Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

All-*trans* retinoic acid promotes smooth muscle cell differentiation of rabbit bone marrow-derived mesenchymal stem cells*

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Received Dec. 27, 2009; Revision accepted Apr. 7, 2010; Crosschecked June 13, 2010

Abstract: Bone marrow-derived mesenchymal stem cells are multipotent stem cells, an attractive resource for regenerative medicine. Accumulating evidence suggests that all-*trans* retinoic acid plays a key role in the development and differentiation of smooth muscle cells. In the present study, we demonstrate, for the first time, that rabbit bone marrow-derived mesenchymal stem cells differentiate into smooth muscle cells upon the treatment with all-*trans* retinoic acid. All-*trans* retinoic acid increased the expression of myocardin, caldesmon, 22-kDa smooth muscle cell-specific protein (SM22α), and SM-myosin heavy chains in rabbit bone marrow-derived mesenchymal stem cells, as detected by reverse transcription polymerase chain reaction (PCR). Immunostaining of SM22α and SM-myosin heavy chains using monoclonal antibodies also indicated smooth muscle cell differentiation of rabbit bone marrow-derived mesenchymal stem cells following the treatment with all-*trans* retinoic acid. In addition, more than 47% of bone marrow-derived mesenchymal stem cells demonstrated the contractile phenotype of smooth muscle cells. Western blot results showed that SM-1 and SM-2 were highly expressed in the differentiated cells. These results suggest that all-*trans* retinoic acid may serve as a potent agent for functional smooth muscle cell differentiation in tissue engineering.

Key words: Bone marrow-derived mesenchymal stem cells, Smooth muscle cells, All-*trans* retinoic acid, Rabbit
doi:10.1631/jzus.B0900415 **Document code:** A **CLC number:** Q25

1 Introduction

Bone marrow-derived mesenchymal stem cells (BMSCs) possess multipotency and can differentiate into osteoblasts, adipocytes, chondrocytes, neurons, hepatocytes, endothelial cells, and smooth muscle cells (SMCs) (Wakitani *et al*., 1995; Pittenger *et al*., 1999; Reyes *et al*., 2002). In vitro, BMSCs treated with transforming growth factor beta (TGF-β) or thromboxane A_2 differentiate into SMCs (Wang *et al*., 2004; Kim *et al*., 2009). In vivo studies have also shown a regeneration of vasculature in infarcted myocardium following treatment (Davani *et al*., 2003; Yoon *et al*., 2005). Moreover, BMSCs can be easily obtained and expanded in vitro, which makes them an excellent source of SMCs for in vitro and in vivo studies.

All-*trans* retinoic acid (atRA) plays a key role in SMC development and maturation. SMCs are heterogeneous cells; however, they can roughly be classified as "contractile cells" and "synthetic cells." Contractile SMCs express a series of specific contractile proteins, such as smooth muscle α-actin (SM α-actin), 22-kDa smooth muscle cell-specific protein (SM22α), desmin, calponin, myosin heavy chain (SM-MHC), and smoothelin (Frid *et al*., 1992; van der Loop *et al*., 1996; Owens *et al*., 2004). These contractile proteins are down-regulated in synthetic SMCs, which exhibit a higher proliferative activity.

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* Project supported by the Science and Technology Department of Zhejiang Province (No. J20020579-30116) and the Central Hospital of Huzhou City (No. H20010984-32536), Zhejiang Province, China

In addition, atRA has been shown to inhibit the proliferation of vascular SMCs by inhibiting the expression of cyclins and cyclin-dependent kinases (Kosaka *et al*., 2001). SMCs are more likely to exhibit a contractile phenotype when treated with atRA, and expression of SMC specific contractile proteins is significantly enhanced (Neuville *et al*., 1999; Axel *et al*., 2001). Retinoids are also very important during normal embryonic development (Ross *et al*., 2000). In vitro atRA treatment induces smooth muscle differentiation of P19 cells (Manabe and Owens, 2001), and recently our lab induced functional SMCs from embryonic stem cells (Huang *et al*., 2006).

