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The differentiation potential of adipose tissue-derived mesenchymal stem cells into cell lineage related to male germ cells

[*Potencial de diferenciação de células-tronco mesenquimais derivadas do tecido adiposo em células da linhagem germinativa masculina*]

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ABSTRACT

The adipose tissue is a reliable source of Mesenchymal stem cells (MSCs) showing a higher plasticity and transdifferentiation potential into multilineage cells. In the present study, adipose tissue-derived mesenchymal stem cells (AT-MSCs) were isolated from mice omentum and epididymis fat depots. The AT-MSCs were initially compared based on stem cell surface markers and on the mesodermal trilineage differentiation potential. Additionally, AT-MSCs, from both sources, were cultured with differentiation media containing retinoic acid (RA) and/or testicular cell-conditioned medium (TCC). The AT-MSCs expressed mesenchymal surface markers and differentiated into adipogenic, chondrogenic and osteogenic lineages. Only omentum-derived AT-MSCs expressed one important gene marker related to male germ cell lineages, after the differentiation treatment with RA. These findings reaffirm the importance of adipose tissue as a source of multipotent stromal-stem cells, as well as, MSCs source regarding differentiation purpose.

Keywords: *Gdnf*, retinoic acid, somatic stem cell, testicular cell-conditioned medium

RESUMO

O tecido adiposo é uma fonte apropriada de células-tronco mesenquimais (MSCs), as quais demonstram ampla plasticidade com capacidade de transdiferenciar em diversas linhagens. No presente estudo, as células-tronco mesenquimais derivadas do tecido adiposo (AT-MSC) foram isoladas de tecido adiposo localizado nas regiões próximas ao omento e testículos de camundongos. Primeiramente, as AT-MSCs foram comparadas com base na expressão de marcadores antigênicos de superfície e no potencial de diferenciação nas três linhagens mesodérmicas. Além disso, AT-MSC, de ambas as fontes, foram cultivadas com meio de diferenciação contendo ácido retinóico (RA) e / ou meio condicionado testicular (TCC). As AT-MSCs expressaram marcadores de superfície mesenquimais e diferenciaram nas linhagens adipogênica, condrogênica e osteogênica. Após o tratamento com RA, somente as AT-MSCs isoladas do tecido adiposo depositado na região do omento expressaram um único importante marcador relacionado às células da linhagem germinativa masculina. Estes resultados reafirmam a importância do tecido adiposo como fonte de células-tronco estromais-multipotentes, bem como, uma fonte de MSCs para estudos de diferenciação.

Palavras-chave: Gdnf, *ácido retinóico, células-tronco somáticas, meio condicionado testicular*

INTRODUCTION

An area of biological research that generates great optimism is the use of stem cells for the treatment of diseases (Young and Black, 2004; Nayernia *et al*., 2006). A reliable source of stem cells are the adipose tissue-derived mesenchymal stem cells (AT-MSCs), which are well characterized, easy to isolate, and have already been used for therapeutic applications (Mitchell *et al*., 2006; Gimble *et al*., 2007). AT-MSCs are multipotent cells and can be induced to differentiate into all three germ layers,

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developing into bone, cartilage, muscle, heart, and neural cells (Zuk *et al*., 2002). Therefore the AT-MSCs potential to transdifferentiate into male germ cells should be investigated aiming future cell therapy for infertility.

Adipose tissue is an organ of great heterogeneity and plasticity (Kim and Moustaid-Moussa, 2000). The cellular complexity of adipose tissue can be divided into two different cell fractions: mature adipocytes and the stromal-vascular fraction (SVF). The SVF is highly heterogeneous, containing fibroblasts, endothelial cells, vascular smooth muscle cells, monocytes, hematopoietic cells, and mesenchymal stem cells (Prunet-Marcassus *et al*., 2006; Schäffler and Büchler, 2007). This cellular fraction can be isolated from adipocytes and maintained in culture. The AT-MSCs expand manifold in culture, reaching high passage numbers and retaining differentiation potential (Bunnell *et al*., 2008; Zhu *et al*., 2008).

