Establishment and adipocyte differentiation of polycystic ovary syndrome-derived induced pluripotent stem cells

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Abstract

Objective: To establish a new biological cell model and approach to mimic abnormal lipid metabolism of polycystic ovary syndrome (PCOS) *in vitro*.

Materials and methods: Epithelial cells from PCOS patients were reprogrammed to pluripotency by retroviral transduction using defined factors. Morphology, growth characteristics, karyotype, gene expression and differentiation *in vitro* and *in vivo* were detected by identification protocol of human embryonic stem cells (ESCs). PCOS-induced pluripotent stem cells (iPSCs) were then induced to differentiate into adipocytes. Ability of the adipocytes for glucose consumption was compared with those from non-PCOS-iPSCs.

Results: iPSCs were successfully generated from PCOS patients' adult cells. Formed iPSC clones had the same characteristics of human ESCs. PCOS-iPSCs were induced to differentiation into normal karyotype adipocytes. Compared to non-PCOS-iPSCs, PCOS-iPSCs had more glucose consumption ability during adipocyte differentiation and development *in vitro*.

Conclusions: This protocol provides a new biological cell model and approach for studying pathogenesis of PCOS and discovering potential drugs to treat it.

Introduction

Polycystic ovary syndrome (PCOS) is a complex endocrine and metabolic disorder syndrome affecting 5–10% of women in the reproductive age group (1). PCOS leads to not only reproductive defect but also long-term metabolic abnormality. Recently, many family surveys have indicated that PCOS is usually associated with cardiovascular diseases, diabetes mellitus and lipid metabolism disorder. Therefore, PCOS affects patient's both reproductive functions and health. However, the pathogenesis of PCOS is not clear. The phenomenon of PCOS familial aggregation suggests that genetic factors play an important role in its aetiology (2). Although the studies of familial PCOS have suggested an autosomal dominant trait, the mode of inheritance has not been formally established, including a cell model with family history and genetic tendency for PCOS. Some scholars believe that the pathological changes begin in the embryonic stage and there are different sites of spatial and temporal regulation of genes in development stage of PCOS (3,4). Because of ethical and legal restrictions for human embryo studies at different stages, and because of limited access to human embryos of PCOS with inheritance, the pathological study of PCOS cannot be performed from visual angle of dynamics and continuous cell and embryo development. An effective cell model is essential to exploring the pathogenesis of PCOS.

The generation of iPSCs from mouse embryonic and adult fibroblasts was first reported by Yamanaka and his colleagues (5). It has attracted enormous attention due to their vast potential in regenerative medicine, pharmaceutical screening and basic research. Subsequent studies demonstrated that human, rat and rabbit iPSCs could be generated using the same or slightly different sets of reprogramming factor (6–8). Some of disease-model

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iPSC cell lines have been established (9–12). However, up to now, there have been no reports about the successful establishment of PCOS-derived iPSCs. Therefore, to generating PCOS-derived human iPSCs helps to explore the pathogenesis, virulence genes, drug discovery and further clinical therapy of PCOS.

Abnormal lipid metabolism is one of important characteristics of PCOS patients. Adipocyte differentiation and development are involved in it. Adipose cells can be induced to differentiate from embryonic stem cells, and are important for research on lipid metabolism and obesity (13,14). Here, we established iPSCs from the somatic cells of PCOS patients with family history and successfully demonstrated the first iPSCs from PCOS disease model. Subsequently, to evaluate the potential of these novel human iPSC lines for exploring the pathogenesis, drug discovery and further clinical therapy of PCOS, we have tested their capacity for adipocyte differentiation, and demonstrated the PCOS-iPSCs have higher glucose consumption ability and lower insulin response during adipocyte differentiation and development. We established a cell model to mimic abnormal lipids metabolism of PCOS patients *in vitro*.

Materials and methods

Ethics

The study was approved by Institutional Review Board and Ethics Committee of the Nanchang Institute of Medical Sciences. The patients enrolled in this study were informed and consented.