Hence, we were interested in whether atRA influences SMC differentiation of BMSCs. Although other studies have demonstrated that atRA has an anti-proliferative effect on BMSCs and that this effect was correlated with up-regulation of the cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p16^{INK4A}$ (Oliva *et al*., 2003), the direct effect of atRA on the SMC differentiation of BMSCs has not definitively been studied. In the present study, we demonstrate, for the first time, that atRA significantly inhibits the proliferation of rabbit BMSCs (RBMSCs) and up-regulates the expression of SMC specific proteins.

2 Materials and methods

2.1 Isolation and culture of RBMSCs

Aspirates were obtained from femoral bones of 3–6 months old New Zealand rabbits. The method of isolating RBMSCs from aspirates was described previously (Pittenger *et al*., 1999). Briefly, mononuclear cells were obtained by density gradient with ficoll. Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% (v/v) fetal bovine serum (FBS) was used for initial culture and expansion. Medium was changed every 2 d. Cells were grown to confluence after initial culture for 10–14 d, and then passaged by digestion with 0.05% (v/v) trypsin. The mesenchymal differentiation potential of RBMSCs was confirmed by Liu *et al*. (2009).

2.2 Test of cell proliferation and viability

Cell proliferation was tested by cell counting. RBMSCs were seeded in a 96-well plate at a density of 6×10^5 cells/cm^2 and incubated with atRA ranging in concentrations from 0.5 to 100 μmol/L. The effect of dimethyl sulfoxide (DMSO) was also tested. Normal cultured RBMSCs served as a negative control. Cells were digested with 0.05% trypsin and counted in a blood cell counting chamber after incubation with atRA or DMSO for 24, 48, 72, or 96 h.

Viability was tested by methyl thiazolyl tetrazolium (MTT) assay. The pattern of incubation was the same for the test of proliferation. Following atRA treatment, cells were incubated with DMEM containing 100 μg MTT for 4 h. Next, formazan was dissolved in 150 μl DMSO and absorption was measured with an enzyme-linked immunosorbent assay (ELISA; Bio-Rad, CA, USA) at 490 nm.

Data are expressed as mean±standard error (SE) and graphs were made using Orgin 8.0.

2.3 Smooth muscle differentiation of RBMSCs

Based on results from the cell proliferation and viability experiments, a concentration of 50 μmol/L atRA was chosen for smooth muscle differentiation. RBMSCs at passage 3 were seeded at a density of 6×10^3 cells/cm^2 and incubated with DMEM supplemented with 10% (v/v) FBS and 50 μmol/L atRA for 4 d. The medium was changed every day. One batch of the differentiated cells was further cultured in DMEM supplemented with 15% FBS, and the proliferative ability of these cells was tested.

The atRA (Sigma, USA), dissolved in DMSO at a concentration of 10 mmol/L and stored at −80 °C, served as a stock solution. Control cultured cells were supplemented with an equal volume of DMSO.

2.4 Reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed to investigate SMC marker gene expression of RBMSCs treated with atRA. The total RNA of cell samples was isolated using Trizol (Invitrogen, USA), then complementary DNA was synthesized by using M-MLV (Takara), and finally PCR was performed. The primer sequences of serum response factor (SRF) were designed based on the homologous genes of human and mouse. All RT-PCR experiments were repeated three times. The information on primer sequences is listed in Table 1.

2.5 Immunocytochemistry

The protocol for immunocytochemistry was

Table 1 List of primer sequences information

Gene name	Primer sequences	Accession number
SM22α	Sense: AAACCCGTCGGACCGT; anti-sense: AACAAATGGAGCAGGTGG	AY336996
SM-MHC	Sense: TCAACCAAACCCAACCCA; anti-sense: CACCCATGTCATGTAAACGAAT	NM_001082308
Caldesmon	Sense: GCTTGCTGTGCTCCTGCTT; anti-sense: GAGGTCCACTTCTGAGACTTCC	NM_001082256
SRF	Sense: GGCGCTACACGACCTTCA; anit-sense: AGCCAGTGGCACTCATTCT	NM_003131.2
Myocardin	Sense: TTGGGAAACAATGGAGTG; anti-sense: TTGAGATCCGTGACATCC	AY566292
GAPDH	Sense: GCCCAGAACATCATCCCT; anti-sense: ACTGTTGAAGTCGCAGGAGA	L23961