Animals have diverse fat sources anatomically divided into subcutaneous and internal fat depots (Strem *et al*., 2005; Bunnell *et al*., 2008). According to the fat pad location, adipose tissue shows different metabolic properties, genes expression profiles, antigenic features, and differentiation potential, influencing AT-MSCs characteristics (Tchkonia *et al*., 2002).

AT-MSCs *in vitro* differentiation has demonstrated promising results (Zuk *et al*., 2002) and several substances are used for differentiation of mesenchymal stem cells (MSCs) into diverse cell lineages (Schäffler and Büchler, 2007; Mosna *et al*., 2010), Including retinoic acid (RA), a vitamin A (retinol)–derivative, which is widely used as a differentiation inducer of MSCs in male germ cells (Nayernia *et al*., 2006; Huang *et al*., 2010) Tan *et al*., 2016). Furthermore, the conditioned medium obtained from the cell culture supernatants is also a strategy applied in differentiation treatments. The soluble factors present in conditioned medium have been employed for chondrogenic, myogenic, neural, and germ cell differentiation of bone marrow- and adipose tissue-derived MSCs (Silva *et al*., 2013; Stern-Straeter *et al*., 2013; Han *et al*., 2014). However, the effect of RA and testicular cell conditioned medium in AT-MSCs differentiation into germ cells should be elucidated.

Analyses of marker gene transcripts allow investigation of AT-MSC differentiation and facilitate the selection of efficient differentiation inducers (Phinney and Prockop, 2007; Schäffler and Büchler, 2007; Hou *et al*., 2014). The genes commonly investigated to confirm MSCs differentiation into male germ cell are *Vasa*, *Stella*, *Dazl, Stra8, Nanos2, Plzf,* and, in addition, *Gdnf* which is used as a marker for Sertoli cells (Hou *et al*., 2014; Chen and Liu, 2015; Ikami *et al*., 2015).

Taking these observations into consideration, the aims of the present study were to compare AT-MSCs, isolated from two different adipose depots in mice, regarding surface markers and trilineage differentiation potential, as well as, to evaluate the expression of relevant gene markers of male germinative cells in AT-MSCs from both sources, after treatments with RA and testicular cell-conditioned medium.

MATERIAL AND METHODS

Adipose tissue from 11 BALB/c mice (8 weeks old), was collected from the omentum and epididymis regions. The tissue was, separately, minced and digested in collagenase solution (1mg/mL) (SIGMA-ALDRICH, USA) for 30min at 37°C. After digestion, the collagenase solution was inactivated with growth medium (Dulbecco's Modified Eagle Medium-F12 + 10% fetal bovine serum + 100µg (100IU) of penicillin and, streptomycin + 0,25µg of amphotericin B) (all Gibco, USA) and the cells were centrifuged (200*xg*, 10min) to obtain a pellet. The resulting pellet was resuspended in growth medium, and 2 x 10^4cells/cm^2 were cultured in cell culture plates (Corning, USA) in a 5% CO_2 incubator (Thermo Scientific, USA) at 37°C. The cells were maintained in growth medium and the passages were carried out using trypsin solution (0.25%) (SIGMA-ALDRICH, USA) once they achieved 70-80% confluency.

Testicles from 11BALB/c mouse of 8 weeks old were removed, separately minced and enzymatically digested in collagenase solution (1mg/mL) for 30 minutes at 37°C. Tissue digestion was completed with addition of growth medium for collagenase inactivation, and then

the tissue homogenate was centrifuged for 10min at 200*xg*. The resulting pellet was resuspended in growth medium, and $2x10^4$cells/cm^2 were cultured in 5% CO_2 at 37°C. Seven days after cultures were started, and every 3 days afterward for 30 days, testicular cell-conditioned medium (TCC) was collected. After collection, the TCC was centrifuged (200*xg*, 10min), and the supernatant was filtered (0.22µm) and stored at -20°C until use.

