that rat BAT xenotransplantation significantly improved the follicle and oocyte quality of the PCOS mice.

Impacts of RTM BAT transplantation on the transcriptional profiles of F0 PCOS mouse ovaries

To find mechanistic clues for how rat BAT xenotransplantation could restore ovarian function, we examined the transcriptome in the ovaries of the four groups via mRNA sequencing. Both Venn diagrams and mRNA expression heat maps of the differentially expressed genes (DEGs) showed that the mRNA expression profiles were dramatically altered in the PCOS group as compared with the control group, and that rat BAT transplantation significantly recovered the profiles close to that of the control group, as shown by changes in many genes (Fig. 6A, B and Supplementary Dataset 1). We found that 230 genes had similar expression patterns among the control, PCOS+RTM, and PCOS+MTM groups, but with different patterns as compared to the PCOS group. The results of real-time PCR of selected overlapping genes supported the reliability of the sequencing results (Fig. 6C, D, E, F and G). KEGG analysis showed that these genes were involved in multiple signal pathways essential for follicle and oocyte quality and survival (Fig. 6H, red arrow pointed; Supplementary Table 2), including ovarian steroidogenesis, the PI3K–AKT signaling pathway, the ErbB signaling pathway, the MAPK pathway, ECM–receptor interaction, and longevity-regulating pathway. Notably, key metabolism-related pathways were also included in these DEGs. These include amino acid biosynthesis and metabolism (phenylalanine, tyrosine, and tryptophan biosynthesis; protein digestion and absorption; cysteine and methionine metabolism) (Fig. 6H, black arrow pointed, Supplementary Table 2). These results support the fact that, at transcriptome level, rat BAT xenotransplantation can rescue both follicle and oocyte quality and the overall abnormal metabolism of PCOS mice.

DEGs in our study overlapped with DEGs in multiple human PCOS transcriptome studies

One purpose of the present BAT xenotransplantation study is to provide a reference for treating PCOS patients.
Therefore, it is important to compare transcriptome findings in our mouse PCOS model to that from PCOS patients. Interestingly, many DEGs from our study overlapped with DEGs from several recent human PCOS transcriptome studies (Kaur et al. 2012, Wissing et al. 2014, Lan et al. 2015, Lu et al. 2018) (Supplementary Dataset 2). These DEGs are involved in pathways significant for the pathogenesis of PCOS (Fig. 7A, B, C and D and Supplementary Dataset 3, Supplementary Table 3). For example, as described in the ‘Introduction’ section, the typical symptoms of PCOS are abnormal metabolism and fertility. Our literature search found four reports that covered the endocrine and metabolic diseases KEGG pathway (Fig. 7A, B, C and D, red arrow-pointed), of which
three identified the metabolism KEGG pathways for significant alterations in the ovaries of PCOS patients (Fig. 7A, B, C and D, red dotted line square). Particularly, Cybb, Kmo, Lipa, Ndufs8, and Plcg2 were identified as metabolism-related DEGs by Kaur et al. (2012), Lan et al. (2015), and us. The other overlapping KEGG pathways involved in oocyte and ovary quality included cell growth and death, replication and repair, development, transport and catabolism, signal transduction, and cancers (black arrow-pointed).
and catabolism, signal transduction, and cancers (Fig. 7A, B, C and D, black arrow-pointed). These comparisons provide circumstantial evidence supporting the fact that our PCOS model recapitulates some characteristics of human PCOS, and that RTM may improve PCOS symptoms by adjusting multiple molecular pathways.

Transcriptome recovery of PCOS F0 mother by RTM BAT transplantation had a positive impact on F1 offspring

We were interested to see whether the overall improvement of the PCOS F0 mother through rat BAT xenotransplantation could have positive impacts on the F1 offspring at transcriptome level. Both Venn diagrams and mRNA expression heat maps of the DEGs showed that F1 offspring from the PCOS group had dramatically altered mRNA expression profiles as compared with the control group, which were partially recovered by rat BAT transplantation (Supplementary Fig. 6A, B, C, D, E, F, G, H, Supplementary Dataset 4 and Supplementary Table 4). Examination of the common biochemical blood indices in the F1 mice showed that several indices, including ALT, CHOL, ALT/AST ratio, UA, CK, and BUN, in the F1 RTM group were all at levels similar to that of the control group and were distributed within a much narrower range than that in the F1 PCOS group (Supplementary Fig. 7). These results support the fact that, at transcriptome level, the improvement of the F0 PCOS mother by rat BAT xenotransplantation had a positive impact on the follicle and oocyte quality, and the overall health of the F1 PCOS offspring.

Discussion

In this study, we successfully rescued the impaired fertility and abnormal metabolism of PCOS model mice through rat BAT xenotransplantation. Recently, it was reported that BAT allotransplantation partially recovered the fertility of PCOS model rats (Yuan et al. 2016). However, the application of BAT allotransplantation in clinical settings faces the difficulty of limited sources of BAT in terms of both donor and tissue quantity. We recently reported that single xenotransplanted BAT will cause immunorejection in PCOS mice. Fortunately, we did not observe significant changes in the main rejection marker HLA-A following RTM BAT xenotransplantation. BAT contains many innate adipose-derived stem cells, which can elicit inflammatory and oxidative responses through specific interactions between cytokines and related receptors (Villarroya et al. 2013, 2017, Wang et al. 2015). In the present study, NRG4 was significantly increased by rat BAT xenotransplantation. In cultured murine and human macrophages, NRG4 binds to ERB4, a receptor tyrosine kinase, to induce the apoptosis of pro-inflammatory macrophages (Schumacher et al. 2017). Thus, increased NRG4 expression could contribute to the absence of immune rejection by inhibiting inflammation.

