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Function of TGF-beta and p38 MAKP signaling pathway in osteoblast differentiation from rat adipose-derived stem cells

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Abstract. – BACKGROUND: Adipose-derived stem cells (ADSCs) are multipotent progenitors that can commit to osteoblast, chondrocyte, adipocyte and several other lineages. The proper utilization of stem cells for clinical application requires an integrated understanding of multiple signal inputs that control maintenance of stemness, proliferation and differentiation.

MATERIALS AND METHODS: In this study, ADSCs in rat were isolated from subcutaneous tissues of abdomen and inguinal fat pads, purified and expanded *in vitro*. Bone morphogenetic protein 2, TGF-beta 1, SB203580 (P38 MAPK inhibitor), Noggin (BMP inhibitor) and SB431542 (TGF-beta inhibitor) were used for differentiation into osteoblasts.

RESULTS: Both TGF-beta signaling pathway and p38 MAKP signaling pathway could affect the differential direction of the ADSCs. PCR assays indicated that both TGF-beta signaling pathway and p38 MAKP signaling pathway played a crucial roles in osteoblasts differentiation of the ADSCs, the members included Smad 1, Smad 5, Smad 8, P38, ASK1, MKK3, MKK6, Runx 2, collagen type 1, and osteopontin.

CONCLUSIONS: This research provides a theoretical basis and experimental evidence for therapeutic application of rat ADSCs to treat bone injury.

Key Words:
Rat, ADSCs, Osteoblast, TGF-beta signaling pathway, p38 MAKP signaling pathway.

Abbreviations

ADSCs = adipose-derivated stem cells; BMSCs = bone marrow stem cells; TGF-β = transforming growth factor-beta; MAPK = mitogen-activated protein kinase; TRAF = TNF receptor associated factor; ERK = extracellular signal-regulated kinase; JNK = Jun N-terminal kinase; DMEM = Dulbecco's modified eagle medium; bFGF = basic fibroplast growth factor; PBS = phosphate buffered saline; BMP-2 = bone morphogenetic protein 2; ALP = alkaline phosphatase; DAXX = Death associated Protein 6; p38 = mitogen activated protein kinase; ASK 1 = Apoptosis signal-regulating kinase 1; MKK3 = MPA kinase kinase 3; RUNX2 = Runt-related transcription factor 2; GAPDH = Glyceraldehyde 3 phosphate dehydrogenase.

Introduction

Adult stem cells hold great promise in tissue repair and regeneration. The basic principle involves an appropriate cell source and a biocompatible and biodegradable scaffold to produce a construct that mimics the target site structurally and functionally. Adipose tissue is derived from the embryonic mesenchyme and contains a stroma, in which the ADSCs can be induced to multiple lineages in specific culture system similar to bone marrow stem cells (BMSCs)[1-4]. Many groups working independently have shown that adult stem cells derived from white adipose tissues can differentiate along multiple pathways *in vitro*, including the adipocyte, chondrocyte, endothelial, epithelial, hematopoietic, support, hepatocyte, neuronal, myogenic, and osteoblast lineages[3,5-9]. The skeletal tissue is composed of various types of mesenchymal cells such as osteoblasts, chondrocytes, myoblasts and bone marrow stromal cells including adipocytes. Bone formation is carried out by the osteoblasts, and bone resorption is carried out by the osteoclasts. ADSCs are easy to isolate, culture, and manipulate *in vitro* and have great plasticity, for these reasons they have become an important tool in cell replacement therapy and are considered as candidates for bone injury.