SM22α: 22-kDa smooth muscle cell-specific protein; SRF: serum response factor; GAPDH: glyceraldehyde phosphate dehydrogenase

described previously (Owens *et al*., 2004). Cells were seeded in 24-well plates, fixed with 4% (w/v) paraformaldehyde for 1 h, and then incubated with phosphate buffered saline (PBS) containing 5% (v/v) goat serum for 30 min. Next, PBS containing 0.3% (v/v) Triton was applied and 1 μg of primary antibody in 100 μl of PBS was incubated with the cells for 1 h. Rhodamine-labelled goat anti-mouse immunoglobulin G (IgG) antibody (Sigma) was used at a concentration of 10 μg/ml. Primary antibodies used in this study included SM22α (Abcam, UK) and SM-MHC (Boster, China). The cell nuclear stain Hoechest 33258 (Sigma) was also used. All steps were performed at 37 °C and PBS was used as the negative control.

2.6 Flow cytometry analysis

The ratio of fully differentiated cells was determined using rhodamine-conjugated anti-SM22α and anti-SM-MHC (SM-1 and SM-2) antibodies. RBMSCs treated with 50 μmol/L atRA for 4 d were harvested and incubated with anti-SM22α and anti-SM-MHC antibodies. Then cells were analyzed using a flow cytometer (Beckman Coulter-FC 500 MCL, USA) and data analyses were performed using FlowJo 2.0 software.

2.7 Western blot

A total of 5×10^5 cells were collected and proteins were extracted using RIPA buffer (50 mmol/L Tris-HCl (pH 7.4), 1% (v/v) Triton X-100, 0.2% (w/v) sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L ethylene glycol tetraacetate (EGTA), 0.1% (w/v) sodium dodecyl sulphate (SDS), and 1 mmol/L phenylmethanesulfonyl fluoride). Then the bicinchoninic acid (BCA) protein reagent was used to determine the protein concentration. A total of 100 μg proteins were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes which were incubated with 2% (w/v) bovine serum albumin for 1 h at 37 °C. The PVDF membranes were incubated with a primary mouse anti-rabbit SM-MHC antibody overnight at 4 °C. The secondary antibody (horseradish-peroxidase (HRP) conjugated anti-mouse IgG antibody; Epitomics Inc., USA) was added at a dilution of 1:4000. After washing with PBS twice, an electrochemiluminescence (ECL) system was used for the detection of protein bands. β-actin served as an internal control.

2.8 Contractility assays

Vaso-active agent-induced contractile activity of SMC-differentiated RBMSCs was also tested (Touyz *et al*., 1999). At Day 4 of culture, the differentiated cells were washed with PBS and 10^{-6} mol/L angiotensin II in DMEM supplemented with 10% FBS was added and incubated for 5 min. The same field was imaged before and after angiotensin II stimulation.

3 Results

3.1 Inhibition of cell proliferation and viability by atRA

The effect of atRA on cell proliferation and viability was investigated. As Fig. 1 shows, the proliferation and viability of RBMSCs were inhibited by atRA in a concentration-dependent manner. Treatment with DMSO, the solvent used for atRA delivery, barely had an effect on the proliferation and viability of RBMSCs, compared to normal cultured cells. Thus, the anti-proliferative effect is mainly produced by atRA. Although 100 μmol/L atRA showed the strongest inhibitory effect on cell proliferation and viability, cell death was observed (data not shown). Thus, 50 μmol/L atRA was employed in our induction system. The experiment was repeated with five batches of RBMSCs from three animals.

