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# Reversine Promotes Porcine Muscle Derived Stem Cells (PMDSCs) Differentiation Into Female Germ-Like Cells

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## ABSTRACT

A small molecular chemical-Reversine has been shown to promote cell reprogramming and induce dedifferentiation of multiple terminally differentiated mesodermal origin cells, and then differentiate into other cell types within mesodermal lineages as well as neuroectodermal. However, the possibilities of these cells to give rise to germ cell lineages have not been examined. The objective of the current study was to detect the effect of Reversine on PMDSCs differentiation into germ cells. PMDSCs from fetal porcine skeletal muscle and their potential of differentiation into germ cells in vitro were investigated. The phenotype, proliferation potential, characteristic markers of the first adhesion cells (pp1), and the purified 2 times cells (pp3) were analyzed by growth curve, FACS, and RT-PCR, respectively. Then, the purified cells were induced with 10% or 20% bovine follicular fluid (FF), the results showed that some of the induced pp3 cells were similar as porcine oocyte, and expressed germ cell and oocyte markers analyzed by semi-quantitative RT-PCR and immunofluorescent staining. Reversine clearly increased the potentiality of PMDSCs differentiation into large round germ-like cells in FF induction medium analyzed by morpholgogy, QRT-PCR and immunofluoresce. The BrdU labeled PMDSCs might differentiate into female germ-like cells in recipient's kidney capsule, which were positive for germ cell and meiotic markers (Dazl, Vasa, Figla, Stra8, Scp3) and oocyte markers (Zp2, Zp3). These findings provided an efficient model to study the mechanism of cell proliferation and germ cell differentiation in livestock promoted by Reversine. J. Cell. Biochem. 113: 3629–3642, 2012. 

**KEY WORDS:** REVERSINE; MUSCLE-DERIVED SATELLITE CELLS (MDSCs); TRANSDIFFERENTIATION; CELL REPROGRAMMING; PORCINE

Germ cells are highly specialized cell populations that are indispensable for the continuation and evolution of each species. Human germ cell development is difficult to study because the important early events occur after embryo implantation in the womb [Nagano, 2007]. Recent reports showed that murine and human embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) can differentiate into primordial germ cells (PGCs), and subsequently develop into early stage gametes (oocytes and sperms) with the capacity to fertilization [Hübner et al., 2003; Toyooka et al., 2003; Geijsen et al., 2004; Lacham-kaplan et al., 2006; Hua and Sidhu, 2008, 2011; Panula et al., 2011].

Muscle derived satellite cells (MDSCs) lie outside the sarcolemma, within the basal lamina, and surround the mature functional cells of skeletal muscle, which is the multinucleated myofiber, are the progenitors of skeletal muscle [Lipton and Schultz, 1979], which give rise to myoblast cells that undergo multiple rounds of division before terminal differentiation. Their progeny-myoblasts, fuse to form new multinucleated myofibers [Cossu et al., 1980; Bischoff, 1994; Yablonka-Reuveni and Rivera, 1994; Cornelison and Wold, 1997]. Postnatal growth and regeneration of adult skeletal muscle is dependent on satellite cells [Mitchell et al., 2010]. Skeletal muscle development during embryogenesis and regeneration in adults

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requires a delicate balance between myogenic differentiation and self-renewal [Buckingham, 2006].

Chen et al. [2004] have screened a library of small heterocyclic compounds and identified a purine derivative-Reversine, which can promote the plasticity of lineage-committed progenitor cells of mouse myotubes (myoblasts, C2C12 cells). C2C12 myoblasts treated with Reversine can differentiate into myotubes, even differentiate into other mesodermal lineages, osteoblasts and adipocytes, respectively, when cultured with the appropriate medium [Chen et al., 2004]. Furthermore, Reversine has been shown to promote the reprogramming of primary murine and human fibroblasts into myogenic competent cells, which can be then converted into skeletal muscle both in vitro and in vivo [Anastasia et al., 2006]. More recently, it has been found that Reversine can increase the plasticity of C2C12 myoblasts at the single-cell level and is active in multiple cell types, including 3T3E1 osteoblasts and human primary skeletal myoblasts [Chen et al., 2007]. Reversine functions as a dual inhibitor of non-muscle myosin II heavy chain and MEK1, and both activities are required for the function of Reversine in dedifferentiation of C2C12 myoblasts. Inhibition of MEK1 and epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) results in accumulation of G2/M phase cells and a decrease in the global acetylation of histone H3 at lysine 9 [Chen et al., 2007]. In addition, inhibition of PI3K blocks the effect of Reversine on the dedifferentiation potential of C2C12 myoblasts toward other cell types of mesodermal lineages [Lee et al., 2009]. Although dedifferentiation of C2C12 myoblasts by Reversine and the subsequent redifferentiation toward other mesodermal lineages as well as neuroectodermal [Lee et al., 2009] have been shown, the potential of Reversine on dedifferentiation of germ cells has not been examined [Chen et al., 2007; Saraiya et al., 2010].

Over-expression of transcription factors may induce terminally differentiated cells reprogramming into induced pluripotent stem cells (iPSCs) [Takahashi and Yamanaka, 2006]. Follicular fluid (FF), hormone, cytokines and microenvironment, small molecules may transdifferentiate terminally differentiated cells reprogramming into induced cells [Uhlenhaut et al., 2009; Efe et al., 2011; Hua and Sidhu, 2011; Pang et al., 2011; Sekiya and Suzuki, 2011]. However, it is little known that at present whether follicular fluid is effective in the derivation of germ-like cells from satellite cells. Porcine is a convenient domestic species for biological investigation and application because of its diversity and valuable products, and also relative short gestation period. Porcine is viewed as the most promising model for human being because it has the similar metabolism, physiology as human being except for primate. In this study, we investigate to isolate and culture of PMDSCs, characterizated their potentiality. Further these cells were treated with FF and Reversine, and induced them differentiate into germ-like cells.

## MATERIALS AND METHODS

### ISOLATION AND CULTURE OF PMDSCs

Porcine primary muscle derived satellite cells were isolated from semitendi-nosus and semimembrinosus muscles of 2–3 month fetus (hybrid York boar). The dissected muscles were minced and incubated in 2 ml 1% collagenase I (Invitrogen, Carlsbad, CA) in DMEM (Invitrogen) and incubated for 30 min in a 37°C humidified atmosphere containing 5% $CO_2$ followed by repeated trituration using a 10 ml pipette and subsequent incubation for 90 min (37°C) until tissue fragments were homogenized. After triturating, tissue debris was further degraded in 0.25% (w/v) trypsin (Invitrogen) for 30 min at 37°C. Tissue fragments were further homogenized by repeated trituration using 10 ml pipettes, and were neutralized with DMEM/F12 containing 15% FBS, centrifuged, and washed two times with DMEM/F12, and the residues were resuspended in growth medium (DMEM/F12 [Invitrogen], 15% FBS [FBS, Hyclone, Logan, UT], 2 mM L-glutamine [Invitrogen], 1% nonessential amino acids [Invitrogen], 0.1 mM β-mercaptoethanol [Sigma–Aldrich Chemical Co., St. Louis, MO], 100 mg/ml penicillin/streptomycin) seeded into uncoated 3.5 cm-well plates. To remove fast-adhering fibroblasts (pp1) from the primary muscle stem cell cultures, the cells were plated into new plates after placed for 2 h in uncoated plates (37°C, 5% $CO_2$) sequentially for two times. After purified two times, the non-adhering purified primary muscle derived satellite cells (pp3, referred to as PMDSCs) were collected and seeded into tissue plates for experimental procedures.

### CELL CYCLE ANALYSIS

For cell cycle analysis, the single cells of pp1 and pp3 were suspended and fixed with 70% ice-cold ethanol for 30 min, and then centrifuged at 500 g for 10 min and washed in ice-cold PBS. The cell pellets were resuspended in 0.5 ml PBS containing 50 μg/ml propidium iodide (Sigma–Aldrich Chemical Co.) and 100 μg/ml RNase (Invitrogen), incubated at 37°C for 30 min, and then analyzed using a FACS can flow cytometer (Becton Dickinson, San Jose, CA) [Groschel and Bushman, 2005].

### CELL PROLIFERATION CRUVE

The cells firstly (pp1) adhered and purified (pp3) were respectively seeded into 24-well plates at a density of $5 \times 10^3$ cells/well. The proliferation ability of the cells was evaluated by cell number count with an interval of 24 h. The number of cells was determined for eight consecutive days (n = 3). Cell population doubling times (PDT) was calculated with the following formula: $PDT = [\log_2/(\log N_t - \log N_0)] \times t$, where $N_t$ = number of cells after t hours of culturing, $N_0$ = number of cells seeded (Cao et al., 2011).