The diagnostic criterion and the characteristics of PCOS patients in the study

In this study, the PCOS patients with family history were diagnosed by the Rotterdam criteria (15). The subjects who had two of the following three items were diagnosed with PCOS: (i) oligo- and/or anovulation; (ii) hyperandrogenism or the clinical manifestations of hyperandrogenism, such as hirsutism and acne and (iii) polycystic ovaries on ultrasonography with exclusion of related disorders, such as hyperprolactinemia, thyroid disease, congenital adrenal hyperplasia, Cushing syndrome, tumour-secreting androgen, 21-hydroxylase deficiency, atypical adrenal hyperplasia and application of exogenous androgen. According to these criteria, seven patients with PCOS were selected. The patients' age, body mass index (BMI), the family history, blood lipid level, androgen levels, infertility status of PCOS and non-PCOS groups were summarized (Table S1).

Generation of PCOS-derived iPSCs

The epithelial cells in urine from the PCOS patients with familial history were collected as adult cells for reprogramming. The iPSC clones were reprogrammed from the adult cells as described previously (9,10). The vectors used in our experiments are from commercial kit (Invitrogen, Carlsbad, CA, USA: catalog numbers A13780-01). It is four individual viral vectors. One CytoTune-iPS Reprogramming Kit of four tubes supplies sufficient reagents to transduce cells in two wells of a six-well plate (5×10^5 cells/well) at an multiplicity of infection = 3. Briefly, 10% confluent epithelial cells were transduced with OCT3/4, SOX2, KLF4 and c-MYC-expressing lentiviral vectors in MEF medium (see user guider of kit) without serum. After overnight viral infection, infected adult cells were seeded onto mitomycin-C-treated mouse EF feeder cells. The medium was changed with fresh complete MEF medium every day. At day 5, the medium was replaced with iPSC medium, which contained HEScGRO (catalog number SCM020; Millipore, Billerica, MA, USA) and antibiotics, but no additional basic fibroblast growth factor. Cells were cultured with fresh HESC GRO-based iPSC medium every day. Putative PCOS-derived iPSC colonies were observed within 21 days after viral vector transduction. Generated colonies were collected and mechanically dissociated. Dissociated cell clumps were plated into new wells with feeder cells for expanding.

Immunocytochemistry and alkaline phosphatase staining

Undifferentiated PCOS-derived iPSC clones were fixed for 30 min at room temperature in 4% paraformaldehyde in PBS and then permeabilized with TritonX-100 for 30 min. After 30 min of blocking with 5% foetal bovine serum in PBS with 0.1% Tween-20, cells were stained with primary antibodies overnight at 4 °C, and after PBS washing, incubated with secondary antibodies (1 h at room temperature at a dilution of 1:200). After staining, cells were analysed using confocal laser-scanning microscopy (LSM 510 confocal scanning laser system; Zeiss, Thornwood, NY, USA). For detection of alkaline phosphatase activity, the alkaline phosphatase detection kit (Millipore, Billerica, MA, USA) was used. Briefly, human iPSCs in culture for 5 days were fixed with 4% paraformaldehyde for 2 min at room temperature. The cells were rinsed with TBST buffer (20 mM Tris-HCl, pH 7.4, 0.15 NaCl, 0.05% Tween-20) and stained with naphtha/fast red violet solution (mixture of fast red violet, naphthol AS-BI phosphate solution and water in a 2:1:1 ratio) for 15 min in the dark. Cells were rinsed with TBST and covered with PBS; alkaline

phosphatase-positive cells were imaged using a light microscope.

In vitro differentiation assay

For *in vitro* differentiation, PCOS-iPSCs colonies cultured on feeder layer were mechanically cut into small pieces of 50–100 cells and continued to grow as aggregates in suspension in ES medium without bFGF for 10 days to form embryoid bodies. Embryoid bodies were then transferred onto gelatin-coated six-well plate for adherent culture for 15 days in the same medium, followed by immunocytochemistry analysis.