Four AT-MSCs cultures from omentum and epididymis fat depots were analyzed by flow cytometry for the stem cells surface markers CD105, CD73, and CD45 at the passages 2 (P2), 4 (P4), and 7 (P7). Briefly, cells were trypsinized, centrifuged, and then 4 x 10^4cells were suspended in Stain Buffer (BD Biosciences, USA) in separate microtubes. Samples were then incubated with the antibodies CD45-FITC (1.5µg/µL), CD73-FITC (1µg/µL), and CD105-PE (1µg/µL) (BD Biosciences, USA) in the dark for 20min at 37°C. After incubation, cells were analyzed using the BD FACSuite flow cytometer (BD Biosciences, USA) and the data obtained were analyzed by FLOWJO software.

Three AT-MSCs cultures, at cell passage number 6 (P6), $4x10^3$cells/cm^2 of omentum and epididymis-derived AT-MSCs were cultured in osteogenic medium (50µM L-ascorbic acid 2-phosphate, 0.1µM dexametasone, and 15mM β-glycerolphosphate) (all SIGMA-ALDRICH, USA), chondrogenic medium (50µM dexametasone, 50µM L-ascorbic acid 2-phosphate, 10ng/mL TGF-β, and 1x insulin-transferrin-sodium selenite) (all SIGMA-ALDRICH, USA), and with adipogenic medium (50µM indomethacin, 1µM rosiglitazone, 1µM dexamethasone, and 1µg/mL insulin) (all SIGMA-ALDRICH, USA), for 21 and 17 days. The media were changed every 3 days and, histological staining was performed after the differentiation periods. Cells induced with osteogenic, adipogenic, and chondrogenic media were first fixed with paraformaldehyde 4% and then stained with alizarin red (pH 4.1), oil red, and alcian blue (pH 2.5) (all SIGMA-ALDRICH, USA), respectively, and observed using an inverted microscope at magnification 100 X (Leica DMI600B, Germany).

Four AT-MSCs cultures derived from the omentum and epididymis fat pads, at P6 and $4x10^3$cells/cm^2, were induced by growth medium supplemented with 10^{-6}M RA (group1) (SIGMA-ALDRICH, USA) , or 50% TCC + 50% growth medium (group 2), or 50% growth medium supplemented with 10^{-6}M RA + 50%TCC (group 3) at 7, 14, and 21 days. Adipose tissue-derived cells maintained with only growth medium were used as a control group (group 4). Each group was tested in duplicate. The media were changed every 3 days.

After each period of differentiation treatments into male germ cells (7, 14, and 21 days), total RNA was isolated from the 4 groups. RNA extraction was performed using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. After extraction, RNA concentration and quality were checked by a NanoDrop1000 spectrophotometer (Absorbance of 260/280nm) (Thermo Scientific, USA). Complementary DNA (cDNA) was synthesized using 1000ng of total RNA, which was first treated with 0.1U of DNase Amplification Grade (Life Technologies, USA) for 5min at 37°C. After DNase inactivation at 65°C for 10min, cDNA was performed in 20µL final volume reaction using the iScript cDNA synthesis Kit (BioRad, USA).

The relative expression levels of specific genes were determined by quantitative PCR (qPCR) conducted in a CFX384 thermocycler (BioRad, USA) using GoTaq qPCR Master Mix (Promega, USA). All primers (Table 1) for analysis of the murine mRNA *Vasa*, *Stella*, *Dazl*, *Stra8, Nanos2, Plzf* , *Gdnf, Gapdh and β-Actin* were designed based on gene sequences deposited in the GenBank database using Primer Express Software (Applied Biosystems, USA).