### MULTIPOTENT DIFFERENTIATION OF PMDSCs IN VITRO

PMDSCs of the 3rd passage were trypsined and resuspended at density of $3 \times 10^5$ cells/ml in DMEM/F12 (Invitrogen), supplemented with 15% FBS (Hyclone), 0.1 mM 2-mercaptoethanol (Sigma–Aldrich Chemical Co.), 2 mM glutamine (Invitrogen) for 3 days. The cells aggregated and formed embryoid bodies (EBs). The EBs with diameter approximately 100 μm were transferred into a 48-well culture plate (5–10 EBs/well) containing the spontaneous differentiated medium, which was normal medium: DMEM/F12 (Invitrogen), supplemented with 15% FBS (Hyclone), 0.1 mM 2-mercaptoethanol (Sigma–Aldrich Chemical Co.), 2 mM glutamine (Invitrogen) [Hua et al., 2011], and cultured for 5–7 days, then changed medium every 2 days. The cultures were observed daily. The expression of three germ layers markers: Pdx1 (endoderm), Desmin and cardiac α-actin (mesoderm), FGF5 and Nestin (ectoderm), Klf4 and Sox2

(pluripotency) and PCNA (proliferation) was examined by RT-PCR. Immunofluorescent staining of Afp and Pdx1 (endoderm), cardiac α-actin, Desmin (mesoderm) and β-III-tubulin (ectoderm) were also performed for EB cultured for 7 days.

Some cultured PMDSCs were induced to differentiate into adipocytes based on previous study [Hua et al., 2011], which were evaluated by Oil-Red O staining. Myotube differentiation was cultured in DMEM/F12 supplemented with 5% horse serum (Hyclone) cultured for 5 days, 0.1 mM 2-mercaptoethanol (Sigma–Aldrich Chemical Co.), 2 mM glutamine (Gibco). Differentiation of neural-like cells was induced in the spontaneous differentiated medium supplemented with 0.1 μM retinoic acid (RA; Sigma–Aldrich Chemical Co.) for 7 days. The induced neural-like cells were identified by immunofluorescent staining of β-III-tubulin and Nestin.

### EFFECTS OF FOLLICULAR FLUID ON THE DIFFERENTIATION OF PMDSCs IN VITRO

The effects of follicular fluid on the differentiation of PMDSCs into germ cells were evaluated on the cultures with the presence of 10% and 20% FF. PMDSCs ($1 \times 10^3$/well, 48 well plate) were seeded, and cultured for 5–18 days, the formation of round large cells were used for evaluation of germ cell differentiation by a morphological assay and immunofluorescence staining with germ cell specific markers including Vasa (1:200, Abcam), Dazl (1:500, Abcam), premeiotic marker, Stra8 (1:500, Abcam), meiotic marker, Scp3 (1:300, Santa Cruz, CA) and oocyte marker ZP2 (1:100, Santa Cruz), ZP3 (1:100, Santa Cruz), pluripotency maker Oct4 (1:500, Chemicon, Temecula, CA) [Toyooka et al., 2003; Clark et al., 2004; Lacham-Kaplan et al., 2006; Hua et al., 2009]. The results presented in this study were from 9 differentiation and 5–7 fetuses were used.

### EFFECTS OF REVERSINE ON THE PROLIFERATION AND DIFFERENTIATION OF PMDSCs

PMDSCs were cultured in vitro in the normal, DMSO, and Reversine (1 μM) for 4 days and the number of cells was counted. This concentration (1 μM) appeared to be optimal in the range studied. Control cells were treated with the same volume of dimethylsulfoxide (DMSO) in which Reversine was dissolved. The effects of Reversine were based on the morphology of PMDSCs, BrdU incorporation assay and the expression of the related markers. The experiments were repeated for at least three times.

PMDSCs were seeded at a density of $5 \times 10^3$/well (35 mm plate) and treated with 1 μM Reversine (Sigma–Aldrich Chemical Co.) in DMEM/F12 for 48 h, and then cultured under 20% FF conditions for an additional 10 days in the absence of Reversine. The formation of round large female germ-like cells were used for evaluation by morphological assay, QRT-PCR and immunofluorescence staining with germ cell specific markers to analyze the effects of Reversine on the differentiation of PMDSCs into germ cells [Dyce et al., 2006; Danner et al., 2007; Linher et al., 2009; Hua et al., 2011].

### RT-PCR

RNA was extracted from $1.0 \times 10^6$ PMDSCs and induced cells using Trizol (TIANGEN BIOTECH Co. Ltd., Beijing, China) according to the manufacturer's instructions. The mRNA was reverse transcribed to cDNA using Advantage RT- PCR Kit (TaKaRa, Biotech. Co. Ltd., Dalian, China) based on the manufacturer's instructions. Semi-quantitative RT-PCR was carried out with 0.5 μl cDNA, 30 pM each of forward and reverse primers, two units Platinum Taq polymerase (Takara, Dalian, China) in a volume of 15 μl. The reaction was performed at 94°C for 2 min, followed by PCR amplification at 95°C for 30 s, 52–58°C for 30–45 s (annealing), and 72°C for 60 s (primer extension) for 30 cycles. The PCR mixture was incubated at 72°C for 10 min at the end of the temperature cycles. The PCR products were resolved on 2.0% (w/v) agarose gels containing 1 mg/ml ethidium bromide and the products were viewed and photographed under UV light. β-actin was used as an internal control. The primers used for RT-PCR are shown in Table I. All primers were designed to span exons to distinguish cDNA from genomic DNA products.

### QRT-PCR

The QRT-PCR reactions were set up in 15 μl reaction mixtures containing 7.3 μl 1× SYBR, 0.1 μl PremixExTaqTM (BOER, Biotech. Co. Ltd, Hangzhou, China), 0.3 μl sense primer, 0.3 μl antisense primer, 6.5 μl distilled water, and 0.5 μl template. The reaction conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for10 s, and 58°C for 20 s. The expression level of GDF9, Scp3, Dazl, Vasa, and Figla was tested for the effects of Reversine on the differentiation of PMDSCs into germ cells [Hua and Sidhu, 2008]. All data were normalized to β-actin in each well. The primers used for QRT-PCR are shown in Table I.

### WESTERN BLOTTING

Total cell extracts were prepared from PMDSCs in the normal, DMSO or Reversine cultured (1 μM, 4d), and proteins were extracted in 1 × SDS–PAGE sample loading buffer. Total cell proteins were resolved by SDS–PAGE, transferred to PVDF membrane, and probed with β-actin (1:1,000, Beyotime, Haimen, Jiangsu, China), PCNA (1:1,000, Millipore). Horse-radish peroxidaseconjugated anti-mouse IgG was used as a secondary antibody (1:1,000, Beyotime). The detection was performed using the BM-Chemiluminescence blotting substrate (Roche, Shanghai, China).

### BrdU INCORPORATION ASSAY

The proliferation of PMDSCs was assayed by BrdU incorporation, performed similarly to previous reports [Dyce et al., 2004], but with some modifications. First, PMDSCs in the normal, DMSO or Reversine cultured (1 μM, 4d) were treated with 30 μg/ml BrdU (Sigma, St. Louis, MO) for 6 h and then subjected to BrdU immunostaining. More specifically, cells were fixed in 4% PFA for 15 min at room temperature and washed three times, for 10 min each with PBS (pH 7.4) containing 0.1% Triton X-100. The cells were then washed for three times in PBS (pH 7.4) alone. Anti-BrdU (1:100; Santa Cruz) dissolved in 0.1 M PBS (pH 7.4) containing 5% normal goat serum was added and the cells were incubated overnight at 4°C. Cells were washed in PBS (pH 7.4) three times, and then incubated with the secondary antibody (FITC, Millipore 1:500) for 1 h at room temperature. Three more washes were carried out and cells were visualized under a Leica fluorescent microscope and analyzed for BrdU uptake. The rate of BrdU positive in the absence or presence of

