Effect of pregnancy on the proliferation of rat adipose-derived stem cells

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ABSTRACT. Stem cell therapy faces many problems including poor survival rates and low viability. Enhancing the biological functions of stem cells improves efficacy of therapies. Estrogen, whose levels are elevated during pregnancy, affects the properties of bone marrow mesenchymal stem cells. Evidence suggests that adipose-derived stem cells (ADSCs), which are a type of adult mesenchymal stem cells, can be used in regenerative medicine. In fact, ADSCs from pregnant animals have been used in clinical therapies. However, the effect of the donor's reproductive status on proliferation of ADSCs is unknown. We investigated the effect of 17β-estradiol (E2) and progesterone (P) on the *in vitro* proliferation of ADSCs from laboratory rats. ADSCs were obtained from five different groups of 15 rats each - non-pregnant, pregnant, in perinatal period, non-pregnant and treated with E2, and non-pregnant and treated with P. Adhesion and viability of ADSCs were determined by MTT assay, and cell cycle was followed by flow

cytometry. The proliferation rate of ADSCs from pregnant rats was significantly higher than those from the non-pregnant rats ($P < 0.05$); however, there was no statistically significant difference in proliferation rates during different phases of pregnancy ($P > 0.05$). Additionally, ADSCs from pregnant rats possess higher adhesion property in early stage (P1 passage) and higher proliferation rate than ADSCs from non-pregnant rats. Interestingly, ADSCs from non-pregnant rats that were treated with E2, but not those treated with P, showed higher proliferation rates than those from their untreated counterparts. These results suggest that the proliferative capacity and residence time in different cell cycle phases of ADSCs can be regulated by extrinsic factors such as estrogen concentration.

Key words: Adipose-derived stem cells; Pregnancy status; Proliferation; Cell cycle; 17β-Estradiol; MTT assay

INTRODUCTION

Mesenchymal stem cells are in the limelight now because of their potential use in tissue engineering (Hattori et al., 2006). Adult mesenchymal stem cells derived from the adipose tissue (ADSCs) can differentiate into mesodermal cells (adipocytes, chondrocytes, osteocytes, and myocytes) (Zuk et al., 2001). Recently, ADSCs were demonstrated to form cells of the neuronal lineage, including neurons (Kang et al., 2004), oligodendrocytes (Safford et al., 2004), and Schwann cells (Kingham et al., 2007; Xu et al., 2008) and cells of the epidermal lineage (Trottier et al., 2008). ADSCs have unique advantages over bone marrow mesenchymal stem cells (BMSCs): a) adipose tissue is readily accessible and can be extracted in a large volume with limited morbidity, such as by liposuction (Trottier et al., 2008); b) the procedure of extracting ADSCs causes negligible pain to the subjects, but at the same time yields high number of stem cells; c) unlike bone marrow, ADSCs do not require specific batches of sera and growth factor supplements for expansion (Strem et al., 2005); d) ADSCs proliferate rapidly and stably in cell culture, with multiplication rate remaining stable until 13-15 generations (Lee et al., 2004). The use of ADSCs has minimized the time required for culturing and generation of cells with therapeutic value. These advantages make ADSCs a better clinical alternative to BMSCs.

However, successful application of ADSCs in clinical therapy faces challenges. Factors that influence both the proliferation rate and differentiation capacity of ADSCs, such as donor age, type, and location of adipose tissue (Lei et al., 2007), methods of cell isolation, and culturing conditions (Oedayrajsingh-Varma et al., 2006; Peptan et al., 2006) limit the success of ADSCs as therapeutic tool. For example, several studies have reported that hormones affect the proliferation and differentiation capacities of human or mouse MSCs (Kuo et al., 2013; Yazawa et al., 2014). Since the levels of sex hormones fluctuate during a woman's life, proliferation and differentiation of ADSCs might also be affected (Gallego et al., 2010; Hirschberg, 2012; Rossi et al., 2014). However, there are no evidence-based studies till date on this subject.