In vivo differentiation assay

For *in vivo* differentiation assay, three independent experiments were performed to each new established PCOS-derived iPSCs to confirm the properties of the new cells. At the same time, the negative control experiment was designed with fibroblast and done along each independent experiment. The PCOS-iPSCs were mechanically cut into small clumps (approximately 50–100 cells each). About $0.5\text{--}1 \times 10^6$ cells were collected and injected into the rear leg of 6- to 8-week-old NOD/SCID mice. About 8–10 weeks later, mice were sacrificed. The xenografts were removed and fixed in 4% paraformaldehyde (Sigma, St Louis, MO, USA) for 24 h. After embedding in paraffin, tumours were sectioned, and standard histopathological analysis was performed following haematoxylin and eosin (H&E) staining.

Rt-PCR

Total RNA was isolated from PCOS-iPSCs using TRIZ reagent (Sigma), and cDNA was synthesized between 10 ng and 5 µg total RNA in a 20 µl reaction. RNA was reverse transcribed in a thermocycler using virus reverse transcriptase along with random primer according to the manufacturer's protocol supplied by Fermentas Company. PCR amplification for different genes was performed using Taq DNA polymerase (Promega, Madison, WI, USA). The PCR products were subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide at 0.5 µg/ml. PUC Mix marker, 8 (MBI-Fermentas, St Leon-Roth, Germany; Nukleotide) was used to estimate size of the amplified bands. The primers were used for RT-PCR (Table S2).

Differentiation and identification of adipocytes

The detailed protocol for stem cell culture and adipocyte differentiation has been described previously (13). After

amplification and passage of PCOS-iPSCs, the colonies were cultured in DMEM culture medium supplemented with 100 ml/l of knock-out serum replacement for embryonic bodies (EBs). EBs were collected at day 2 and plated in gelatin-coated dishes. A day after, the cells were treated with RA (1 µM) and AsA (12.5 µg/ml) for 3 days. From day 7 to day 11, the cells were treated with an adipogenic cocktail containing: rosiglitazone (0.5 µM), insulin (0.5 µg/ml), T3 (3 nM) and AsA (12.5 µg/ml). At day 12 of the differentiation, the cells were replated using accutase and treated with the previously mentioned adipogenic cocktail. The cells were treated with a second adipogenic cocktail from day 15 to day 20: IBMX (0.5 µM), dexamethasone (0.1 µM), insulin (20 µg/ml), rosiglitazone (0.5 µg/ml), indomethacin (0.06 mM) and AsA (25 µg/ml). Finally from day 21, the medium was supplemented with insulin (10 µg/ml), rosiglitazone (0.5 µg/ml), AsA (25 µg/ml) and T3 (3 nM) until the end of the differentiation (day 27). Twenty-seven days later, identifications, including cellular morphology, oil red O staining, were carried out to identify the adipocyte cells.

Glucose consumption ability and insulin response ability of the adipocytes

Twenty-seven days after induction, the previous culture medium in the 24-well plate was replaced by 200 µl of high-glucose DMEM (4.5 g/l) supplemented with 0.2% bovine serum albumin per well with or without 10 nmol/l of insulin. The adipocyte differentiation experiment was divided into four groups. Group 1, adipocytes differentiated from non-PCOS-derived iPSCs without insulin. Group 2, adipocytes differentiated from non-PCOS-derived iPSCs with insulin. Group 3, adipocytes differentiated from PCOS-derived iPSCs without insulin. Group 4, adipocytes differentiated from PCOS-derived iPSCs with insulin. Each group, there were eight wells of parallel samples, and glucose concentration was determined with glucose oxidase method after 24-h incubation. The wells without cells were served as control. Glucose consumption is equal to glucose concentration in control well minus glucose concentration in each well with cells incubated for 24 h. The cell number was corrected with MTT method: The medium of each well was replaced with 100 µl of 0.05 mg/ml MTT stock solution (Sigma). After 4 h of incubation, DMSO was added (100 µl/well). The absorbance was determined using the Multimode Plate Reader at 490 nm (EnSpire; PerkinElmer Co. Inc., Waltham, MA, USA). Viable cells were accounted by a percentage of under treatment relative to the differentiated adipocytes after 27-day induction. Compared with the adipocytes differentiated

from non-PCOS-derived iPSCs, the glucose consumption ability and insulin response ability of adipocytes differentiated from PCOS-derived iPSCs were observed.