TABLE I. The Primer Sequences

| Gene | Forward primer | Reverse primer | Annealing temp (°C) | Product size (bp) (°C) |
|---|---|---|---|---|
| β-actin | 5′-gcggcatccacgaaactac-3′ | 5′-tgatctccttctgcatcctgtc-3′ | 138 | 58 |
| Oct4 | 5′-gaagctggacaaggagaagct-3′ | 5′-catgctctccaggttgcctc-3′ | 247 | 58 |
| Nanog | 5′-gcgaatcttcaccaatg-3′ | 5′-tttctgccacctcttac-3′ | 407 | 54 |
| C-myc | 5′-ctggtgggcgagatcatca-3′ | 5′-cactgccatgaatgatgttcc3′ | 304 | 54 |
| Klf4 | 5′-cactgtctcatcaggagtca-3′ | 5′-cgacggtgcacgaggagaca-3′ | 525 | 55 |
| PCNA | 5′-agtggagaacttggaaatggaa-3′ | 5′-gagacagtggagtggcttttgt-3′ | 154 | 58 |
| Sox2 | 5′-ccaggagaaccccaagatgc-3′ | 5′-gggtgccctgctgctgcgagta-3′ | 518 | 58 |
| Desmin | 5′-accgcttcgccaactacat-3′ | 5′-tcactttcttaaggaacgcga-3′ | 378 | 58 |
| MyoD | 5′- cgtgcaagcgcaagacca-3′ | 5′- gatggcgttgcgcaggat -3′ | 164 | 62 |
| Myog | 5′-aggctacgagcggactgag-3′ | 5′-gcagggtgctcctcttcag-3′ | 226 | 55 |
| Pdx1 | 5′-gagcccgaggagaacaagc-3′ | 5′-tgacagccagctccaccc-3′ | 121 | 58 |
| α-actin | 5′-gctgtcttcccgtccat-3′ | 5′-tgtgagttacaccatcccc-3′ | 213 | 55 |
| Fgf5 | 5′-tacagagtgggcatcggtttc-3′ | 5′-cgcggtccctgttatttaac-3′ | 433 | 55 |
| Nestin | 5′-cctcacccttgcctgctac-3′ | 5′-gtcctggatttccttcctgtt-3′ | 539 | 58 |
| Zp1 | 5′-tgggaccagaagggaagc-3′ | 5′-cgtttgttcacatcccagtg-3′ | 162 | 60 |
| Zp2 | 5′-tcgacatgccgaactgcac-3′ | 5′-ggcagcactgttgtcatgactc-3′ | 131 | 56 |
| Zp3 | 5′-ggcatgtgacagaagaagca-3′ | 5′-agagtcagggacaccaccac-3′ | 151 | 58 |
| Vasa | 5′-agaaagtagtgatactcaaffaccaa-3′ | 5′-tgacagagattagcttcttcaaaagt-3′ | 199 | 58 |
| Scp3 | 5′-ctagaattgttcagagccagag-3′ | 5′-gttcaagttctttcttcaaag-3′ | 247 | 58 |
| Figla | 5′-gataaaaaatctcaaccgtgg-3′ | 5′-agtcgcacctttaaggatatc-3′ | 344 | 59 |
| Dazl | 5′-atgttaggatggatgaaactgagatta-3′ | 5′-ccatggaaatttatctgtfattctact-3′ | 178 | 59 |
| Gdf9 | 5′-tagtccacccacacacctga-3′ | 5′-ccagaagcctgagaaccaag-3′ | 197 | 57 |

Reversine was made by manual counting under fluorescent microscope [Mussmann et al., 2007].

### IMMUNOFLUORESENCE ANALYSIS

Cells were fixed in 4% PFA, treated with 0.1% Triton X-100 for 10 min at room temperature. After blocking with 10% FBS for 30 min, the cells were incubated with primary antibodies to Pdx1 (1:500, Chemicon), α-actin (1:500, Sigma–Aldrich Chemical Co.), Desmin (1:50, Zhongshan Biotech. Co. Ltd., Beijing, China), β-III-tubulin (1:500, Santa Cruz), Nestin (1:500, Santa Cruz), Afp (1:500, Chemicon), Scp3 (1:300, Santa Cruz), Zp3 (1:100, Santa Cruz), Dazl (1:500, Abcam, Cambridge, MA), Stra8 (1:500, Abcam), and Oct4 (1:500, Chemicon), respectively for overnight at 4°C, followed by fluorescein- or phycoerythrin-coupled goat anti-mouse or rabbit IgG secondary antibodies (1:500, Millipore, Billenca, MA, CA) for 30 min. Between incubations, slides and plates were washed with PBS for three times. The nuclei of cells were counterstained with Hoechst33342, then observed and analyzed with Leica microscope.

### OVARIAN TISSUE AGGREGATION AND KIDNEY CAPSULE TRANSPLANTATION

Adult mouse ovaries were collected and minced after the removal of adherent tissues. The specimens were digested with 1 mg/ml collagenase at 37°C for 15 min with shaking at intervals. After centrifuged at 1,500 rpm for 5 min, the specimens were incubated in a dissociation solution that consisted of a mixture of 0.2% (v/w) trypsin (Invitrogen) and 1.4 mg/ml DNase (Invitrogen) for 10 min at 37°C. The ovarian cell suspension from one female mouse ovary per graft was then mixed with $1 \times 10^7$ PMDSCs (cultured 10 days in 20% FF condition and treated with 30 μg/ml BrdU for 2 days). Cell suspensions were pelleted into grafts and transplanted under the left kidney capsules of busulfan-treated female Kunming mice [Nicholas et al., 2009; Hu et al., 2012].

### ESTRADIOL MEASUREMENTS

The medium was pooled from culture dishes and stored at −80°C for hormone analysis. Estradiol (E2) was assayed using an Estradiol Correlate-enzyme immunoassay kit according to the manufacture's protocol [Hillier et al., 1994].

### STATISTICAL ANALYSIS

The effects of Reversine on the differentiation of PMDSCs were evaluated based on fluorescence staining and QRT-PCR. The data are presented as mean ± SEM (the standard errors of the mean) from three independent experiments, three replicates were evaluated for each experiment. Statistical comparisons were assessed with analysis of Student's test. $P < 0.05$ was considered as a statistic significant difference, and $P < 0.01$ was considered highly significant difference.

## RESULTS

### CHARACTERIZATION OF PMDSCs

The primary PMDSCs were isolated from fetal porcine skeletal muscle. The PMDSCs can be purified through repeated differential adhesion, using a culture condition described in this report, and most of the cells were of mononuclear cells [Rebelatto et al., 2008]. Compared the first adhesion cells (pp1) with the purified two times cells (pp3), the pp1 cells exhibited as spindle-shaped fibroblasts (Fig. 1A,B), while pp3 presented as typical spindle-shaped fibroblasts or triangle-shaped in their phenotypes (Fig. 1C). The purified PMDSCs were capable of growing up to a 60–80% confluence in 5 days (Fig. 1D). The cell cycle analyzed by FACS showed that there were 13.6% S-stage cells in the pp1, whilst 28.8% in the pp3, confirmed that the PMDSCs had a stronger proliferative capacity than pp1(Fig. 1E). Immunophenotype of cultured PMDSCs derived from fetal porcine skeletal muscle were characterizated by flow cytometry revealed PMDSCs were similar as mesenchmyal stem cells (MSCs) [Rebelatto et al., 2008; Lv et al., 2011].

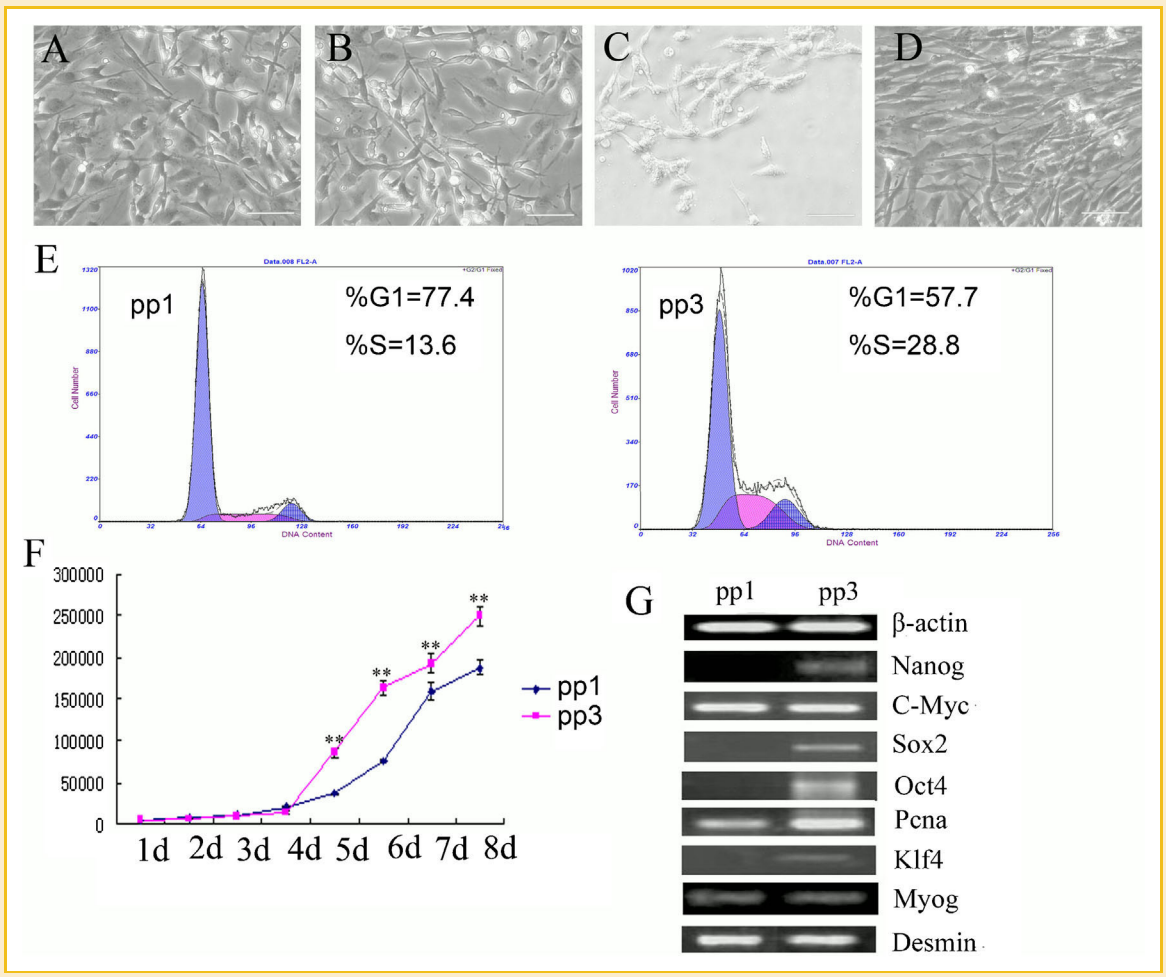


Fig. 1. Characteristics of PMDSCs. A: The first adhesion cells (pp1) at 2 days after culture, the cells exhibited as spindle-shaped fibroblasts; B: The second adhesion cells (pp2) at 2 days after culture; C: The third adhesion cells as well as the purified cells (pp3) at 2 days after culture, the pp3 cells exhibited as typical spindle-shaped fibroblasts or triangle-shaped in their phenotype; D: The purified cells (pp3) growing up to a 60–80% confluence at 5 days, scale bar = 100 μm; E: The cell cycle analysed by FACS showed that there were 13.6% S-stage cells in pp1, whilst 28.8% in pp3; F: The growth curves of pp1 and pp3, $^{**}P < 0.01$; G: The pp1 expressed C-myc, PCNA, Desmin, Myog, while the pp3 expressed both these muscle markers and pluripotent markers including Nanog, Sox2, Oct4, and Klf4 analyzed by RT-PCR.