In this study, we used an *in vitro* system to investigate the effects of pregnancy and its different phases on proliferation, progression through cell cycle and differentiation of ADSCs

derived from adult rats. We also examined the effect of hormones related to pregnancy, namely, 17β-estradiol (E2) and progesterone (P), on the abovementioned parameters.

MATERIAL AND METHODS

Ethics statement

Kunming Medical University Review board approved this study. All animals were cared for in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Kunming Medical University, People's Republic of China. All surgery was performed under general anesthesia, and efforts were made to minimize animal suffering.

Treatment groups

A total of 75 Wistar rats were included in this study. The five treatment groups were: non-pregnant, pregnant, in perinatal period of pregnancy, non-pregnant treated with a physiological concentration of 0.01 nM E2, and non-pregnant treated with a physiological concentration of 0.01 nM P. The male and female rats were put together in one cage, and vaginal plug formation was monitored. Vaginal smears were observed for presence of sperms to confirm copulation. The day sperms were observed in the vaginal smear was counted as the first day of pregnancy; ADSCs were separated on the seventh day.

Isolation of adipose-derived stem cells

Six-to-eight-week-old female rats were sacrificed using diethyl ether. ADSCs were isolated from rat peritoneal adipose tissue and collected in phosphate-buffered saline (PBS) at 4°C. Each sample of adipose tissue was washed extensively with sterile PBS to remove contaminants. Then, the tissues were digested at 37°C for 1 h with 0.075% type I collagenase (Gibco, Grand Island, NY, USA) in plain Dulbecco's Modified Eagle Medium/nutrient Mix F-12 (DMEM/F12) (Gibco, USA). After addition of fetal calf serum (Gibco, Paisley, Scotland, UK) to a final concentration of 10% (v/v) for neutralizing the collagenase, the solution was filtered through a 250-μm nylon mesh and centrifuged at 1200 *g* for 8 min at 4°C. The pellet was washed twice with ice-cold PBS and centrifuged at 1000 *g* for 10 min. Finally, the cell pellet was resuspended in adipose-derived stem cell medium: DMEM/F12, 10% FBS (Gibco, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, USA). The cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Culture medium was replaced every 24 h during the initial three days of culturing. ADSCs were cultured and expanded in basal medium and used for experiments at passage 3 (P3).

Detection of surface markers on rADSCs

Rat ADSCs (rADSCs) at passage 3 were harvested and resuspended in culture medium at a density of 1 x 10^6 cells/mL. These cells were directly stained with antibodies against CD90, CD34, and CD44 (BD Bioscience, Heidelberg, Germany). Next, 2 x 10^5 cells were incubated with the appropriate antibody (Dilutions 1:200) in 0.1 mL PBS containing 0.1% bovine serum albumin for 30 min at room temperature. Labeled cells were analyzed four times

by flow cytometry. Prior to this analysis, samples were washed, and dead cells and cell debris were removed. Data were analyzed using the Cell Quest software (BD Biosciences) and the percentage of cells that were positive for a marker are reported as means ± standard deviation (N = 4).

Detection of osteogenesis

rADSCs were cultured on 6-well plates at a density of 1 x 10^5 cells per well. Cells were cultured for 15-19 days at 37°C and 5% CO_2 in osteogenic differentiation medium (Cyagen Biosciences Inc.). The medium was replaced twice a week. After two weeks of incubation, samples were harvested. RT-qPCR was performed to detect osteogenic differentiation.

Alizarin red staining (ARS)

The cells grown in T25 flasks were washed with PBS and fixed in 10% (v/v) formaldehyde (Sigma, USA) at room temperature for 20 min. The monolayer was washed twice with excess distilled water prior to the addition of 2% ARS (Sigma) (pH 4.1) per flask. The flasks were incubated at room temperature for 20 min while being shaken. After aspiration of the unincorporated dye, the flasks were washed four times with distilled water while being shaken for 5 min.