The transforming growth factor-beta (TGF-β) superfamily is comprised of over forty members, such as TGF-βs, Nodal, Activin, and bone morphogenetic proteins (BMPs)[10]. TGF-β/BMPs have widely recognized roles in bone formation during mammalian development and exhibit versatile functions in the body[11,12]. Disruptions of TGF-β/BMP signaling have been implicated in

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multiple bone diseases including tumor metastasis, brachydactyly type A2, and osteoarthritis[13-20]. TGF-β is abundant in bone and plays a critical role in bone remodeling[21], which is a complex process and relies on the interplay between bone resorption and formation that involves osteoclasts, osteoblasts, and osteocytes[22]. In the early stages of osteoblastic differentiation, TGF-β can provide competence, but at late stages of osteoblastic differentiation, TGF-β acts as an inhibitor[23]. This maturation stage-dependent effect of TGF-β was also confirmed in highly pure CD14 osteoclast precursor cells[24]. MAPK family members, which are proline-directed serine/threonine kinases, function in various signaling cascades, including TRAF-mediated ones[25,26]. MAPK family members are classified into three groups: the ERK, JNK, and p38 MAPK groups. p38 MAPK was originally identified as the target of pyridinylimidazole compounds that inhibit the production of inflammatory cytokines in monocytes[27]. Pyridinylimidazole SB203580, a specific inhibitor of p38 MAPK[28], has been widely used to investigate the roles of p38 MAPK in the regulation of cell differentiation and function[25,26,29]. Using SB203580, p38 MAPK-mediated signals were shown to be involved in osteoclastic bone resorption induced by IL-1 and TNF in fetal rat long bones[29,30]. These results suggest that p38 MAPK-mediated signals regulate osteoclast differentiation or function, or both.

To further understand how TGF-beta and p38 MAPK regulates osteoblast differentiation, we explored the roles of TGF-beta and p38 MAPK mediated signals in the differentiation of osteoclasts from rat ADSCs in the present study.

Materials and Methods

Isolation, Culture and Identification of Rat ADSCs

Adipose tissues were separated from subcutaneous tissues of abdomen and inguinal fat pads of adult rat. All animal experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Hebei Medical University. All operations were conducted aseptically. The adipose tissues were washed 3 times with phosphate buffer saline (PBS) containing 100 IU/ml penicillin and 100 μg/ml streptomycin to remove connective tissue membrane and capillaries. The adipose tissues were chopped into small pieces (about 1 mm^3) and digested with 0.1% (m/v) type collagenase (Sigma, St, Louis, MO, USA) at 37°C for 1 h. Enzymatic digestion was then neutralized with fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA). The suspension was filtered with an 80 μm Cell Strainer and centrifuged at 300 g for 5 min at room temperature. Then the supernatant was discarded and the cells were resuspended with a complete medium containing L-DMEM, 10% (v/v) FBS, 10 ng/ml bFGF, and 2 mM L-glutamine, 1% B-27 (m/v) (all from USA Gibco, except bFGF from Peprotech, Rocky Hill, NJ, USA). The cell suspension was plated and incubated at 37°C with 5% CO_2. The culture medium was changed every three days and the non-adherent cells were removed[31]. When the cells reached 70-80% confluence, then 0.25% (m/v) trypsin and 0.02% EDTA (Gibco, Carlsbad, CA, USA) were added to dissociate the cells from plates, and trypsinization was terminated with the complete medium. Immunofluorescence was used to detect the special surface antigen of rat ADSCs for identification. The surface antigen of ADSCs included CD13, CD29, CD44, CD71, CD73, CD90, CD105, CD31 and STRO-1.

Immunofluorescence Staining

ADSCs were fixed with 4% paraformaldehyde in PBS for 15 min, followed by three rinses with PBS. The cells were then permeabilized with 0.1% Triton X-100 for 15 min. After rinsing three times with PBS, the cells were blocked with goat serum or 4% bovine serum albumin (BSA) in PBS for 30 min. The cells were then incubated with primary antibodies in a humidified chamber at 4°C overnight. After three washes with PBS for 5 min each, the cells were incubated with FITC labeled secondary antibodies at room temperature for 1 h, the cells were rinsed three times with PBS for 5 min each. Finally, nuclei were labeled by incubation with 4,6 diamidino-2-phenylindole (DAPI) (Sigma, St, Louis, MO, USA). The cells were examined by a phase contrast fluorescence microscope (Olympus, Tokyo, Japan).