The cell growth curve assay showed that pp1 multiplied nearly 38 folds in 8 days, while pp3 showed a stronger proliferative capacity than pp1, which multiplied nearly 50 folds in 8 days. The mean population doubling time (PDT) in pp1 and pp3 was 41.06 h versus 36.95 h, respectively (Fig. 1F). The pp1 proliferated for four passages whilst the PMDSCs (pp3) proliferated up to 10 passages and maintained typical phenotypes. RT-PCR showed that pp1 expressed C-myc, PCNA, Desmin, Myog, while the PMDSCs (pp3) expressed both these markers and pluripotent markers: Nanog, Sox2, Oct4, and Klf4 (Fig. 1G).

## MULTIPOTENT DIFFERENTIATION OF PMDSCs

To test for spontaneous differentiation of the PMDSCs, 1st- to 3rd-passage cells were cultured using a modified method [Zhang et al., 2011]. The cells were plated at a density of $3 \times 10^5$ cells/ml in a suspended dish, the cells aggregated and formed embryoid bodies (EBs) in suspension culture in bacteriological dishes (Fig. 2A). The expression of Afp and Pdx1 (endoderm), cardiac α-actin, Desmin (mesoderm), and β-III-tubulin (ectoderm) were detected by immunofluorescent staining on 7 day EBs (Fig. 2A). In addition, RT-PCR analysis also supported the expression of PDX1 (endoderm), Desmin and cardiac α-actin (mesoderm), FGF5 and Nestin (ectoderm), Klf4, Sox2 and C-Myc (pluripotency) and PCNA (proliferation) in the EBs on Day 7 of cultures (Fig. 2B). Under 5% horse serum conditioned medium cultured 5 days, the treated PMDSCs exhibited typical myotuble and showed multinuclear cells, whilst no myotuble-like cells were observed in without horse serum group (Fig. 2C). The cultured PMDSCs can differentiate into neuron-like cells that were positive for β-III-tubulin and Nestin by immunofluorescent staining (Fig. 2D) and differentiated into cells positive for Oil Red-O staining (adipocyte-like cells; Fig. 2E).

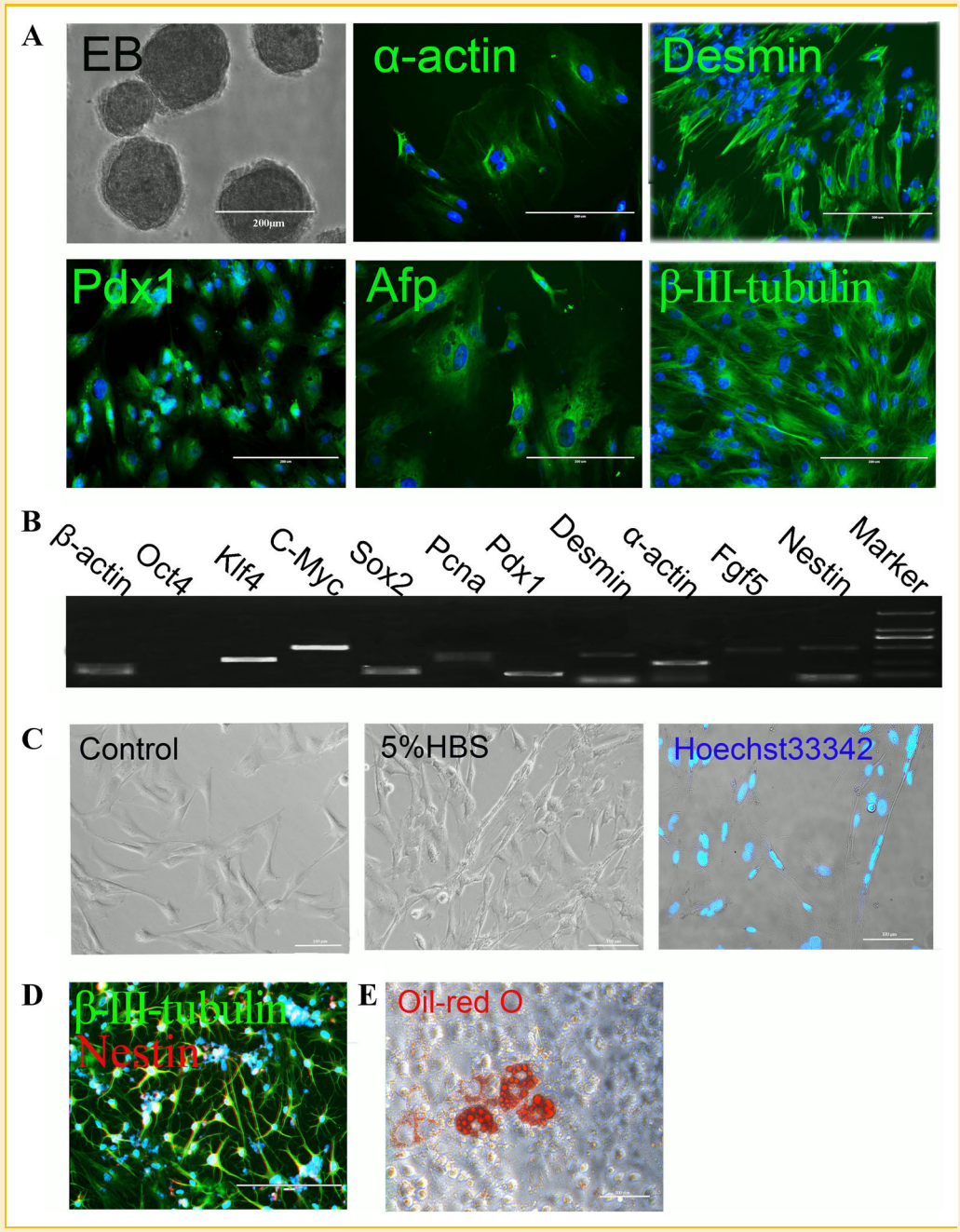


Fig. 2. PMDSCs spontaneously differentiated into three germ layer types. A: Afp (endoderm), Pdx1 (endoderm), cardiac α-actin (mesoderm), Desmin (mesoderm), and β-III tubulin (ectoderm) positive cells analysed by immunofluoresce staining, scale bar = 200 μm; B: The expression of Pdx1 (endoderm), Desmin and cardiacα-actin (mesoderm), Fgf5 and Nestin (ectoderm), Klf4, Sox2 and C-Myc (pluripotency), and Pcna (proliferation) was detected by RT-PCR; C: Under 5% horse serum conditioned medium cultured 5 days, PMDSCs differentiated into muscle tuble (right), control (left), and showed multinuclear cells by hoechst33342 staining, scale bar = 100 μm; D,E: PMDSCs differentiated into cells positive for β-III-tubulin and Nestin (neural-like cells) and oil-red staining (adipocyte-like cells), (D) scale bar = 200 μm, (E) scale bar = 100 μm.