Karyotype

Human iPSCs were cultured overnight in routine culture medium containing 0.06 ng/ml colcemid (Sigma). After washing three times in PBS, the cells were incubated in culture medium containing 0.05% trypsin and 0.53 mM EDTA (Gibco-BRL, Grand Island, NY, USA) at incubator 10 min and harvested using standard procedures, followed by standard G-banding for karyotyping.

Statistical analyses

Statistical analyses were performed using SPSS version 21.0. Data were represented as the mean $\pm$ SEM and analysed by *t*-test. A *P*-value of < 0.05 was considered statistically significant.

Results

Generation of iPSCs from the adult cells of PCOS patients

The epithelial cells in urine from PCOS patients with familial history were collected as adult cells for

reprogramming after informed consent. The time schedule for PCOS-special iPSC induction is summarized (Fig. 1a). Adult epithelial cells were cultured in serum-free medium two passages. The epithelial cells were transduced with lentiviral vectors expressing four pluripotency factors *Oct4*, *Sox2*, *Klf4* and *c-myc* at day 0 (Fig. 1b). At day 1, the transfected epithelial cells were removed on feeder cells for further induction. About day 14–20, iPSC-like colonies were formed. No iPSC-like colonies were formed in cells transduced with three factors without *c-myc*. Human ES cell-like colonies were picked based on morphological criteria, such as compact colonies, high nucleus-to-cytoplasm ratios and prominent nucleoli (Fig. 1c). The PCOS-specific iPSC clones were positive for alkaline phosphatase (Fig. 1d). These colonies were cultured and expanded on feeder cells.

To detect the impact of the source and density of feeder cells, we tested if the reprogramming is influenced by the source or density of feeder cells. Mouse EFs from the Kunmingbai and the ICR mouse, as well as the density of MEF cells were tested on the reprogramming process. In our experiment, the AP-positive colonies were generated from 10^5 human PCOS epithelial cells on Kunmingbai-MEF and ICR-MEF at the density of $7.5 \times 10^4/\text{cm}^2$. There were not statistical differences in the number of AP-positive colonies on Kunmingbai-MEF (62.67 ± 2.51) and ICR-MEF (57 ± 2.6) (The results are mean values of three

Figure 1. The generation of PCOS-special iPSCs from adult epithelial cells. (a) Timeline of virus-mediated reprogramming in adult epithelial cells. (b) The morphology of adult cells after virus transfection. (c) The typical PCOS-iPSC colonies after day 25. (d) PCOS-iPSCs displayed AKP-positive.

experiments.) (Fig. 2a). At the density of 2.5×10^4 , 5.0×10^4 and $7.5 \times 10^4/\text{cm}^2$ on ICR feeder cells, the AKP-positive colonies generated from 10^5 human PCOS adult cells were 48.33 ± 3.05 , 56.33 ± 2.51 and 62.67 ± 2.51 respectively. The AKP-positive colonies were generated with significantly higher efficiency on feeder cells at density of $7.5 \times 10^4/\text{cm}^2$ (Fig. 2b).

From seven diagnosed PCOS patients with familiar history, 12 PCOS-derived iPSCs cell lines were established and seven PCOS-derived iPSCs cell lines were come from the different patients (Table S1). At the same time, two no PCOS-derived iPSCs cell lines were established also as a control in our study.

The established PCOS-derived iPSCs have the similar characteristics of human ESCs

Human PCOS-specific iPSC clones cultured on feeder cells or feeder-free conditions exhibited morphology similar to that of human ES cells, characterized by large nuclei and scant cytoplasm, with distinct borders. PCOS-iPSC clones were positive for OCT4, NANOG, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, and negative for SSEA-1 by immunofluorescence (Fig. 3a). After reprogramming, the karyotype of PCOS-special iPSCs was normal (Fig. 3b). The results of RT-PCR indicated that the cells expressed special markers, such as *Oct-4*,

Figure 2. The impact of the source and density of feeder cells on PCOS-iPSC reprogramming. (a) The number of AKP-positive colonies on different source of feeder cells at the same density (mean $\pm$ SD, $n = 3$; $*P < 0.05$). (b) The number of AKP-positive colonies on different density of feeder cells at the same source (mean $\pm$ SD, $n = 3$; $*P < 0.05$; $**P < 0.01$).