Cell proliferation assay

Proliferation rates were determined by 3-(4,5,-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation assay. ADSCs were harvested on days 1, 3, 5, 7, and 9. In brief, the cultures (1 x 10^4 cells seeded per well on a 24-well plate) were washed with PBS and 200 μL MTT reagent was added. Following incubation for 2 h in the incubator (5% CO_2 at 37°C), the supernatant was removed and incubated with 400 mL dimethyl sulfoxide for 10 min. Absorbance at 570 nm was measured in an enzyme-linked immunosorbent assay plate reader (Infinite M200 Pro, Tecan Group Ltd., Switzerland).

Cell adhesion assay

A total of 40,000 cells were seeded onto 96-well plates to form a confluent monolayer. Differently treated cells were incubated for 90 min before being subjected to washing twice with PBS to remove non-adherent cells. Then, adherent cells were fixed in 4% formaldehyde (v/v), stained with 0.5% (w/v) crystal violet and counted under a microscope.

Matrigel adhesion assay

Matrigel (Sigma) was formulated at a concentration of 10 μg/250 μL with serum-free DMEM medium; subsequently, 2 μg/50 μL matrigel was added to each well and seasoned overnight. The plates were washed with PBS to remove excess matrigel. Various treated cells were collected and resuspended in serum-free DMEM medium. A total of 4 x 10^3 cells were seeded in each well in triplicates, and plates were incubated at 37°C in 5% CO_2 incubator. Unattached cells were washed away by rinsing in PBS thrice. The attached cells were fixed with methanol for 15 min, and then stained with Giemsa for 15 min. The number of adherent cells was counted under a microscope after washing with water.

Detection of cell cycle phases

The distribution of cells in different phases of the cell cycle was evaluated by staining cells with propidium iodide (Invitrogen, St. Louis, MO, USA), followed by flow cytometry. In brief, 10,000 cells were harvested with trypsin 48 h, washed with PBS, and fixed with 4% formaldehyde for 20 min on ice. Cells were washed with PBS, then resuspended in 0.2% Triton-X plus 1% BSA in PBS solution, and incubated at room temperature for 15 min. Cells were washed with 1% saponin in PBS wash solution, then resuspended in PBS/propidium iodide/RNase A solution and incubated for 30 min, and detected on a FACS Calibur flow cytometer (BD Bioscience). Results were analyzed using the Cell Quest Pro software (BD Bioscience).

RNA extraction and RT-qPCR analysis

Total RNA was isolated from cells using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Subsequently, cDNA was synthesized from 5 μg of RNA using MLV reverse transcriptase (Fermentas) according to the manufacturer's instructions. The measurement of gene expression levels on 10 ng cDNA was performed using the LightCycler® 480 System. The real-time PCR (KAPA Biosystem) conditions consisted of 1 cycle at 95°C for 3 min followed by 40 cycles at 95°C for 3 s and at 60°C for 30 s. Primers designed on glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal standard. Primers used were as follows: alkaline phosphatase (*ALPL*), sense, 5'-CTTGACTGTGGTTACTGCTG-3', antisense, 5'-GAGCGTAATCTACCATGGAG-3'; collagen type I (*COLI*), sense, 5'-GAGCGGAGAGTACT GGATCG-3', antisense, 5'-TACTCGAACGGGAATCCATC-3'; Runt-related transcription factor 2 (*RUNX2*), sense, 5'-CCAAGAAGGCACAGACAGAA-3', antisense, 5'-ATACTGGGATGAG GAATGCG-3'; osteocalcin (*BGLAP*), sense, 5'-CTAGCAGACACCATGAGGAC-3', antisense, 5'-CAGGTCCTAAATAGTGATACC-3'; and GAPDH, sense, 5'-GGGTGTGAA CCACGAGAAAT-3', antisense, 5'-CCTTCCACAATGCCAAAGTT-3'.