### PMDSCs DIFFERENTIATION INTO FEMALE GERM-LIKE CELLS IN VITRO

To investigate the potentiality of PMDSCs differentiation into germ cells in the presence of FF, PMDSCs were cultured in the differentiation medium with different concentration of FF between 10% and 20%. The PMDSCs firstly grew and proliferated as a monolayer in the culture dish. A dose dependent FF induced round cells and the formation of germ-like cells, and the morphologically distinct round cells (about 20 μm) appeared in 20% FF medium after culture in vitro for 5 days (Fig. 3C). On day 8, a population of "shiny" round cells formed, growing either as single cells or clusters (Fig. 3E). The clusters grew larger on Day 13 (Fig. 3F,G). A

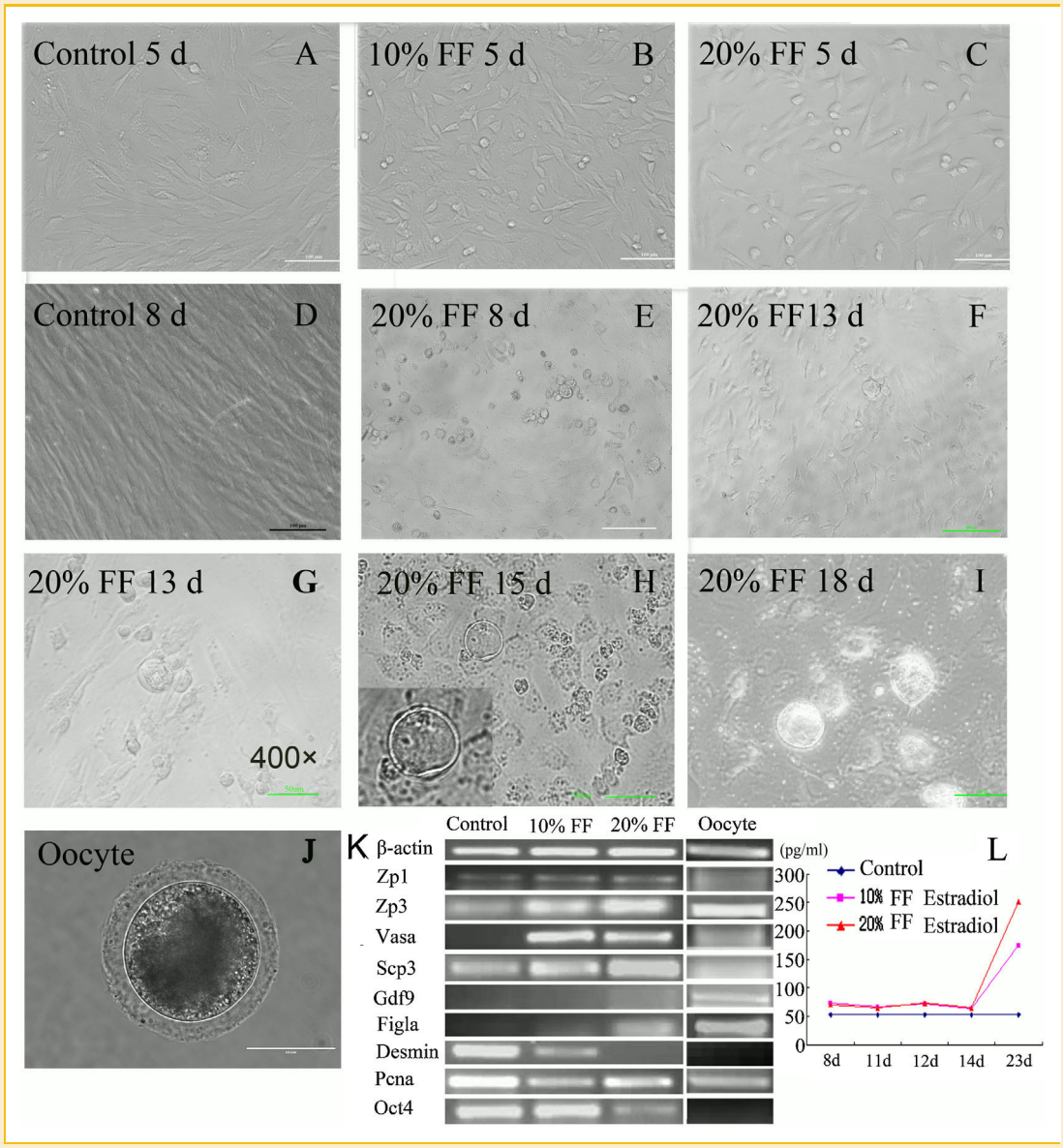


Fig. 3. The potentiality of PMDSCs differentiation into germ cells. A–C: PMDSCs cultured on 5 day, (A) control, (B) cultured in 10% FF, (C) cultured in 20% FF, the round cells were about 20 μm, scale bar = 100 μm; D–E: PMDSCs cultured on 8 day, (D) the cells growing up to a 90% confluence as typical spindle-shaped fibroblasts in the absence of FF, (E) a population of "shiny" round cells formed, growing either as a single cell or in clusters in 20% FF, scale bar = 100 μm; F,G: The round cells grew on 13 day in 20% FF, (F) scale bar = 100 μm, (G) scale bar = 50 μm; H: On 15 day, larger round cells were 30–40 μm, similar as oocyte-like cells, scale bar = 50 μm, the magnification of the large round cells in the lower left corner; I: On 18 day, large oocyte-like cells grew up to 40–60 μm, scale bar = 50 μm; J: The porcine ovary was used as positive control, scale bar = 50 μm; K: The expression of germ cell and oocyte markers was increased in 20% FF induced cultured compared with that in the absence of FF normal medium and in the presence of 10% FF; L: The estradiol measurements in FF induced PMDSCs. The level of estradiol in 20% FF cultured was higher than that of 10% FF and control.

subpopulation of the round cells gradually detached and grew as a separate non-adherent population of single and clustered cells. The morphology is similar as the typically oval or round shape, with large nucleus, which are known to be larger in size compared with somatic cells on Day 15 (Fig. 3H) and resemble porcine PGCs dissociated from gonadal ridges [Shim and Anderson, 1998] or murine PGCs [De Felici and McLaren, 1982]. The large cells grew with time to reach a diameter of 40–60 μm on Day 18 (Fig. 3I). And a few of them formed oocyte-like structures. The frequency of large round cell production was varied by cultures, the batches of follicular fluid and FBS. In contrast, the control cells exhibited typical spindle-shaped fibroblasts in the absence of FF (Fig. 3A,D). The results of semi-quantitative RT-PCR and immunofluorescent staining demonstrated that the expression of the germ cell specific markers including Figla, Vasa, Gdf9; meiotic marker Scp3; and oocyte marker ZP1, ZP3, were elevated in FF-treated cultures in comparison with the untreated group, whilst mesoderm maker Desmin, pluripotency maker Oct4 and proliferation marker PCNA, were declined in FF-treated cultures (20%) compared with the untreated group and 10% FF-treated cultures (Fig. 3K). These results

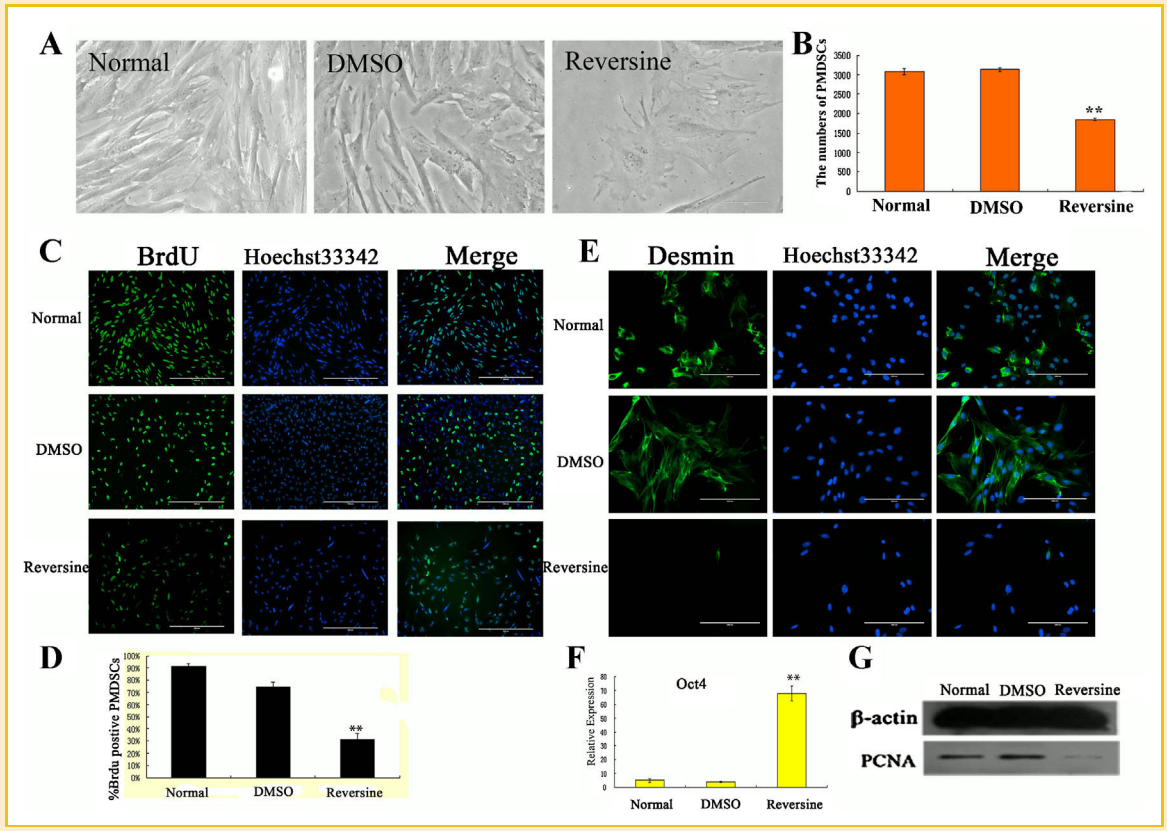


Fig. 4. The effect of Reversine on PMDSCs. A: The PMDSCs were cultured for 4 days, the morphology of the Reversine treated cells became larger than the other two groups, scale bar = 100 μm; B: The number of the PMDSCs; C: BrdU incorporation assay demonstrated that the mitiosis index with Reversine was significantly lower than that without Reversine medium, scale bar = 200 μm; D: The percentage of positive cells treated with Reversine, DMSO and normal from C; E: Immunofluorescene staining analysis showed PMDSCs were positive for Desmin and the percentage of positive cells was decreased in Reversine, scale bar = 200 μm; F: QRT-PCR analysis showed that the expression of pluripotency marker Oct4 had an up-regulated trend in Reversine treated cells; G: Western blotting analysis showed the expression of PCNA had a downward trend in Reversine treated compared with the other group cells. $^{*}P < 0.05$, $^{**}P < 0.01$.

strongly indicated that a small fraction of PMDSCs were able to differentiate into female germ-like cells in the presence of a higher concentration of FF. The estradiol measurements showed that the level of estradiol in 20% FF cultured was higher than that in the presence of 10% FF and the untreated group (Fig. 3L).