Melting-curve analyses were performed to verify product identity. To optimize the qPCR assay, serial dilutions of the cDNA templates were used to generate a standard curve. The standard curve was constructed by plotting the log of the dilution factor against the Ct value obtained during amplification of each dilution. Reactions with a coefficient of determination (R2) higher than 0.98 and efficiency between 95% and 105% were considered optimized. The relative standard curve method was used to quantify transcripts in each sample. Samples were run in duplicate, and results were expressed relative to the average Ct values for the *Gapdh* and *β-Actin* genes as

internal controls. A sample of mRNA extracted from mouse testicles was used as a positive control for validating primers and amplicons. Variation in expression levels was analysed by ANOVA and multiple comparisons between days or groups were performed by LSMeans Student's t test using the JMP Software.

Table1. List of primer sequences and accession numbers for gene sequences used for the mRNA gene expression analysis

Gene	Forward primer	Reverse primer	Accession number
Dazl	CGAAGCATACAGACAGTGGTCTCT	TAAGCACTGCCCGACTTCTTCT	010021.5
Stra8	TTGCCGGACCTCATGGAAT	GTGTCACTTCATGTGCAGAGATGAT	009292.1
Stella	CGGTGCTGAAAGACCCTATAGC	GGCTCACTGTCCCGTTCAAA	139218.1
Vasa	GGCTGTGTTTGCATCTGTTGAC	ATCAACTGGATTGGGAGCTTGT	001145885.1
Nanos2	AGGTAGCTGAGGAGCCCAACTC	TGCTTGCAGAAGTTGCATATGG	194064.2
Plzf	CGAGCTTCCGGACAACGA	AAATGCATTCTCAGTCGCAAAC	001033324.2
Gdnf	GATTCGGGCCACTTGGAGTT	GACAGCCACGACATCCCATAA	010275.2
Gapdh	CAGCCTCGTCCCGTAGACAA	GTAGACCATGTAGTTGAGGTCAATGAA	008084.2
β-Actin	TCGTGGGCCGCTCTAGGCAC	TGGCCTTAGGGTTCAGGGGG	007393.3

All procedures using BALB/c mice in the present study were approved by the Institutional Committee for Ethics in Animal Experiments at the Federal University of Santa Maria, RS, Brazil, approval number 087/2014.

RESULTS AND DISCUSSION

Flow cytometry analysis showed that in omentum-derived AT-MSCs a decrease in double-positive CD105/CD73 cells, and an increase in CD105 and CD73 single-positive cells in P2, P4, and P7 (Figure 1A and Table 2 occurred. While in epididymis-derived AT-MSCs there was an increase in cells expressing the double-positive mesenchymal surface markers, as well as an increase in CD105 single positive cells in the same passages mentioned above (Figure 1B and Table 2). In both adipose tissue-derived cultures, cells expressed insignificant levels of the hematopoietic linage marker CD45 (Figure 1A, B and Table 2).

AT-MSCs cultures from two different fat pads in mice, omentum and epididymis, were analyzed by flow citometry and, although the classical mesenchymal surface markers were expressed in cells from both sources, their levels differed between cells from the two adipose sources and among passages (Figure 1A, B and Table 2). These results indicated that adipose tissue isolated from mouse omentum and epididymis regions have different subsets of MSC The immunophenotype results presented in this study are in accordance with studies that relate the variable expression of surface markers to differences in tissue sources, the method of isolation and culture, and species differences (Silva *et al*., 2013; Prunet-Marcussus *et al*., 2006; Chamberlain *et al*., 2007; Schäffler and Büchler, 2007). Interestingly, previous studies have demonstrated that the expression of surface antigens, also shows significant variation during subculture. For example, studies comparing freshly isolated human adipose-derived cells and serially passaged showed a progressive increase in mesenchymal markers like CD90, CD73, and CD29 (Mosna *et al*., 2010). Similar increases occurred in the present study in the expression of MSC surface markers CD105 and CD73 in AT-MSCs derived from epididymis at P2, P4, and P7. Taken together the flow cytometry results obtained in the present study illustrate that stem cell surface antigen expression profiles vary with different cell sources and passages.