Statistical analysis

Data are reported as means ± SE. Statistical analyses were performed by GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) and statistical difference was analyzed by one-way ANOVA, followed by the Dunnett test. $P < 0.05$ was considered significant. All assays were performed in triplicates.

RESULTS

Isolation, culturing and identification of rADSCs from rats in different stages of pregnancy

We isolated and cultured ADSCs from rats in different stages of pregnancy (Figure 1a). At first, a large number of red blood cells and biconcave, disc-shaped, large, round cells suspended in medium were seen under the microscope along with lipid droplets (Figure 1b). After 24 h, non-adherent cells were removed by replacing the medium; only a few adherent fibroblast-like cells were observed using an inverted microscope (Figure 1b-f).

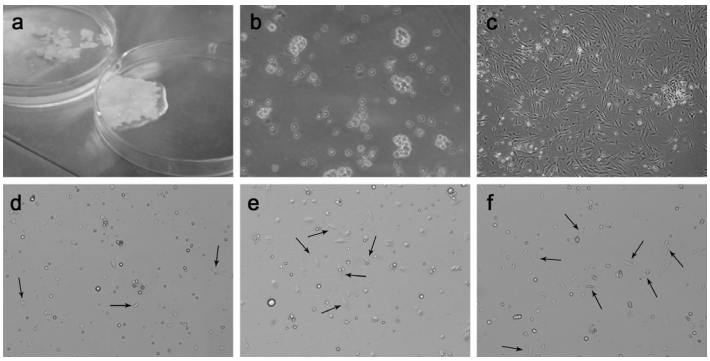

Figure 1a-f. Morphology of rADSCs in different periods of pregnancy. **a.** Adipose tissue. **b.** Primary culture contains many double concave cells, wheel-shaped cells, and few lipid droplets. **c.** ADSCs showed typical spindle-shaped morphology. **d.** After 24 h of primary culturing, cells of the non-pregnant group seldom showed fusiform cells (40X magnification). **e.** After 24 h of primary culturing, cells obtained from mid pregnancy group showed many fusiform cells (40X magnification). **f.** After 24 h of primary culturing, cells obtained from the perinatal pregnancy group showed many fusiform cells (40X magnification). The arrows indicate the fusiform cells in panels **d-f**.

We analyzed the surface antigenic characteristics of rADSCs at P3 using flow cytometry (data represents cells from pregnant rat). The results showed that the cells were positive for MSC markers, CD44 (99.3 ± 0.4, 97.5 ± 1.6 and 97.2 ± 3.7%, in non-pregnant, pregnancy, and perinatal period, respectively) and CD90 (80.2 ± 4.0, 77.5 ± 5.2 and 76.7 ± 4.7%, in non-pregnant, pregnancy, and perinatal period, respectively); by contrast, they were negative (0.0 ± 0.0, 2.2 ± 1.0 and 3.1 ± 1.4%, in non-pregnant, pregnancy, and perinatal period, respectively) for hematopoietic stem cell marker, CD34 (Figure 2). No significant differences were found in different ADSCs. These results indicated that rADSCs had been successfully isolated and cultured.

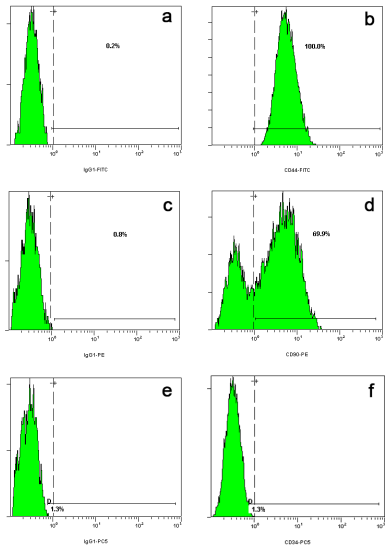

Figure 2a-h. FACS analysis and immunohistochemical staining to characterize rADSCs. rADSCs were uniformly positive for expression of MSC cell surface markers CD44 (**b)** and CD90 (**d**), while negative for CD34, a hematopoietic stem cell marker (**f**). Panels **a**, **c** and **e** depict staining results with the IgG control.