### EFFECTS OF REVERSINE ON PMDSCs

PMDSCs were grown for 4 days in the presence of 1 μM Reversine in growth medium as reported by Ding's group for C2C12 cells [Chen et al., 2004]. After 4 days of Reversine treatment, PMDSCs acquired a drastically different morphology compared with normal and DMSO treated-cells (Fig. 4A), appearing considerably larger in size, flatter, less contrasted, and more adhesive to the culture plate. No clear cell death was observed, although growth inhibition was noticed, as confirmed by cell count (Fig. 4B), 56% proliferation reduction compared to normal cells, observed by BrdU incorporation assay (Fig. 4C,D). Moreover, immunofluorescent staining demonstrated that the expression of skeletal muscle marker (Desmin) was decreased compared with the normal and DMSO cells, whilst, the expression of Oct4 was increased up to seven times by QRT-PCR analysis (Fig. 4F). And the expression of PCNA was markedly dropped by Western blotting analysis (Fig. 4G), implying that the pluripotency of cells was increased, whilst the proliferation was reduced after treatment by Reversine.

### REVERSINE PROMOTED PMDSCs DIFFERENTIATION INTO FEMALE GERM-LIKE CELLS

To characterize the multipotency of the Reversine-treated cells, PMDSCs at 2nd passage were treated with 1 μM Reversine for 48 h, and we found that the Reversine treated cells grew larger in morphology about two times than the other two groups, and the number of Reversine treated cells were fewer than others (Fig. 5A–C). Then, the cells were cultured in 20% FF conditions for an additional 10 days in the absence of Reversine (Fig. 5D–N). At 3–10 days, there appeared large round or oval female germ-like cells. The number of germ-like cells appeared in Reversine-treated group was significantly larger than DMSO treated or normal group under the same induction conditions (Fig. 5O), and the expression of Zp3 and Stra8 were markedly enhanced by Western blotting analysis (Fig. 5P). QRT-PCR analysis showed that the expression level of Gdf9, Dazl, Vasa, Scp3, Figla, and Zp2 was increased significantly in Reversine cultures compared with DMSO and normal (Fig. 6). Furthermore, these large round female germ-like cells expressed the germ cell specific markers including Vasa, Dazl; premeiotic marker:

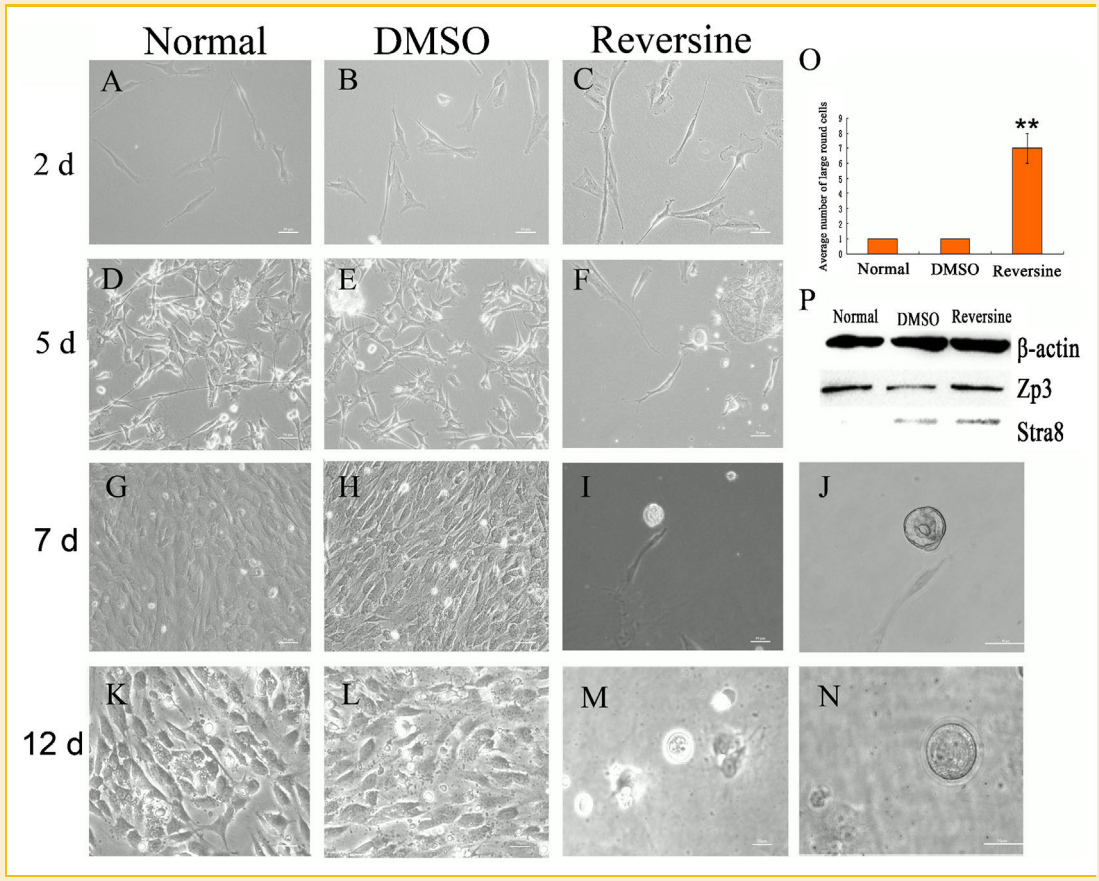


Fig. 5. Reversine promoted PMDSCs differentiation into female germ-like cells. A–C: PMDSCs were cultured for 48 h, (A) normal cultured, (B) DMSO treated, (C) 1 μM Reversine treated for 48 h, cells were larger than the other two groups; D–N: PMDSCs were cultured in 20% FF medium for an additional 10 days in the absence of Reversine, (D–F) on 3 day, the number of cells in Reversine treated was fewer than that in the other groups; G–J: On 5 day, round cells exhibited in Reversine treated group, whilst rare round cells appeared in the other two groups; K–N: Large round cells showed on 10 day, which were about 40–60 μm, few round cells were formed in the other two groups, scale bar = 50 μm; O: The number of large female germ-like round cells was formed in the Reversine treated PMDSCs; P: Western blotting analysis showed the expression of Zp3 and Stra8 had been increased in Reversine treated compared with the normal and DMSO treated cells. $^{**}P < 0.01$.

Stra8, meiotic marker: Scp3, and oocyte marker: Zp2, Zp3; whilst no Oct4 expression in these induced cells was based on immunofluorescent staining (Fig. 7A). The expression pattern of the induced female germ-like cells was similar as porcine oocyte (Fig. 7B). However, the uninduced PMDSCs were negative for germ cell marker: Vasa, Dazl, premeiotic marker-Stra8, and Oocyte marker-Zp2 analyzed by immunofluorescence (Supplemented Fig. 1).

### DIFFERENTIATION OF PMDSCS FOLLOWING THE CO-TRANSPLANTATION OF MOUSE OVARIAN CELLS AND PMDSCs

We transplanted the co-aggregates of both mouse ovarian cells and PMDSCs under the kidney capsule of recipient mice for 4 weeks. Two out of eight grafts were detected (Fig. 8A), and we found that the ovary of the transplanted side was recovered compared with the other side ovary. The weight of the transplanted side ovary was 5.0 mg, while the other side was 3.8 mg (Fig. 8B). HE staining analysis presented primary follicular formed in the transplanted side and atretic follicles were seen in the other side. Furthermore, garland-like structurs were seen in the grafts (Fig. 8B).

We observed BrdU postive cells in the grafts and these cells simultaneously expressed germ cell and meiotic markers including Stra8, Dazl, Vasa, and Scp3. Meanwhile, some cells of the co-aggregates were positive for germ cell and oocyte markers: Figla, Zp2, and Zp3 (Fig. 8D).