The trilineage differentiation protocols were used to induce AT-MSCs, from both mouse fat depots, into bone, cartilage, and fat to further confirm their mesodermal differentiation capacity, and both adipose tissues were able to differentiate into all three linages. Osteogenic and chondrogenic differentiations occurred at 21 days. With chondrogenic differentiation, cells developed a multilayered matrix that was strongly stained with alcian blue, indicating an abundance of glycosaminoglycans in the extracellular matrix (Figure 2 A and B). AT-MSCs in osteogenic medium differentiated into osteoblasts, with calcium accumulation indicated

by positive staining with alzarin red (Figure 2 A and B). In adipogenic differentiation, both adipose tissue-derived cultures needed 17 days to differentiate and showed an accumulation of lipid-rich vacuoles within cells. These vacuoles were positively stained by oil red staining (Figure 2 A and B). The AT-MSCs grown in culture medium (undifferentiated) did not show any lipid droplets and maintained their typical fibroblast-like shape (Figure 2 A and B). Therefore, AT-MSCs from both the omentum and epididymis fat sources showed the potential to differentiate into the three mesodermal: adipogenic, osteogenic, and, chondrogenic, lineages.

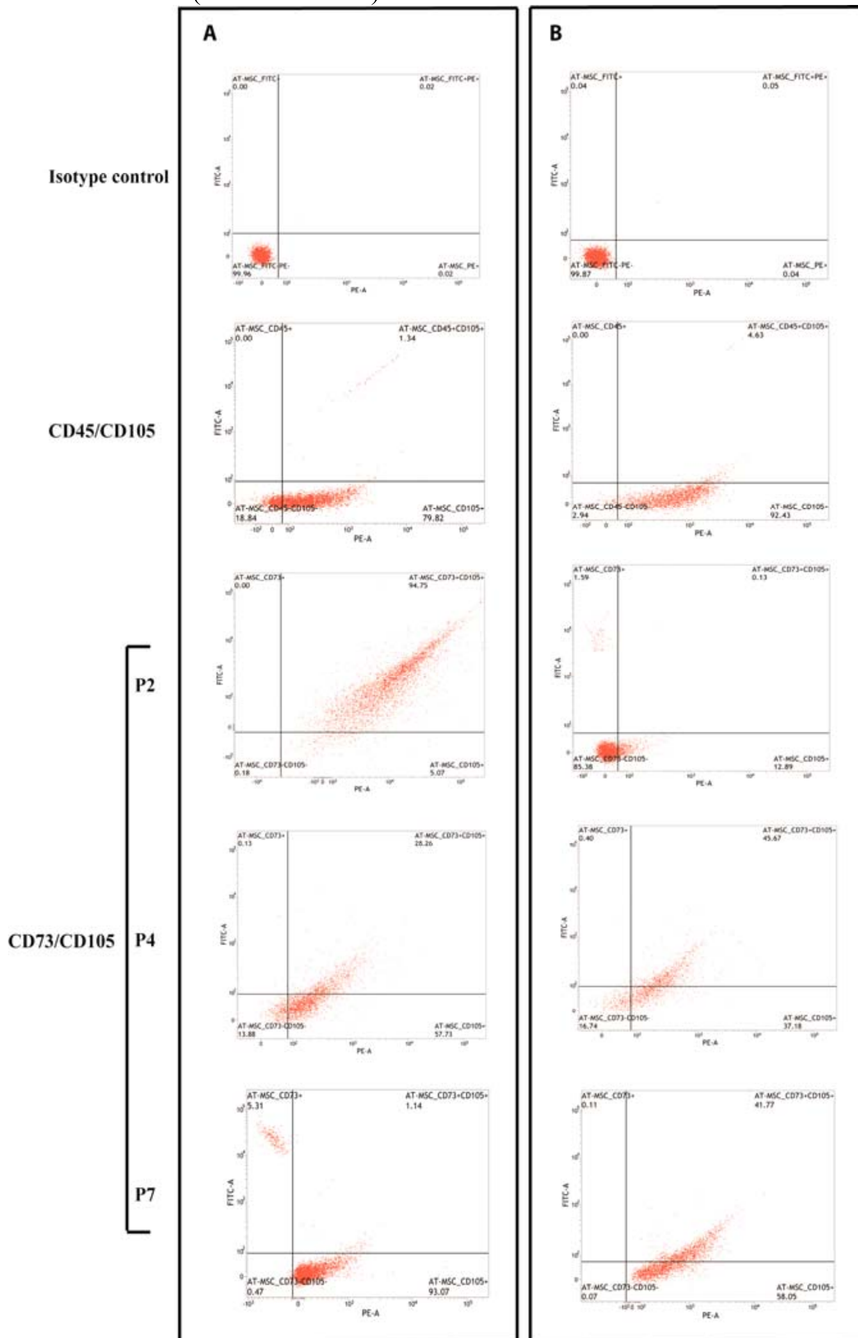

Figure 1. Flow cytometric analysis of surface markers CD73 FITC), CD105 (PE) and CD45 (FITC) in mice omentum A) and epididymis B) derived AT-MSCs at cell passage 2 (P2), 4 (P4) and 7 (P7).

Table 2. Flow cytometric analysis of MSC markers CD73 and CD105, and hematopoietic marker CD45 expression in mice omentum (A) and epididymis (B) derived AT-MSCs at cell passage 2 (P2), 4 (P4) and 7 (P7)

Cell surface marker	A	B	A	B	A	B
	P2		P4		P7	
CD 45^{+}/$CD105^{-}$	0.0%	0.04%	0.28%	0.13%	0.0%	0.0%
CD 45^{-}/$CD105^{+}$	99.31%	12.55%	77.07%	76.73%	79.82%	92.43%
$CD45^{+}$/$CD105^{+}$	0.67%	0.18%	8.38%	8.43%	1.34%	4.63%
$CD73^{+}$/$CD105^{-}$	0.0%	1.59%	0.13%	0.40%	5.31%	0.11%
$CD73^{-}$/$CD105^{+}$	5.07%	12.89%	57.73%	37.18%	93.07%	58.05%
$CD73^{+}$/$CD105^{+}$	94.75%	0.13%	28.26%	45.67%	1.14%	41.77%