PCR

RNA was extracted from ADSCs using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse transcribed, followed by 30 PCR cycles using RNA PCR kit ver 3.0 (Taraka, China). Information of gene specific primer pairs was listed in Table I. PCR was performed in 50 μl of mixture containing 10 l of 5×PCR Buffer (Taraka, China), 28.5 μl of ddH_2O, 0.25 μl of Ex-Taq (Taraka, China), 0.5 μl of forward and reverse

Table I. Primer sequences used in PCR assay.

Gene	Primer sequence	Tm (°C)	Cycle	Fragment size (bp)
Smad 1	F 5' CGACACATCGGGAAAGGAGTC 3'			
	R 5' TTCACAGACTGCGCCAGTAG3'	55	30	181
Smad 5	F 5' TGCACCATCCTGAACCTGAC 3'			
	R 5' CGACAGGACATGGACAACCA 3'	60	30	114
Samd 8	F 5' AACTGCAGACTGTCCAGACG 3'			
	R 5' TTGGGAATCTGAAAGCCCCC 3'	55	30	115
MKK 3	F 5' GATCGCTGTGTCTATCGTGC 3'			
	R 5' CGAGGCTCCAGACATCAGAC 3'	58	30	112
MKK 6	F 5' CCTGCAGCTTGCATCTTTGT 3'			
	R 5' TCGCTTCTTGCCTTTCGACT 3'	60	30	121
ASK 1	F 5' CATGGCGGTGAAGCGGATA 3'			
	R 5' GGTCACGGTAAACGGACAGT3'	60	30	109
DAXX	F 5' ATTCCGGTGAGGGTCCTAGT 3'			
	R 5' CAAGATGGTGGGGAGGGAAC3'	60	30	143
Runx2	F 5' TAGAGCTCTCCCCTGTGACC 3'			
	R 5' TTTGGGATGAACGTGGAGGG 3'	55	30	159
Collagen type I	F 5' CAGGCTGGTGTGATGGGATT 3'			
	R 5' GACCACGGGCACCATCTTTA 3'	55	30	164
Osteopontin	F 5' AATGAATCCGACGATGCCGA 3'			
	R 5' CACGTGTGAGCTGAGGTCTT 3'	55	30	273
P38	F 5' GCTACTACACGGAATCACCA 3'			
	R 5' GGGCTCCACTGTCACTCA 3'	54.9	30	234
GAPDH	F 5' TTGCTGTCGCCCGTTCG 3'			
	R 5' CCAGCATCACCCCACTTGAT 3'	60	30	232

primers, and 1.5 μl of template cDNA. The cycling conditions consisted of one initial 2-min cycle at 94°C, followed by 30 30-s cycles at 94 (denaturation), one 30-sec cycle at 50-60°C (annealing), and one 2-min cycle at 72 (extension). PCR products were detected by 2.5% agarose gel electrophoresis. Real-time PCR was performed in a 20 l mixture containing 10 l SYBR premix Ex Taq buffer (Takara, China), 0.4 l ROX Reference Dye, 0.8 M each of forward and reverse primers (Table I), 1 μl template cDNA and 7 μl ddH_2O. The cycling conditions consisted of initial 10 sec at 95°C followed by 40 cycles of two-temperature cycling: 5 sec at 95°C (for denaturation) and 34 sec at 60°C (for annealing and polymerization). Each experiment was performed with duplicates in 96-well plate and repeated three times. Gene expression was detected on an ABI 7500 real-time PCR system (San Mateo. CA, USA). The expression level was calculated by the $2^{-\Delta\Delta Ct}$ method to compare the relative expression.