Nanog, *Sox-2*, *TERF1*, *TERF2*, *REX*, *FGF4*, *Cripto*, *Thy1* and *LEFTY1* (Fig. 3c). We then assessed whether expression of the vector transgenes was silenced in derived iPSC clones. RT-PCR analysis was performed to detect OCT4-transgene-specific, KLF4-transgene-specific and c-MYC-transgene-specific transcripts. The presence of exogenous pluripotency genes was evident in most of the clones (data not shown). Morphology and expression of pluripotent genes indicated the establishment of PCOS-specific iPSCs from adult cells.

PCOS-derived iPSCs differentiate into all three germ layers in vitro and in vivo

To examine the differentiation potential, we suspended PCOS-derived iPSCs in low-attachment culture dishes for EB formation. The PCOS-derived iPSCs formed well-shaped EBs. Seven-day-old EBs were replaced on dishes and cultured in ES medium without BFGF. Various cell types were observed to have appeared in the outgrowth in 15 days. Immunocytochemistry analysis using antibodies against β -tubulin (ectoderm), smooth muscle antigen (mesoderm) and Alpha-feta protein (endoderm) showed differentiation to three germ layers (Fig. 4a–c). To test the differentiation potential of PCOS-derived iPSCs *in vivo*, three independent experiments were performed to each new established PCOS-derived iPSCs. PCOS-derived iPSCs were injected into SCID-beige mice for teratoma formation. Four weeks after injection, well-shaped teratomas were formed. Histochemical analysis indicated the existence of tissues from all three germ layers, such as pigment retinal epithelium (ectoderm), epidermal tissues (ectoderm), neural tube (ectoderm), cartilage (mesodermal), adipose tissue (mesoderm), intestines (endoderm) and muscle (mesoderm) (Fig. 4d–f). Our results showed that PCOS-derived iPSCs at either early (P8) or late (P25) passages could produce teratomas containing derivatives of three embryonic germ layers. There was no any discernible difference between these PCOS-derived iPSCs in the ability to form teratomas. These results indicate that established 12 PCOS-derived iPSCs and two non-PCOS-derived iPSCs had been reprogrammed into a pluripotent state and, like human ESCs, possessed the capacity for differentiation into three germ layers.

PCOS-derived iPSCs differentiate into adipocytes

Three PCOS-derived iPSCs and three non-PCOS-derived iPSCs were differentiated into mature adipocytes. On the day 5 of induction, the colonies became bulgy with irregular borderline (Fig. 5a). On the day 11, lipid droplets appeared in the cytoplasm and became more

Figure 3. PCOS-iPSCs showed the characteristics of human ESCs. (a) All colonies stained positive for OCT4, NANOG, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81. (b) G-banding of PCOS-iPSCs at passage 20 showed normal karyotype. (c) RT-PCR analysis of pluripotency-related genes and differentiation-related genes in PCOS-iPSCs, non-PCOS-iPSCs and human ESCs.

and more obvious (Fig. 5b). On day 20, scattered adipocytes could be seen in the visual fields and reached the maximum until the 27th day. Many lipid droplets were present in the cytoplasm, and were red by staining with Oil Red O (Fig. 5c). The differentiation ratio was calculated in PCOS-derived iPSCs and non-PCOS-derived iPSCs. The scanning was performed in 100 field images in at least three independent experiments. Our results suggested that the PCOS-iPSCs have higher efficiency and more strong potential of adipocyte development and differentiation (Fig. 5d).