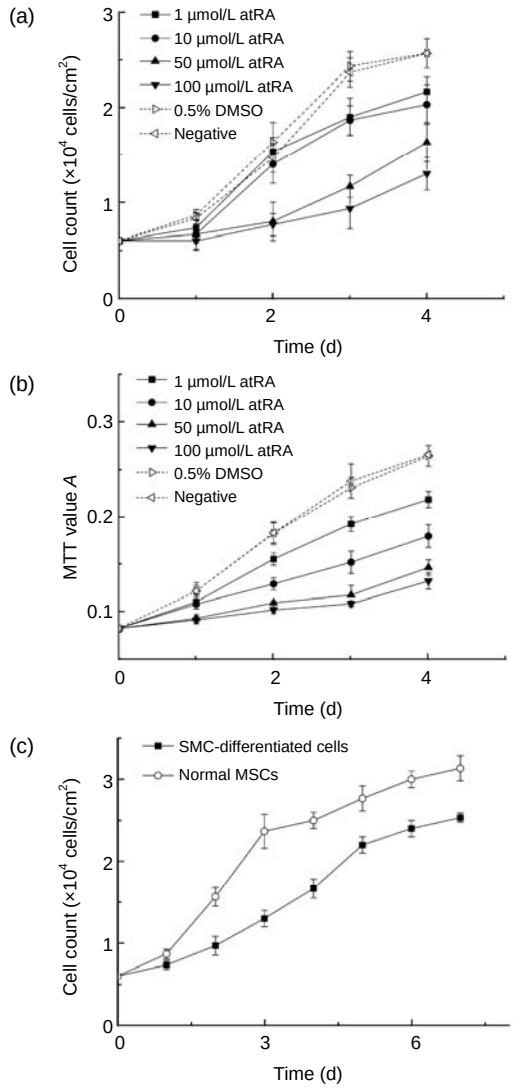

Fig. 1 Inhibition of RBMSCs' proliferation and viability by atRA at concentrations ranging from 1 to 100 μmol/L
(a) The cell densities of RBMSCs treated with different concentrations of atRA and controls (DMSO); (b) MTT assay showing that atRA has an inhibitory effect on cell viability in a concentration-dependent manner; (c) Proliferative ability of SMC-differentiated RBMSCs. Normal cultured RBMSCs served as a control

3.2 Morphological changes of atRA-treated RBMSCs

Based on results from the cell proliferation and viability experiments described above, 50 μmol/L atRA was used for the in vitro differentiation of RBMSCs into SMCs. As Fig. 2 shows, the majority of cells treated with atRA for 4 d had smoother margins than the control cultured RBMSCs treated with DMSO. Moreover, in long-term culture (more than 6 d), the control cultured cells grew in multiple layers. The cells treated with atRA, however, grew in a monolayer, which is not a typical mesenchymal stem cell growth pattern.

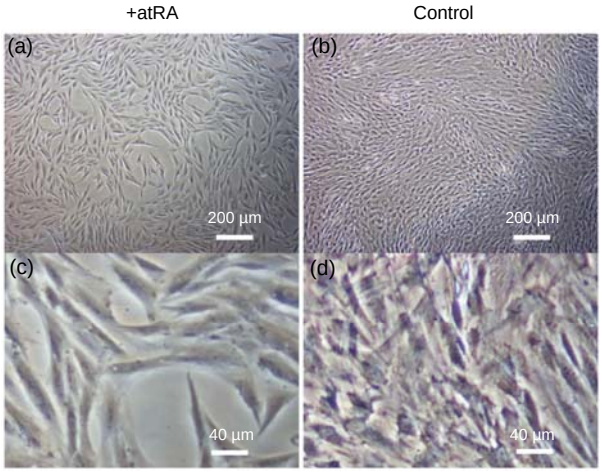

Fig. 2 Cell morphology and growth characteristics of RBMSCs
(a) & (c) RBMSCs cultured in medium supplemented with 50 μmol/L atRA were documented with phase contrast microscopy at two different magnifications; (b) & (d) RBMSCs cultured in control medium at two different magnifications