Metabolism changes are recognized as important symptoms of PCOS. In the present study, we observed multiple metabolic improvements after BAT xenotransplantation. First, the average values of blood UN, ALT, AST, and HDL-C of the RTM groups became much closer to that of the young group than to the aging group. UN reflects the filtration function of the glomeruli. ALT and AST are two primary markers of liver function. HDL-C is related to arterial health. The improvement in these biochemical indices suggests that BAT xenotransplantation can significantly improve the overall health of PCOS mice. Second, the GTT and ITT showed that glucose metabolism in the RTM group was much improved. Third, the levels of ROS, the toxic byproducts of aerobic metabolism, increased significantly in the PCOS group, but were decreased by rat BAT xenotransplantation, suggesting that rat BAT xenotransplantation can successfully correct the metabolism abnormality. Fourth, mTOR activity is important for maintaining normal metabolism, and abnormal mTOR activity levels in PCOS mice are related to abnormal energy production (Roa & Tena-Sempere 2010, Hung et al. 2014). The positive changes in mTOR activity achieved by rat BAT xenotransplantation can partially explain the recovery of energy production in the PCOS model mice.

It should be noted that PCOS is a complicated disease, and endocrine alteration, epigenetic changes, environmental effects, and aging could all contribute to its pathogenesis. Indeed, our ovarian RNA sequencing and KEGG analysis of the top 230 DEGs showed that, in addition to the metabolism-related signal pathways, changes in additional pathways could be involved in the pathogenesis of the PCOS model and the positive impact of RTM BAT transplantation. It is worth noting that many metabolism-related DEGs rescued by RTM BAT
transplantation in the ovary overlapped with the DEGs identified by other transcriptome studies on PCOS. These overlapping DEGs, for example, Ndufs8, Mapk3, Pygl, Plcg2, Cybb, and Lipa, are classified into multiple KEGG pathways, reflecting the complex nature of PCOS pathogenesis.

The health status of the mother can affect the fetus and the next generation through genetic, epigenetic, and other mechanisms. For example, for beneficial impacts, maternal intake of peanuts, milk, and wheat during pregnancy is associated with reduced allergy and asthma in children aged 7–9 years (Bunyavanich et al. 2014). For adverse impacts, maternal thyroid dysfunction during pregnancy causes altered thyroid function parameters in adolescent offspring (Päkkilä et al. 2013). In the present study, changes in several blood biochemical indices of the F1 generation from the PCOS F0 mother had significantly larger variation than that of the control mice. Moreover, RNA sequencing of the F1 ovaries showed that the mRNA expression profiles of F1 ovaries from the PCOS F0 mother were significantly different from those of the control F0 mother. Notably, RTM BAT xenotransplantation in the F0 mother could rescue the F1 metabolic abnormality in the ovaries. Intriguingly, multiple DEGs such as Lhcg, Ptgs2, Nr4a1, Nr4a2, Pla2g5, Hspa1a, Chr1r, Ryr1, Ada, Cd19, Gstn6, Comp, Cox7a1, and Adh1 are involved in insulin resistance/diabetes. Further studies are required to confirm the existence of this phenomenon in human populations.

In conclusion, we realize the limitation of the xenotransplantation in the ovary because of the evolutionary closeness between mice and rats, and the limitation of the DHEA-induced PCOS model in resembling real human PCOS. Nevertheless, for the first time, we provide evidence that one-time RTM BAT xenotransplantation can rescue the low fertility and abnormal metabolism of PCOS mice effectively, and that these rescue effects can even benefit the F1 generation. However, low fertility and abnormal metabolism are not unique features of PCOS and frequently occur in insulin resistance/diabetes as well, so further study is required to investigate in depth how xenotransplanted rat BAT functions in PCOS model mice.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-20-0068.

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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Author contribution statement
Zhi-Xia Yang, Xiao-Wei Xing, Dong Zhang, and Fan Hu designed the research. Zhi-Xia Yang also coordinated between authors for the experiment assignment. Lei Du, Yang Wang, Cong-Rong Li, Liang-Jian Chen, and Jin-Yang Cai performed most of the experiments, data collection and analysis. Zheng-Rong Xia, Wen-Tao Zeng, Zi-Bin Wang, and Xi-Chen Chen assisted them during the whole process. Lei Du and Yang Wang did most of the figures preparation under the supervision of Zhi-Xia Yang, Xiao-Wei Xing, Dong Zhang, and Fan Hu. Dong Zhang wrote the manuscript with the assistance of Lei Du and Yang Wang. Zhi-Xia Yang, Xiao-Wei Xing, and Fan Hu proofread and gave advice. All authors read and approved the final manuscript.

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