Osteogenic Differentiation of ADSCs

The ADSCs were plated to 24-well plates for osteogenic differentiation. When the confluence of cells reached 80% the medium was removed. The cells were washed with PBS for 3 times. Then, DMEM medium containing 5 ng/ml TGF-beta 1, 300 ng/ml BMP-2 (Peprotech, Rocky Hill, NJ, USA) and 10% FBS was added for the induced group (group A)[32,33]. Induced medium containing 5 ng/ml TGF-beta 1, 300 ng/ml BMP-2, SB203580 (10 M, Peprotech, USA) as P38 MAPK inhibitor and 10% FBS as a group B. Induced medium containing 5 ng/ml TGF-beta 1, 300 ng/ml BMP-2, SB31542 (10 μM, Peprotech, USA) as TGF-beta inhibitor and 10% FBS as a group C. Induced medium containing 5 ng/ml TGF-beta 1, 300 ng/ml BMP-2, Noggin (100 ng/ml, Peprotech, USA) as TGF-beta inhibitor and 10% FBS as a group D. The SB431542 (TGF-beta inhibitor) was added into every induced group after 6 h induced osteogenic differentiation for inhibition late function of TGF-beta in stem cell differentiation. The control was cultured with conventional complete L-DMEM medium, and induced group and control medium were collected every 3 h respectively to detected alkaline phosphatase (ALP) concentration using alkaline phosphatase assay kit (Abcam, San Francisco, CA, USA). Two days later, the cells were detected for the formation of calcium node using Alizarin Red staining, and the expression of osteoblasts specific genes and TGF-beta and P38 MAPK signals pathway members via RT-PCR and real time PCR assay.

Results

Morphological Features of Primary Cultures

Approximately 6×10^5 ADSCs were yielded from the inguinal fat pads of each rat. Most of the cells that attached to the culture dish surface exhibited a fibroblast-like spindle shaped at first. The cells proliferated quickly in completed medium to form colonies that grew and merged to a uniform confluent cell monolayer after 5 days. The cells doubled every 41.62 h and were maintained in culture for more than 100 days with no sign of senescence or differentiation.

Phenotypic Characterization of Rat ADSCs

Multiple independent groups have examined the surface immunophenotype of ADSCs isolated from human and other species. The expression profile changes as a function of time in passage and plastic adherence. To characterize the ADSCs, CD marker profile (included CD13, CD44, CD29, CD71, CD73, CD90, CD31 and CD105) and STRO-1, a marker used to isolated multilineage progenitors from bone marrow and other tissue, were examined using Immunofloures-cence. Immunoflourescence staining results showed that different passages of ADSCs expressed antigens CD13, CD44, CD71, CD73, CD90, CD105 and STRO-1, but did not express antigens CD31. CD31 antigen is special marker of endothelial cells. There was no significant difference in the positive rates of different passages ($p > 0.05$) (Figure 1).

Osteogenic Differentiation of Rat ADSCs

After the induction with osteoblast inducers, ADSCs had apparent changes in appearance. From the 5 h after the induction, ADSCs in group A changed from fusiform to three-dimension, becoming larger and changing into polygon. As time went by, triangle or polygonal cells increased, and then grew into multilayers, and many crystal particles could be observed.

Alizarin red staining of cells was positive at 2 days after induction. The positive region was