3.3 Identification of smooth muscle differentiation

RT-PCR showed that RBMSCs express a series of SMC-marker genes following the treatment with atRA. These genes include functional genes, such as SM22α and SM-MHC, and important transcriptional regulators, such as SRF and myocardin (MyoCD). As Fig. 3a shows, in 2 d, atRA has initiated the expression of myocardin, caldesmon, and SM22α, suggesting that RBMSCs had begun to differentiate toward SMCs. At Day 4, the expression of SM22α, caldesmon, and myocardin was significantly enhanced and SM-MHC, a mature gene marker of SMCs, was highly expressed. This result indicates that the RBMSCs have obtained a typical characteristic of contractile SMCs. Compared to control cultured cells, the expression of SRF was barely changed during the treatment with atRA.

Immunofluorescence was used to confirm the SMC differentiation of RBMSCs treated with atRA for 4 d. As Fig. 4 shows, a large portion of RBMSCs

treated with atRA were SM22α- and SM-MHC-positive at this time point. Few of the control cells were positively stained with the anti-SM22α monoclonal antibody and they were completely negative for anti-SM-MHC monoclonal antibody staining. Flow cytometry analysis was used to quantitatively analyze the efficiency of the atRA-induced differentiation of RBMSCs into SMCs. As Fig. 3b shows, over 56% of the differentiated cells expressed SM22α and 47% of the differentiated cells expressed SM-MHC.

Although three isoforms of SM-MHC exist (SM-1, SM-2, and SMemb), only SM-1 and SM-2 were detected in mature SMCs. Western blot analysis was carried out to determine whether fully differentiated makers of SMCs were expressed. As Fig. 3c shows, SM-1 and SM-2 were almost equally expressed in the cells treated with atRA and both were undetectable in control cultured cells. These results suggest that the RBMSCs obtained a mature SMC phenotype.

The ability to contract in response to a vasoactive agent is considered to be a functional characteristic of mature SMCs. As Fig. 3d shows, upon angiotensin II treatment, a large number of the differentiated cells either shortened or narrowed in morphological appearance. This result indicates that the differentiated cells obtained a contractile ability similar to SMCs. The proliferative ability of the differentiated cells cultured in medium without atRA was also tested (Fig. 1c). Results indicate that the cells can be passaged every 7 d and stably maintained in culture for at least another 30 d.