Effect of pregnancy on cell adhesion and proliferation capacity of rADSCs

Under the same culture conditions, the surface adhesion of rADSCs from the pregnant rats was significantly higher than those from the non-pregnant rats ($P < 0.05$) (Figure 3a and b). We further analyzed surface adhesion in an *in vitro* matrigel adhesion assay. Compared to cells from the non-pregnant rats, cells from the pregnant group showed minor differences in adhesive capacity, which was not statistically significant.

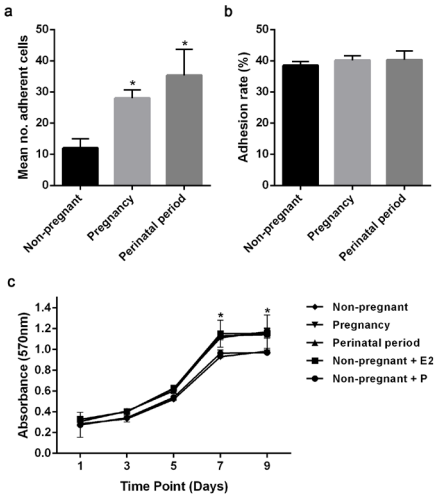

Figure 3a-c. Effect of pregnancy on the adhesive and proliferative properties of rADSCs. Number of adherent cells were counted in early stage (P1 passage) (**a**) and adhesion rates was detected by Matrigel adhesion assay (**b**). The number of cells attached to matrigel was counted and graphically represented. **c.** Proliferation of ADSCs from rats that were non-pregnant, pregnant, in perinatal period of pregnancy, or non-pregnant, but treated with E2 or P, was detected using MTT assay. Bars represent the means ± SD values from three different experiments (*$P < 0.05$, compared to non-pregnant group).

We also investigated the proliferation rates of rADSCs obtained from the five experimental groups to understand the effect of reproductive status on the total number of surviving rADSCs (Figure 3c). We found that cells from the two pregnancy groups (pregnant and perinatal stage of pregnancy), as well as the E2-treated cells, showed significantly higher proliferation rate than those from the non-pregnancy or P-treated groups ($P < 0.05$). There are no significant differences in proliferation between the P-treated cells and the untreated cells ($P > 0.05$).

Effect of pregnancy on cell cycle

To accurately quantitate the distribution of cells in the different phases of cell cycle, we labeled the DNA of rADSCs isolated from the various study groups with propidium iodide and subjected them to flow cytometric analysis. As shown in Figure 4, cell cycle increased in S phase and G2/M phase during pregnancy. The addition of E2 had a similar effect, but no effect was seen in cells treated with P.