Glucose consumption ability and insulin response ability of the adipocytes

In this section, three different iPSC lines from different PCOS patients were used for adipocyte differentiation. On the day 27 of induction, the glucose consumption of

adipocytes differentiated from non-PCOS- and PCOS-derived iPSCs was detected with glucose oxidase method, and the cell number was corrected with MTT method. Our results showed that (Table 1): (i) the glucose consumption of each non-PCOS-iPSC-derived adipocyte in the presence and absence of insulin were 16.23 ± 1.19 and 14.67 ± 0.39 mmol/l, respectively, with significant difference ($P = 0.023$), indicating more glucose consumption of non-PCOS-iPSC-derived adipocytes in the presence of insulin; (ii) the glucose consumption of PCOS-iPSC-derived adipocytes in the presence and absence of insulin were 16.26 ± 0.27 and 15.47 ± 0.20 mmol/l, respectively, with significant difference ($P = 0.0009$), indicating more glucose consumption of PCOS-iPSC-derived adipocytes in the presence of insulin; (iii) at the absence of insulin, there was a significant difference in glucose consumption per cell between non-PCOS-iPSC and PCOS-iPSC-derived

Figure 4. PCOS-derived iPSCs differentiate into all three germ layers *in vitro* and *in vivo*. (a) PCOS-derived iPSCs were induced to differentiate into fetal protein. (b) PCOS-derived iPSCs were induced to differentiate into smooth muscle. (c) PCOS-derived iPSCs were induced to differentiate into nerve cells. (d) Teratoma formation indicated the existence of tissues from ectoderm, such as epidermal tissues and neural tube. (e) Teratoma formation indicated the existence of tissues from mesodermal, such as adipose tissue and muscle. (f) Teratoma formation indicated the existence of tissues from endoderm, such as intestines.

adipocytes ($P = 0.0038$), indicating higher insulin response ability is in PCOS-iPSC-derived adipocytes at the absence of insulin; (iv) at the presence of insulin, there was no significant difference in glucose consumption each cell between non-PCOS-iPSC- and PCOS-iPSC-derived adipocytes ($P = 0.922$), indicating more glucose consumption ability in PCOS-iPSC-derived adipocytes. Results suggested that there were significant differences in glucose consumption ability between non-PCOS-iPSC and PCOS-iPSC-derived adipocytes. In a word, the PCOS-iPSCs have more glucose consumption ability during adipocyte differentiation and development.

Conclusions

The iPSCs were successfully generated from PCOS patients' epithelial cells. The PCOS-iPSCs can be induced to differentiate into adipocytes with normal karyotypes. This provide a new biological cell model and approach for studying pathogenesis of PCOS and discovering potential drugs for PCOS.

Discussion

In this paper, we established human PCOS disease-model iPSCs using a lentiviral vector carrying four

reprogramming factors (*Oct4*, *Klf4*, *C-myc* and *Sox2*) and demonstrated that PCOS-iPSCs can be induced to differentiate into adipocytes. Established PCOS-iPSCs possessed all the key features of human ESCs, such as the expression of pluripotency markers (*Oct4*, *Nanog* and *Sox2*), the long-term self-renewal and the capacity to differentiate into derivatives of all three germ layers. The PCOS-iPSCs have more glucose consumption ability during adipocyte differentiation and development.

In our study, we demonstrated successful iPSC derivation from PCOS patients. It is a prominent feature of this study. One main factor, the density of feeder cells can affect the generation of PCOS-iPSCs. When transfected PCOS epithelial cells were plated on low-density feeder cells ($2.5 \times 10^4/\text{cm}^2$), the efficiency of clone formation is lower than on high density ($7.5 \times 10^4/\text{cm}^2$). The PCOS-iPSCs can be generated efficiently on different feeder cell types, including ICR, Kunmingbai and human embryonic fibroblast cells.

iPSC technology enables the generation of patient-specific pluripotent stem cells that carry patient/disease-associated genotypes. Disease-specific iPSC have been produced from various diseases. These clones provide unique opportunities to study disease-specific pathogenesis *in vitro* and *in vivo*. But as anything has two sides,

Figure 5. PCOS-derived iPSCs differentiate into adipocytes. (a) On the day 5 of induction, the PCOS-derived iPSCs became bulgy with irregular borderline. (b) On the day 7, lipid droplets appeared in the cytoplasm and became more obvious. (c) Many lipid droplets were present in the cytoplasm, and were red by staining with Oil Red O. (d) The lipid droplet number per cell was quantified in PCOS-derived iPSCs, non-PCOS-derived iPSCs and H9 cells. (e) The differentiation ratio was calculated in PCOS-derived iPSCs, non-PCOS-derived iPSCs and H9 cells.