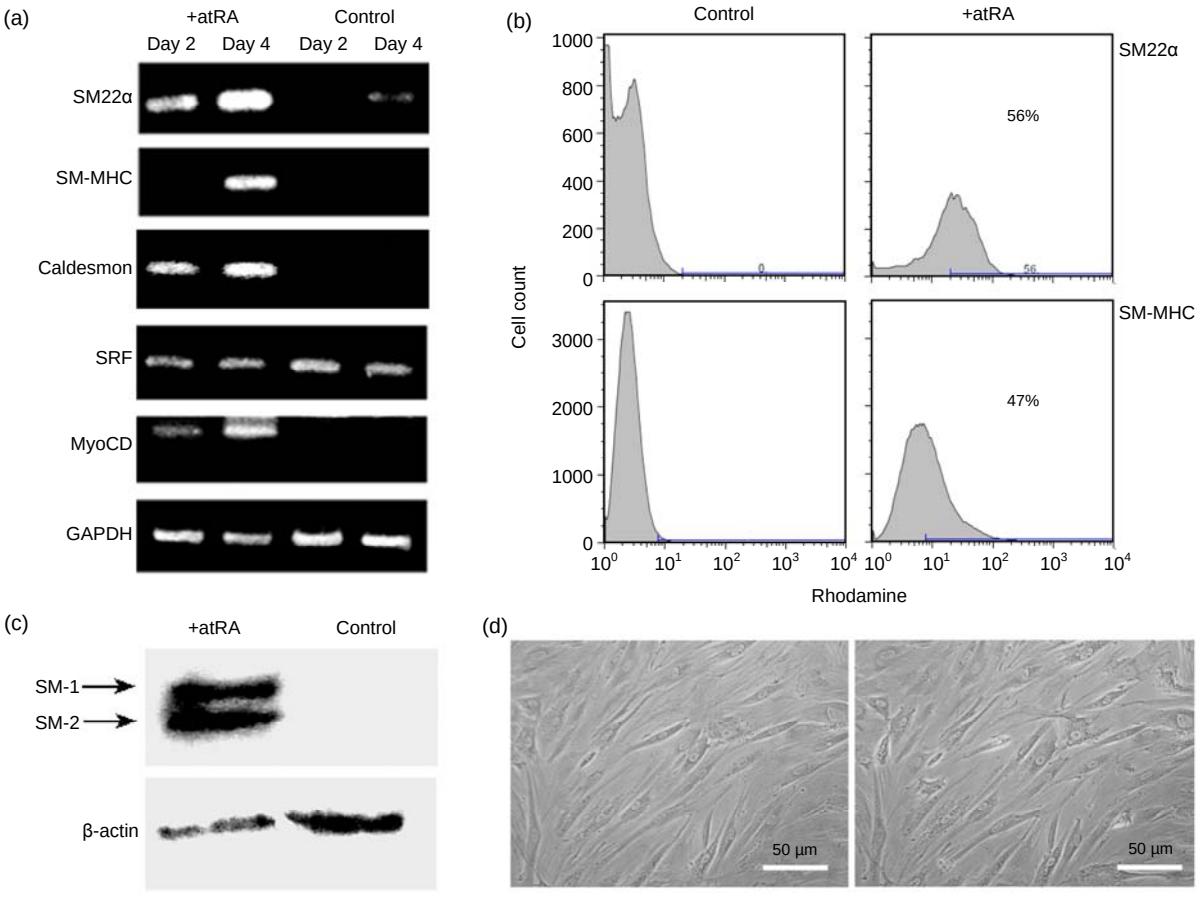

Fig. 3 SMC identification of RBSMCs

(a) RT-PCR showing changes in the expression of SMC-marker genes during the treatment with atRA. The expression of SMC-marker genes, such as SM22α and SM-MHC, was significantly increased on Day 4; (b) Flow cytometry analysis of SMC-differentiated RBMSCs. 56% of the atRA-treated cells expressed SM22α and 47% of the atRA-treated cells expressed SM-MHC; (c) Western blot analysis of differentiated cells. SM-MHC (SM-1 and SM-2) was expressed in RBMSCs treated with atRA for4 d and it was undetectable in control cells; (d) The differentiated cells before (left) and after (right) the stimulation of angiotensin II