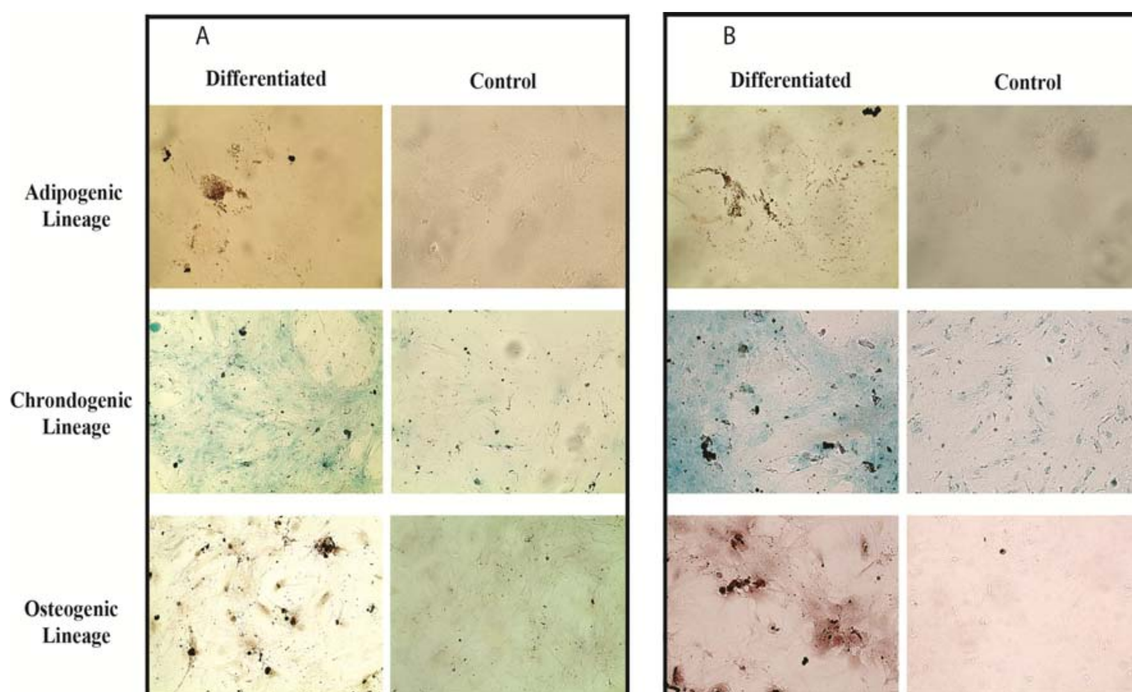

Figure 2. Adipogenic (oil red), osteogenic (alizarin red), and, chondrogenic (alcian blue) differentiation of mice omentum A) and epididymis B) derived- AT-MSCs. (100X magnification).

Since different fat pads have their own metabolic characteristics, fatty acid compositions, and gene expressions (Tchkonia *et al*., 2002; Schäffler and Büchler, 2007), the source of adipose tissue might be expected to influence AT-MSCS characteristics, such as surface markers and differentiation potential. Therefore, additional studies are necessary to properly understand the molecular characteristics, as well as the plasticity, of the AT-MSCs isolated from different fat depots.

A qPCR assay was performed to determine the expression levels of germinative markers (*Dazl, Stella, Stra8, Vasa, Nanos2, Plzf* and *Gdnf*) in RA and TCC MSC-treated cells. Amongst the AT-MSCs sources (epididymal and omental), *Gdnf* was the only gene that demonstrated detectable expression level, and its expression observed only in omentum-derived AT-MSCs treated with RA differentiation media, after incubation periods of, 7, 14, and 21 days (Figure 3). Conversely, no detectable expression was detected in AT-MSCs maintained in TCC medium for any period of treatment (Figure 3) similar to untreated AT-MSCs (Figure 3). The qPCR results demonstrated that RA stimulated *Gdnf* expression in omentum-derived AT-MSCs, therefore, indicating that RA was the main factor involved in *Gdnf* expression.

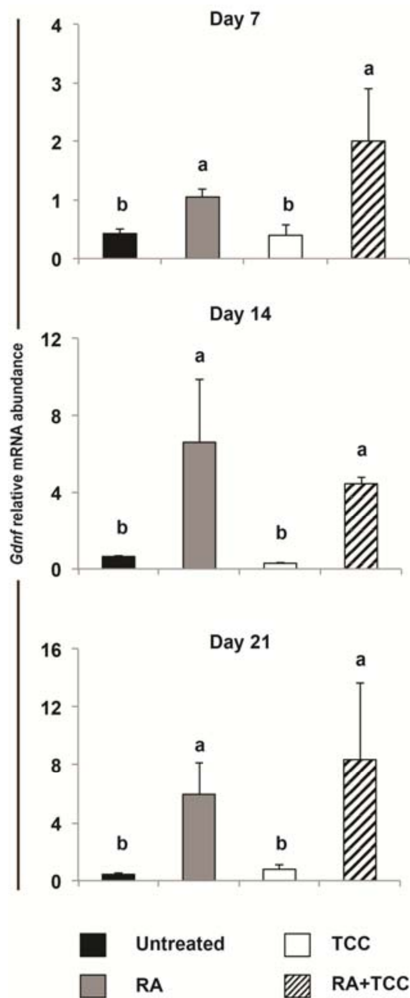

Figure 3. Relative expression of the murine *Gdnf* gene in omentum AT-MSCs treated with retinoic acid (RA), testicular cell-conditioned medium (TCC), or RA+TCC for 7, 14, and 21 days, or with growth medium only for an untreated control group. Values with different superscripts (a, b) are significantly different (P<0.05).