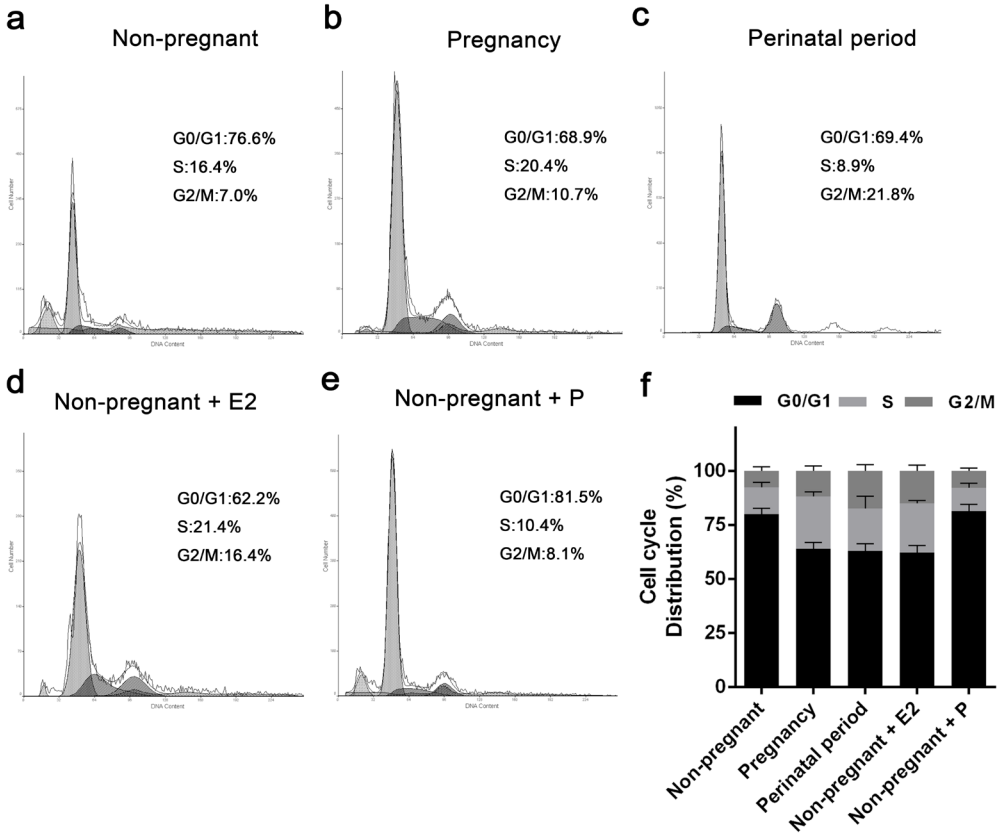

Figure 4. Effect of pregnancy on the cell cycle progression of rADSCs. Flow cytometry analysis of ADSCs obtained from different rats, namely, non-pregnant, pregnant, in perinatal period of pregnancy, non-pregnant and treated for 24 h with E2 or P. The percentage of cells in the G2/M-phase was significantly higher for pregnant rats (middle of pregnancy or in perinatal period) and in non-pregnant ones that were treated with E2 than those from non-pregnant control cells or P-treated cells ($P < 0.01$).

Effect of pregnancy on the osteogenic differentiation capacity of rADSCs

To evaluate the differentiation potential of rADSCs, cells at P3 were induced to differentiate into osteocytes. After rADSCs were cultured in an osteogenic differentiation medium for three weeks, most of the differentiated cells appeared cubical and exhibited a dull-red staining with ARS, which is indicative of calcium mineralization; cells cultured in normal medium (as control) did not exhibit these characteristics (Figure 5a).

Expression levels of osteogenic genes, namely *ALPL*, *BGLAP*, *RUNX2*, and *COLI*, were investigated from five groups by real-time PCR after 15 days of differentiation. No significant differences were observed in the differentiation potential between these five groups. It suggests that rADSCs derived from all donors, namely, pregnant, in the perinatal period of pregnancy and non-pregnant treated with P or E2, had no significant difference in their osteogenic differentiation abilities (Figure 5b).