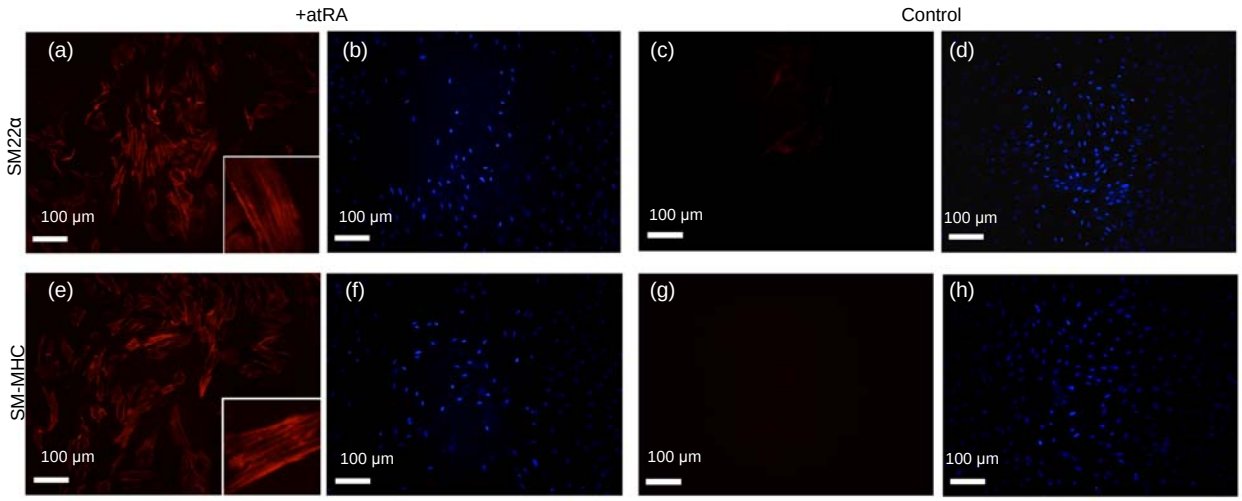

Fig. 4 Immunological staining of cells with anti-SM22α and anti-SM-MHC monoclonal antibodies
(a) & (e) Immunological staining of RBMSCs treated with 50 μmol/L atRA for 4 d with anti-SM22α (a) and anti-SM-MHC (e) antibodies; (c) & (g) Immunological staining of RBMSCs cultured in control medium with anti-SM22α (c) and anti-SM-MHC (g) antibodies. (b), (d), (f), and (h) are Hochest 33258 staining of the same fields of views as (a), (c), (e), and (g)

4 Discussion

SMCs are distributed in blood vessel, gastrointestinal tract, bladder, etc. SMC dysfunction can cause serious health problems, such as atherosclerosis, hypertension, and gastrointestinal disease. Therapies based on cell transplantation may be an excellent solution for regenerating tissues impaired by SMC dysfunction. Thus, finding a reliable source of SMCs for transplantation is important. Previously, functional SMCs were induced from embryonic stem cells in our lab. Teratoma formation and immunologic rejection of derivatives from embryonic stem cells, however, have become major obstacles for their clinical application (Huang *et al*., 2006; Blum and Benvenisty, 2008).

BMSCs are multipotent cells that have the potential to differentiate into SMCs in vivo and in vitro. BMSCs display a strong immunosuppressive effect towards T cells, B cells, natural killer cells, and antigen presenting cells. Furthermore, teratoma formation of BMSCs is rarely reported (Di Nicola *et al*., 2002; Krampera *et al*., 2003; Le Blanc *et al*., 2003; Tse *et al*., 2003). BMSCs can be easily obtained and expanded in vitro. Thus, BMSCs are an excellent candidate for SMC transplantation. The mechanisms of SMC differentiation from BMSCs, however, have not been clearly studied.

Since atRA plays a key role in theSMC development, we were interested in whether atRA would effect SMC differentiation of BMSCs. The present study demonstrates that atRA inhibits proliferation of BMSCs in a concentration-dependent manner and promotes SMC differentiation.

Contractile SMCs were defined by several unique markers, including SM α-actin, SM22α, caldesmon, calponin, SM-MHC, and smoothelin. In our studies, RBMSCs treated with atRA acquired an SMC morphology and the expression of both early and late markers of SMC differentiation, SM22α and SM-MHC, was significantly increased. SM22α, an early marker of contractile SMCs, was also detected in control cultured cells at an RNA level, but its expression level was minimal when compared with the atRA-treated cells. SM-MHC, a late marker of SMC differentiation, was only detected at a genetic level in cells treated with atRA. These results were confirmed by immunocytochemistry at the protein level. Western blot analysis showed that SM-1 and SM-2, both markers of fully differentiated SMCs, were highly expressed in RBMSCs treated with atRA. Moreover, the differentiated cells could contract in response to angiotensin II. These results indicate that atRA induced differentiation of functional SMCs from RBMSCs. This treatment may serve as a potent tool for cell transplantation or tissue engineering requiring

abundant functional SMCs.

In summary, our results demonstrated that atRA exhibits a strong anti-proliferative effect on RBMSCs and promotes SMC differentiation of RBMSCs. Furthermore, atRA treatment increases the expressions of SMC specific transcripts and contractile proteins. Thus, atRA can fully induce the differentiation of RBMSCs into SMCs.

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