Table 1. Glucose consumption of PCOS-derived adipocytes

Group	Insulin	Sample	Glucose consumption	MTT (%)	Glucose consumption/cell
Non-PCOS-iPSCs	Present	5	20.24 ± 0.56	1.25 ± 0.06	16.23 ± 1.19
	Absent	5	18.22 ± 0.52	1.24 ± 0.05	14.67 ± 0.39
PCOS-iPSCs	Present	5	21.53 ± 0.46	1.32 ± 0.04	16.26 ± 0.27
	Absent	5	19.69 ± 0.56	1.27 ± 0.02	15.47 ± 0.20

different cell origin may contain different epigenetic marks that affect the differentiation potentials. These epigenetic marks may be, or not be the reason that PCOS patients have special clinical characteristics. This is what we should pay attention to when we use the cell model to make a research *in vitro*. The adipocyte plays an important role in PCOS patients. Obesity and PCOS often coexist and interact. Increased body fat may change insulin secretion and insulin response ability, and decrease the number of insulin receptors in adipose

tissue, liver and muscle tissue at the cellular level, leading to hyperinsulinism and insulin resistance (16). Hyperinsulinism may also cause hyperandrogenemia, which aggravates the disorder of reproductive function. In our study, PCOS-iPSCs can be induced to differentiate into adipocytes. The PCOS-iPSCs have more glucose consumption ability during adipocyte differentiation and development. These results showed the disorder of this cell type was associated with metabolic disease of PCOS. Some groups obtained the

adipose tissues from PCOS patients and control subjects to culture the primary adipocytes to explore the pathology of PCOS. But it is difficult to mimic dynamically the development of adipocyte. In this sense, iPSC from PCOS patients in our study would be useful for disease modelling, drug discovery and, eventually, autologous cell replacement therapies. We were the first to demonstrate successful iPSC derivation from PCOS patients. In addition, our study established a novel, systemic cell model about adipocyte metabolism for the studying of PCOS patients.

Integration of lentiviral vectors into the host genome inherently harbours the risk of increased tumorigenicity due to insertional mutagenesis (17,18). In addition, integration of reprogramming vectors could lead to sustained expression or reactivation of reprogramming factors, including proto-oncogene *c-MYC*. Expression of reprogramming factors could prevent proper differentiation of iPSC, while reactivation of *c-MYC* expression can increase the risk of tumorigenicity *in vivo* (19). In our study, lentiviral vectors were used for reprogramming iPSCs. PCOS-iPSCs with integration of reprogramming factors and with good differentiation potential to adipocyte were established. In our opinion, the quantity and the degree of integration of reprogramming factors is very important factor. Proper integration of reprogramming factor is beneficial to pluripotency status. However, more integration of reprogramming factor, more tumorigenicity. Notably, various reports have demonstrated the possible cellular reprogramming strategies without using integrating vectors, including adenoviral vectors, episomal vectors or introduction of reprogramming proteins, RNAs or miRNAs (20–26). It gives us a new idea to reprogramme patient cells without using integrating vectors, especially for future clinical applications. We have recently reprogrammed PCOS patient's somatic cells using proteins and generated genomic modification-free, disease-specific iPSC, and are currently in the process of analysing the differences between iPSC made with lentiviral vectors and proteins.

PCOS is a complex endocrine and metabolic disorder syndrome. At the cellular level, the disorder of adipocyte, insulin cells and germ cell differentiation were involved in it. In this article, we provided a new way to explore the pathophysiology of PCOS from cell development and differentiation. However, in the present study, we did not perform the differentiation, and development of insulin cells and germ cells from PCOS-iPSCs, which may also play an important role in the pathophysiology of PCOS. We believed that considerable work was still required. Moreover, we have started some exploring in the area.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1 The information about the PCOS patients included in this study.

Table S2 The primers of RT-PCR.