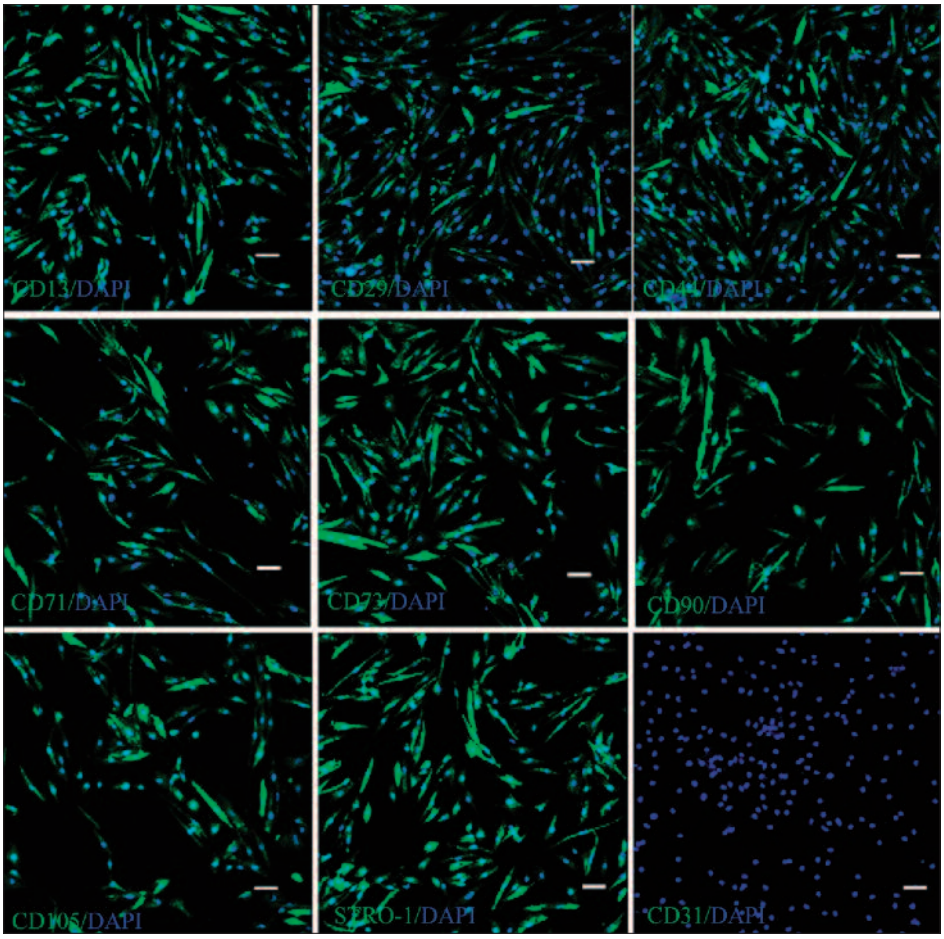

Figure 1. Surface antigen characteristics of rat ADSCs at different passages. Immunoflourescence staining results showed that rat ADSCs at different passages expressed antigens CD13, CD44, CD29, CD71, CD73, CD90 and CD105, but not antigens CD31. (bar = 100 μm).