An active derivative of vitamin A, RA influences germ cell differentiation and is required for the transition to meiosis in both female and male germ cells (Koubova *et al*., 2006). RA alone has usually been used to promote the differentiation of ESCs and MSCs into germ cells (Geijsen *et al*., 2004; Nayernia *et al*., 2006; Drusenheimer *et al*., 2007; Kerkis *et al*., 2007). However, RA has been used in association with other substances to induce neuronal differentiation from cultured mouse AT-MSCs (Bi *et al*., 2010; Pavlova *et al*., 2012).

RA receptors (RARs) are expressed in both Sertoli and germ cells (Esklid *et al*., 1991), and RA functions inside the nucleus, recognizing two different classes of RARs. Both classes (RARs and RXRs) are encoded by distinct genes, and they transduce RA signals by binding directly to RA-responsive elements (Rossi and Dolci, 2013). Therefore, based on the effect of RA on *Gdnf* gene expression showed in this study, AT-MSCs respond to RA stimulation.

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Retinoids are involved in the regulation of testicular functions, which appear to be necessary for spermatogenesis (Livera *et al*., 2002) and previous studies have indicated that RA favors spermatogonial differentiation through direct action on spermatogonia and indirect action mediated by changes in the expression of GDNF secreted by Sertoli cells in the testicular niche influencing the self-renewal of spermatogonial stem cells (SSCs) and inhibiting their differentiation. The *Gdnf* gene is mainly expressed in Sertoli cells, considered, therefore, a marker for this cell line. (Rossi and Dolci, 2013; Chen and Liu, 2015; Ikami *et al*., 2015).

The testes are abundant sources of numerous hormones and growth factors, such as bone morphogenic protein 4 (BMP4), leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), stem cell factor (SCF), growth differentiation factor-9 (GDF9), and testosterone, all of which are needed for the development of male germ cells (Takabayashi *et al*., 2001; Pellegrini *et al*., 2003). Recent studies have shown that TCC supports the differentiation of ESCs into germ cells (Lacham-Kaplan *et al*., 2006). However, in the present study, TCC alone was not effective promoting expression of germinative markers maybe, due the inappropriate concentration of the soluble factors. Therefore, it seems that TCC might be

used in combination with other induction factors or might be concentrated to promote cellular differentiation.

MSCs are considered the most plastic adult stem cells and their potential to differentiate has broadly been demonstrated (Zuk *et al*., 2002; Schäffler and Büchler, 2007); however, there are not studies considering the AT-MSCs differentiation into Sertoli cell. Although previous studies have shown that MSCs maintained in media containing RA differentiate into male germ cells (Nayernia *et al*., 2006; Drusenheimer *et al.*, 2007; Zhang *et al*., 2014) and that TCC contributed to this differentiation (Huang *et al*., 2010; Kaviani *et al*., 2014) additional gene expression and other analyses should be performed to confirm whether the AT-MSCs treated in the present study, differentiate into cell lineage related to male germ cells.

CONCLUSIONS

Adipose tissue from omentum and epididymis fat depots represents source of AT-MSCs with different characteristics. The fat pad location had a significant influence on AT-MSCs differentiation potential. Between the omental and epididymal sources of adipose tissue in mouse, only AT-MSCs isolated from the adipose tissue deposited on omental region expressed *Gdnf*, an important gene related to Sertoli cells, after differentiation treatment with retinoic acid.

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