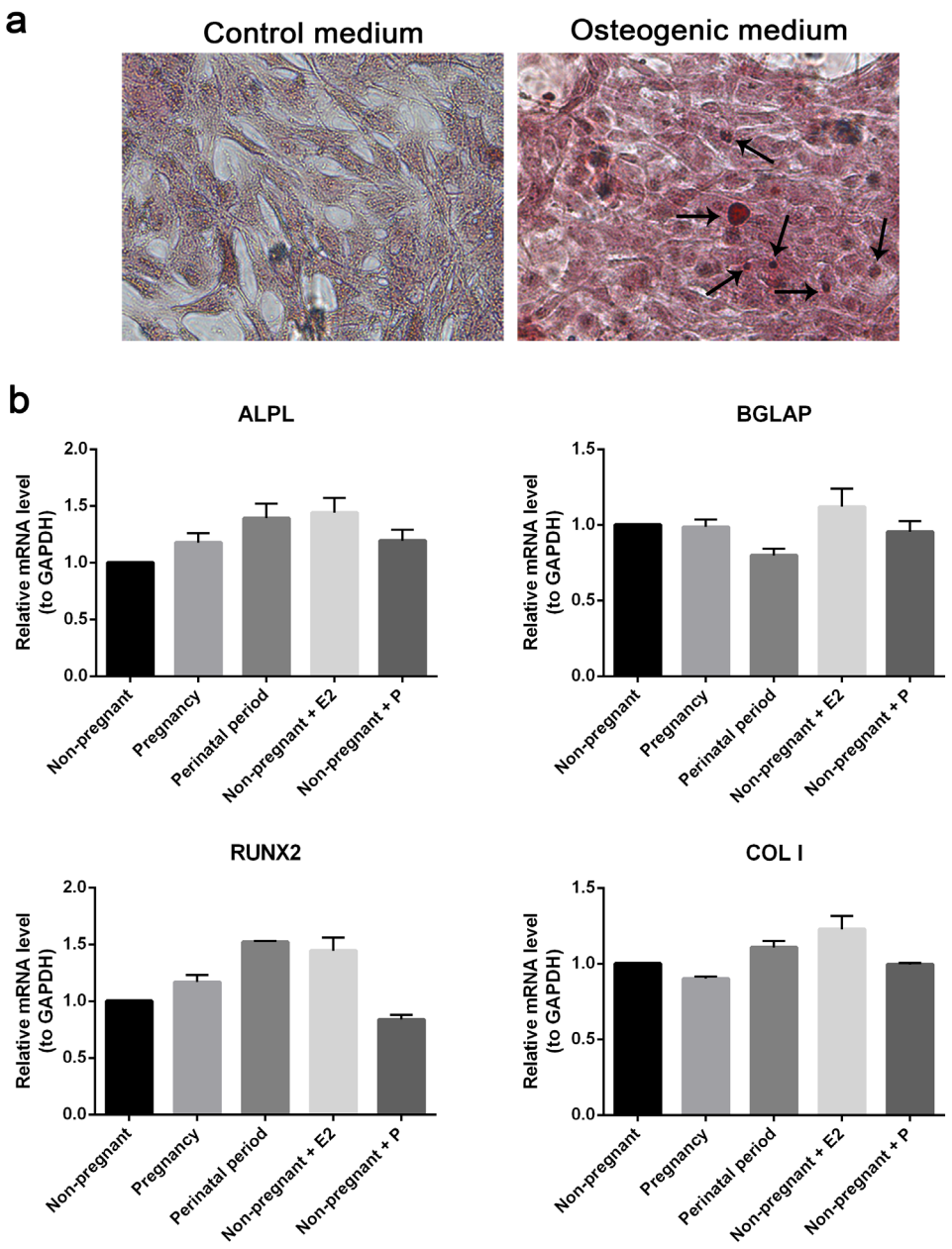

Figure 5. Assessing the expression of genes involved in osteogenic differentiation by RT-qPCR. Fourteen days after bone format induction, red calcium nodules were observed in rADSCs cultured in osteogenic differentiation medium (**a**, right), but not in the normal medium (**a**, left) (observed under 100X magnification) by alizarin red staining. **b.** Expression of different osteogenic markers was measured by RT-qPCR in rADSCs that were cultured in osteogenic differentiation medium for 14 days. Data represents fold changes of target genes relative to the housekeeping gene *GAPDH*. Values are reported as means ± SD in 3 independent experiments from five groups (N = 15 in each groups).