## DISCUSSION

PMDSCs have been studied and successfully isolated from the sarcolemma of the myofiber and basal lamina. These "satellite" cells were fibroblast-like and triangle-shaped in morphology, self-renewal, and capable of differentiation into the connective tissue lineages of the mesoderm including bone and adipocytes [Pittenger et al., 1999; Lee et al., 2009]. Our results showed that an adherent subpopulation of fibroblast-like and triangle-shaped satellite cells isolated and purified from porcine skeletal muscle by simple differential adhesion method. These purified homogeneous porcine PMDSCs exhibited typical MDSC phenotypes determined by the morphology, expression of MDSC markers analyzed by RT-PCR,

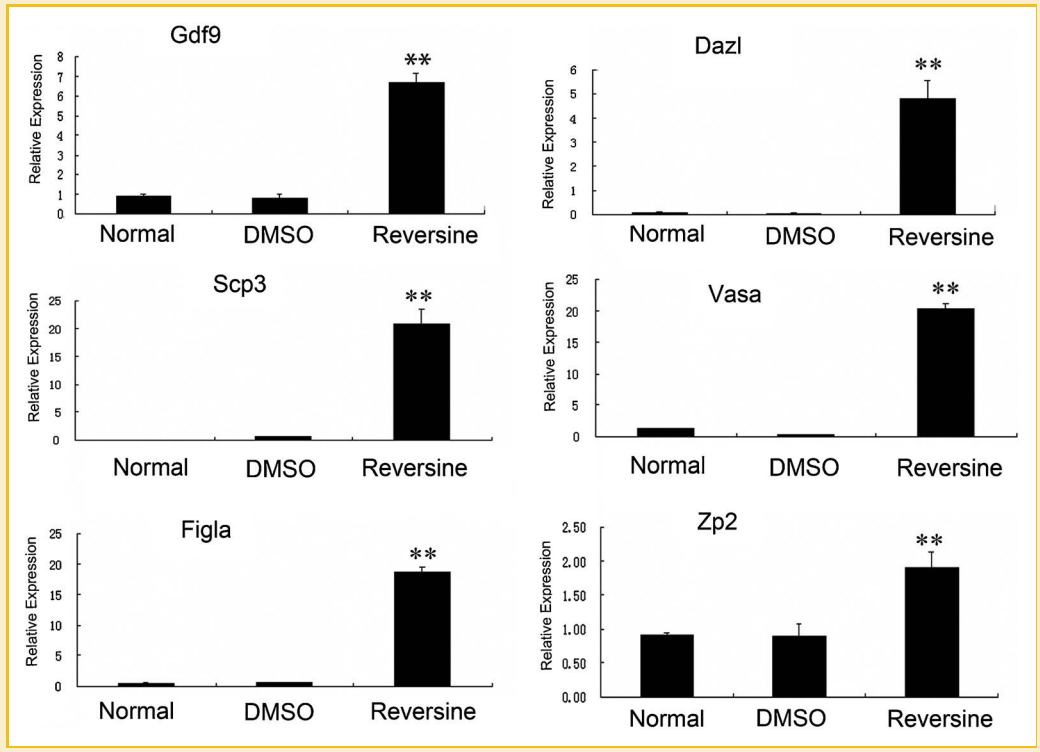


Fig. 6. The effects of Reversine on the differentiation of PMDSCs analyzed by QRT-PCR. The expression of Gdf9, Dazl, Vasa, Scp3, Figla, and Zp2 were increased clearly in Reversine treated compared with that in DMSO and normal groups, $^{*}P < 0.05$, $^{**}P < 0.01$.

histochemical, cytochemical, and immunocytochemical staining [Lee et al., 2004; Hua et al., 2011]. Indeed, the purified PMDSCs (pp3) showed a stronger proliferative capacity than the cells that adhered to dish at the first and second time (pp1 and pp2). Importantly, the expression of pluripotent markers of ESCs and induced pluripotent stem cells, Oct4, Sox2, C-myc, Nanog, and Pcna [Baharvand et al., 2010] were detected in the undifferentiated purified PMDSCs. Additionally, these cells were capable of being differentiated into cell types expressing differentiated markers of Pdx1, Afp (endoderm) and cardiac α-actin, Desmin (mesoderm) and Fgf5, β-III-tubulin (ectoderm). These data clearly suggested that the cultured PMDSCs were multipotent [Hua et al., 2009, 2011], with the potentiality to differentiate into multiple lineages including the mesoderm, ectoderm, and endoderm [Rebelatto et al., 2008].

While it has been shown that PGCs can be differentiated from ESCs in vitro, as well as somatic-derived stem cells [Toyooka et al., 2003; Clark et al., 2004; Geijsen et al., 2004; Lacham-kaplan et al., 2006; Hua and Sidhu, 2008, 2011; Linher et al., 2009]. The current study is the first report to generate PGC-like and oocyte-like cells from mesoderm muscle derived satellite cells. Porcine is an important model for both biomedical and agricultural research. The generation of oocytes from somatic stem cells in vitro may provide a valuable model for identifying factors involved in germ cell formation and oocyte differentiation [Linher et al., 2009].

Follicular fluid is the fluid surrounding the ovum and granulosa cells in the Graafian follicle (ovarian follicle), which contains sex steroids, glycoprotein hormones, proteins, cytokines, mucopolysaccharides, and enzymes. The levels of follicular fluid hormones (FSH, $E_2$, and P), GDF9 and BMP15 may affect the oocyte maturation, and embryo quality [Gode et al., 2011]. The level of GDF9 in the follicular fluid have been suggested to significantly correlate with oocyte nuclear maturation and embryo quality [Gode et al., 2011]. The proteins and cytokines (EGF; insulin-like growth factor, IGF) contained in the FF affected the fate of oocytes and embryos [Dyce et al., 2006; Nayernia et al., 2006].

Previous reports have shown that stem cells may provide a unlimited new potential resource for male and female germ cells that could be used for infertility and sterility [Clark et al., 2004; Lacham-kaplan et al., 2006; Hua and Sidhu, 2008, 2011; Hua et al., 2009; Linher et al., 2009]. However, the application was hurdled by the undefined, complicated, and inefficient methods of induction of germ-like cells derivation of stem cells [Hua and Sidhu, 2008; Hua et al., 2009]. In this study, a small subset of PMDSCs were able to develop into PGCs and oocyte-like cells, which were large round cell masses and positive for specific germ cell markers Scp3 and Vasa. The expression levels of ZP1, ZP3, SCP3, and GDF9 were elevated in the PMDSCs treated with high concentration of FF as compared with that in the low concentration of FF and the controls. These markers were specifically expressed in PGCs and oocytes [Clark et al., 2004; Dyce et al., 2006; Hua et al., 2011; Hua and Sidhu, 2011]. Our results revealed that FF promoted the PMDSCs differentiation into germ-like cells. This suggested that an appropriate concentration of proteins and cytokines in FF may alter the differentiation potentiality of PMDSCs in vitro.