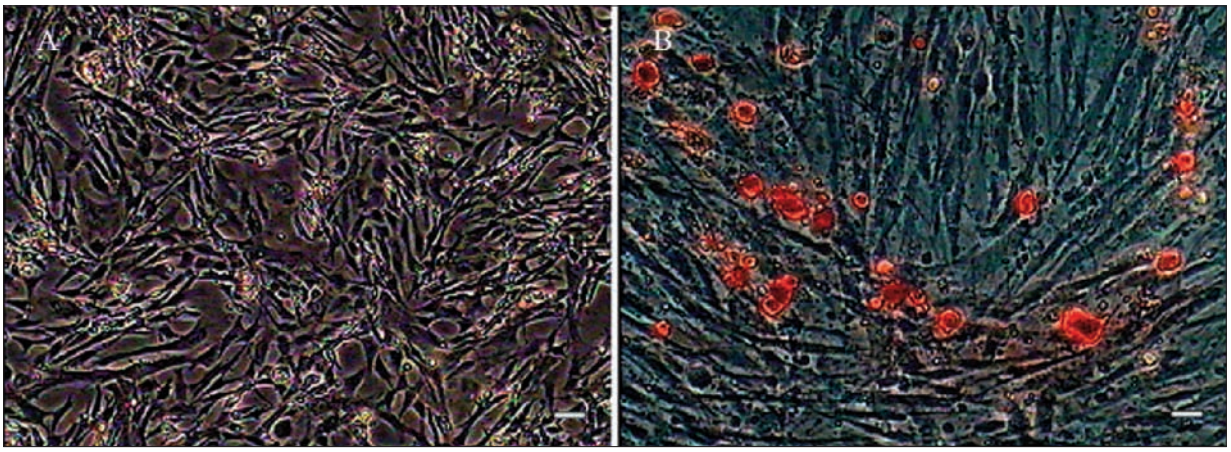

Figure 2. Osteogenic differentiation of rat ADSCs. ***A,*** Rat ADSCs. The cells expanded easily and exhibited fibroblast-like morphology. ***B,*** After induction in osteogenic differentiation for 2 days, the cells metamorphosed from fusiform to tridimensional shapes and the nodules increased in number and size with prolonged inducing time. The nodules were obviously observed following Alizarin Red staining. (bar = 100 μm).

brightly red, showing clear calcium nodules (Figure 2), while the control group was negative. The ALP concentration assay of medium showed that ALP concentration of group A increased with induced time extension, other groups was no significant difference (Figure 3). Real-time PCR and RT-PCR indicated that after incubation with BMP-2 and TGF-beta 1, the specific genes, including Smad 1, Smad 5, Smad 8, ASK1, MKK3, MKK6, p38, Runx2, collagen type 1 and osteopontin were detected, and gene expression level showed a time-lapse increase (Figures 4, 5 and 6).

Discussion

Since bone and cartilage tissue engineering requires large amounts of osteogenic/chondrogenic precursor cells, new sources of progenitor cells are needed. Compared with BMMSC, ADSC have the same ability for osteogenic differentiation, and this ability is maintained with increasing donor age[34,35]. ADSC can easily be isolated from adipose tissue[1,4,36], and they have the potential to differentiate into bone, cartilage and other cells when cultivated under lineage-specific conditions[37,38]. Isolated ADSC can be cryopreserved

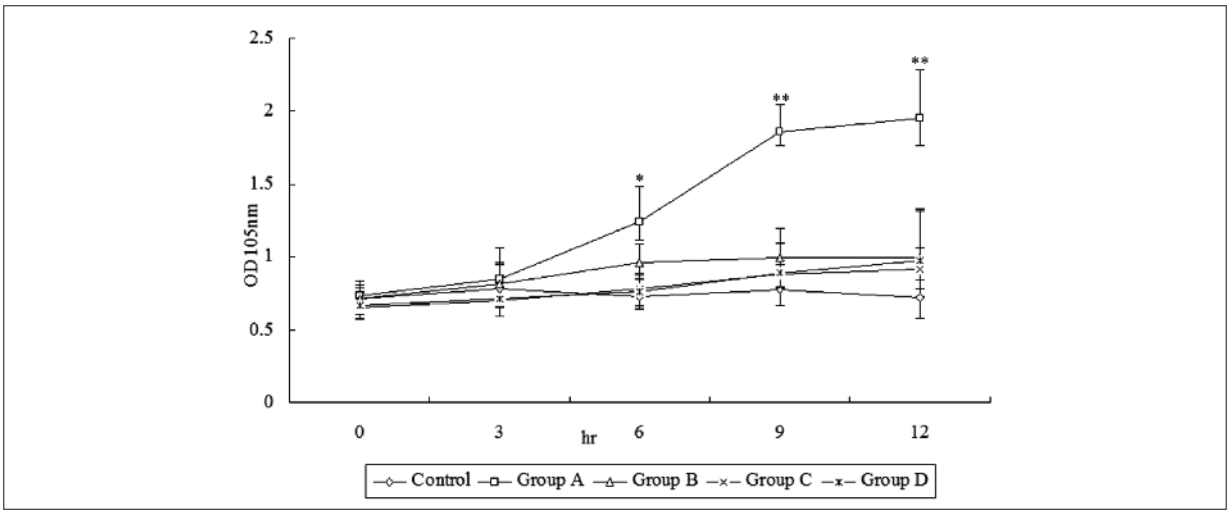

Figure 3. Detection of ALP concentration after Osteogenic differentiation. The ALP concentration assay of medium showed that ALP concentration increased with induced time extension. Group A is significantly different ($p < 0.05$) then other group, this result demonstrated that both TGF beta and p38 MAKP signaling pathway could collaboratively affect the osteogenic differentiation of rat ADSCs.