DISCUSSION

In the present study, we isolated ADSCs from adult rats at different stages of pregnancy. We obtained significantly more ADSCs from the pregnant and perinatal rats than from the non-pregnant rats. No significant difference was observed between cell numbers from rats in the middle of pregnancy and those in the perinatal period. We isolated ADSCs from identical locations and tissue weights of the rats, and used same conditions for primary culture and subculture. Our results indicate that ADSCs differ based on the reproductive stage of the animal, which contributes to a significant difference in the number of ADSCs harvested. Since there is no research on this aspect of ADSC biology, the reason behind these differences is unknown. Visvader and Lindeman (2011) had a similar observation in mouse mammary stem cells, and they indicated that estrogen and progesterone promoted the proliferation and activity of mouse mammary stem cells.

We also compared proliferation rate of rADSCs isolated from the different study groups and found it to be significantly higher in the pregnant group than in the non-pregnant groups; however, no significant difference was observed between mid-pregnancy period and the perinatal period. In women, estrogen levels change greatly during pregnancy. Therefore, we hypothesize that differences in estrogen levels in the pregnant group versus the non-pregnant group could account for the variation. Many studies suggest that pregnancy is associated significantly with stem cell activity. For instance, Strehlow et al. (2003) found that estrogen during pregnancy can effectively prevent apoptosis of bone marrow-derived endothelial progenitor cells. Sugawara et al. (2005) reported that peripheral blood progenitor cells gradually increased with progress of pregnancy and positively correlated to serum estradiol levels. Manotaya et al. (2002) indicated that hematopoietic stem cell number and activity increased during pregnancy. In addition, Ng et al. (2009) found that the proliferation rate of ADSCs from pregnant women was higher than ADSCs isolated from pre- and postmenopausal women. We also detected the effect of progesterone and estradiol on rADSC proliferation. Interestingly, the relationship between cell proliferation and estradiol is consistent with the results obtained with human neural progenitor cells, while this is not the case with progesterone. Wang et al. (2008) found that E2 can induce human neural progenitor cell proliferation, which is mediated by an estrogen receptor beta-phosphorylated extracellularly regulated kinase pathway. The estrogen receptor is the decisive factor influencing cellular response to estrogen (Cobellis et al., 2002), and it was shown to exist in MSCs (Zhou et al., 2001; Wang et al., 2006). Ng et al. (2009) showed that upon binding estrogen receptors on the cell surface, estrogen activated specific genes and eventually affected cellular processes. However, no differences in proliferation rates were observed when ADSCs from pregnant, premenopausal or menopausal women were treated with exogenous estrogen. This may be due to differences in the sources of the cells, and the variation of treatment in the concentration and time of external hormone administration. Similar reasoning was applied to explain the effect of melatonin on ADSC proliferation and differentiation - the concentration of melatonin and the type of the examined cell influenced the rate of proliferation (Sainz et al., 2003; Hong et al., 2006; Chen et al., 2014).

The mammalian cell cycle is composed of four phases: G1, S, G2, and M. Dysregulation of cell cycle progression leads to changes in proliferation. We found that estradiol treatment of ADSCs from non-pregnant rats increased the G2/M phase population, similar to that seen with ADSCs from pregnant rats. Studies have shown hormones, including estrogen, could regulate progression through cell cycle and subsequent cell proliferation by multiple pathways. However, the molecular mechanisms of hormone-induced proliferation ability of ADSCs are unknown and require investigation.

In summary, rADSCs from pregnant lab rats are more proliferative than those from non-pregnant rats. Treatment of rADSCs from non-pregnant rats with estradiol, but not progesterone, showed similar effect. This implies that the differences in proliferation between different reproductive statuses may be due to differences in estrogen levels, and may be related to the cell cycle. Future studies should address the molecular mechanisms of ADSC proliferation and differentiation in response to various internal and environmental stimuli and investigate the effect of ADSCs on animal models of different reproductive statuses.

Conflicts of interest

The authors declare no conflict of interest.

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