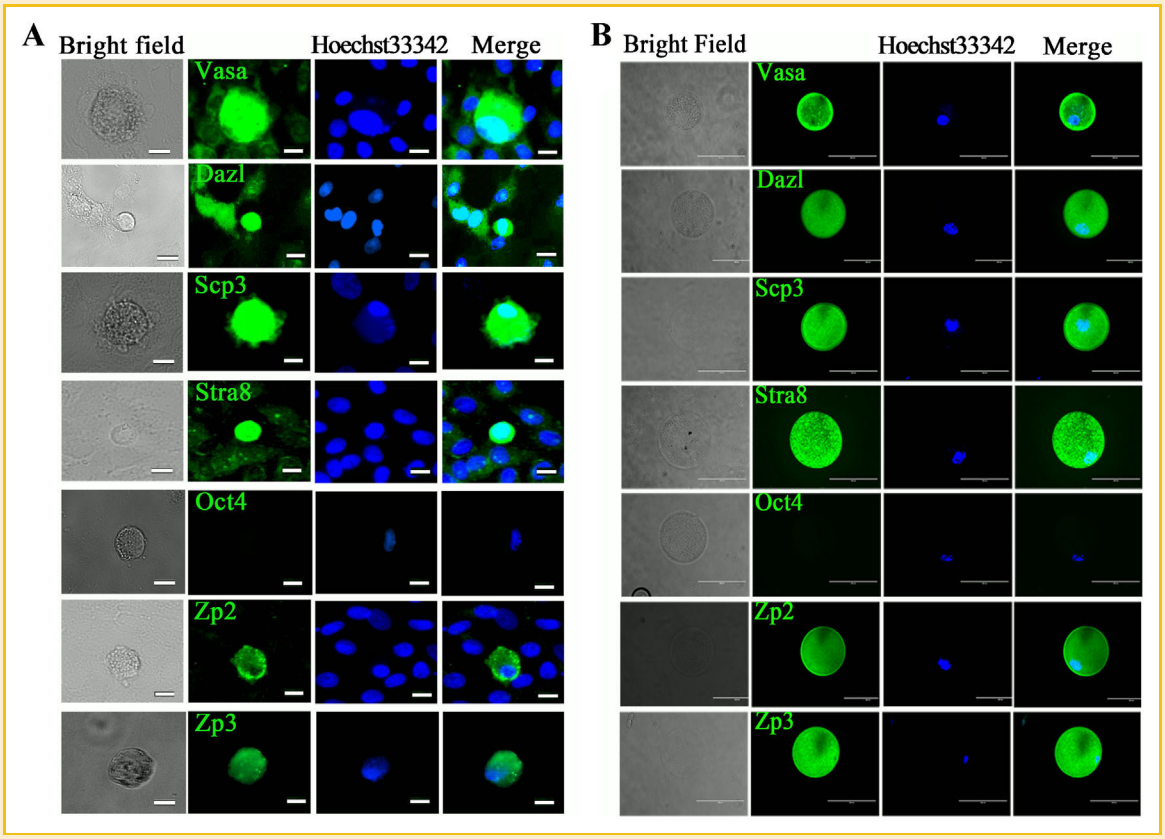


Fig. 7. Oocyte-like cells were formed in 20% FF treated PMDSCs. A: The FF treated large round oocyte-like cells were positive for Vasa, Dazl, Scp3, Stra8, Zp2, and ZP3, negative for Oct4. The nucleus of cells were stained by Hoechst33342, scale bar = 30 μm; B: The porcine oocyte was used as positive control for oocyte markers, immunofluorescence staining showed that porcine oocyte was positive for Vasa, Dazl, Scp3, Stra8, Zp2, and Zp3, and negative for Oct4, scale bar = 200 μm.

The germ cell differentiation potentiality in FF treated PMDSCs was consistent with the previous observations in mouse and human ESCs, MSCs and skin stem cells [Dyce et al., 2006; Danner et al., 2007; Cao et al., 2011, 2012; Hua and Sidhu, 2011; Hua et al., 2011; Qiu et al., 2012]. These results indicated that at least part of PMDSCs were transdifferentiated into germ cells in vitro in the presence of a high concentration of FF, which was consistent with the findings that culture media was able to determine the fate of stem cells [Danner et al., 2007; Roobrouck et al., 2011; Cao et al., 2011, 2012]. These results first demonstrated that the higher concentration of FF affects on the fate of PMDSCs and stimulated them differentiate into oocyte-like cells. However, which factors in FF control the fate of PMDSCs remain unclear [Maniwa et al., 2005].

Reversine, a purine derivative, or a potent Aurora kinase inhibitor was able to increase the plasticity of C2C12 myoblasts [Amabile et al., 2009]. This compound interferes with the normal differentiation pathway to promote C2C12 cells reprogramme into a pluripotent state, and even dedifferentiate human fibroblasts into skeletal muscle cells in vitro and in vivo [Anastasia et al., 2006]. Previous studies showed that Reversine is also capable of reprogramming primary somatic cells into a state of increased plasticity [Anastasia et al., 2006]. In our study, we firstly discovered that Reversine was also promoting PMDSCs differentiating into large round female germ-like cells. The expression of Gdf9, Dazl, Vasa, Scp3, Figla, and Zp2 was increased clearly in Reversine treated compared with that in DMSO and Normal groups analyzed by QRT-PCR. Furthermore, these female germ-like cells expressed the germ cell specific markers including Vasa and Dazl; premeiotic marker: Stra8, meiotic marker: Scp3; and oocyte marker Zp2 and ZP3; whilst no Oct4 expression was detected based on immunofluorescent staining. However, the efficiency of female germ-like cells formation was needed to be improved, and the mechanism lies in it needed to be elucidated further [Dyce et al., 2006; Martin-Rendon et al., 2008; Panula et al., 2011]. The transplanted assay demonstrated that BrdU postive cells might differentiate into female germ-like cells in recipient's kidney capsule and also stimulate the recovery of the transplanted side ovary. These results showed that PDMSCs had similar effects as ESCs, bone marrow cells to influence ovarian physiology, and improve the fertility of mice previously treated with chemotherapeutic agents [Nicholas et al., 2009; Hu et al., 2012; Santiquet et al., 2012].

In summary, this study first demonstrated the isolation and characterization of purified multipotent PMDSCs from the porcine skeletal muscle. These cells were capable of differentiating into female germ-like cells in the presence of 20% FF, and Reversine can promote the PMDSCs differentiation into female germ-like

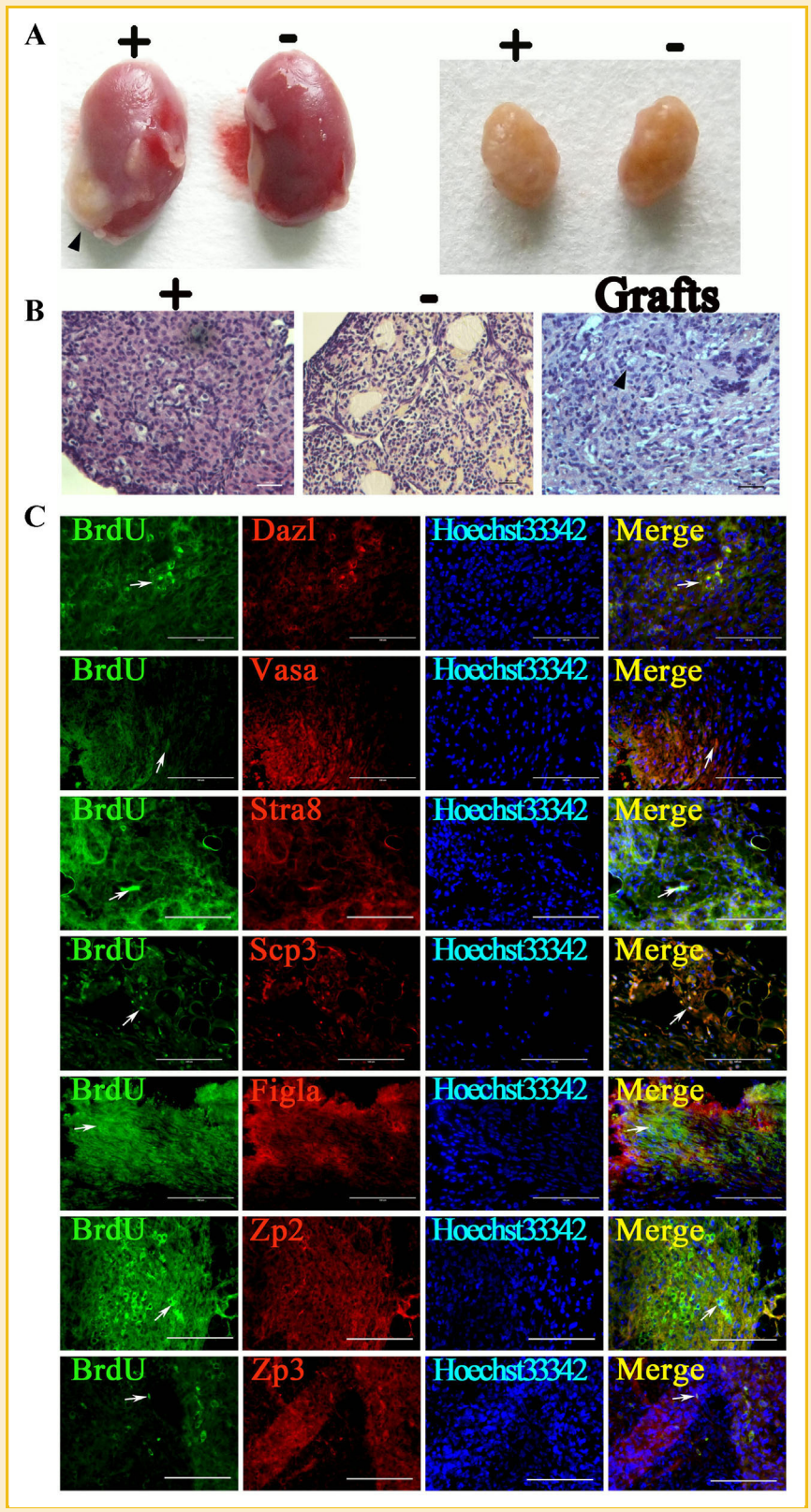


Fig. 8. The PMDSCs grafts in kidney capsule of recipient mice recovered the transplanted side ovary and differentiated into female germ-like cells in an ovarian niche. A: The PMDSCs graft in kidney capsule of recipient mice for 4 weeks, "+" represented the transplanted side, while "−" was un-transplanted side, the transplanted side ovary recovered and larger than the other side, the arrow marked the grafts; B: HE staining analysis presented primary follicular formed in "+" and atretic follicles were seen in"−", and the arrow marked the garland-like structure in the grafts; C: BrdU positive cells were observed in transplanted grafts and these cells simultaneously expressed germ cell markers which were positive for germ cell and oocyte markers. The nucleus was stained with Hoechst33342 (blue), scale bar = 100μm.

cells effectively. Thus, the PMDSCs may be a new resource of stem cells to study germ cell differentiation and cell reprogramming in porcine.

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