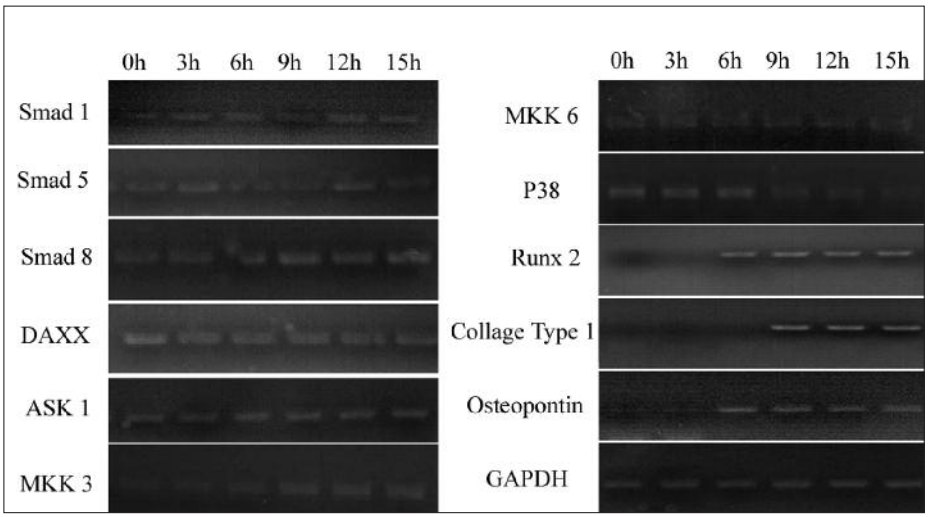

Figure 4. RT-PCR analyses of TGF beta and p38 MAKP signals pathway members and osteoblast markers in osteogenic differentiation.

and expanded easily *in vitro*. Under the conditions commonly used, these cells develop a fibroblast-like morphology. The greatest number of ADSCs can be obtained from cultures plated at low density.

Transforming growth factor β (TGF-beta) and more than 30 related proteins have been identified as members of the TGF-beta superfamily, which includes three isoforms (beta1, beta2, beta3) of TGF-beta, three isotypes of activins, and nearly 20 isoforms of bone morphogenetic proteins (BMP), which are present with special subtypes in all tissues[39]. Transforming growth factor-β is the prototype for a family of proteins, several of which regulate bone and cartilage differentiation. Several lines of evidence have implicated TGF-β, which is deposited in bone matrix by osteoblasts and is released during osteoclastic resorption[40,41]. p38 MAPK is known to play an important role in stress response. It is also involved in cell differentiation[42]. Two of the best studied examples are osteoclasts and myocytes[43,44]. The mechanisms whereby p38 MAPK regulates osteoclast differentiation are not well understood. Previous studies have shown that p38 MAPK activation plays important roles in differentiation of primary calvarial osteoblast, bone marrow osteoprogenitor cells, and some immortalized osteoblast or stromal cell lines. The activation of p38 by TGF-β1 and BMP-2 stimulation in C2C12 cells and the crucial role of p38 in osteoblast differentiation have been reported[45,46]. However, target transcription factors of p38 and TGF-beta pathway responsible for the osteoblast differentiation have been poorly understood. In this study, we provided evidence that one mechanism by which both p38 MAPK and TGF-beta associated regulates osteoblast differentiation is through up-regulating the expression of Smad 1, Smad 5, Smad 8, ASK1, MKK3, p38, Runx 2, Osterix and Osteopontin. Eight Smad proteins are encoded in the human and mouse genomes, four in Drosophila, and three in C. elegans. Smad transcription factors lie at the core of one of the most versatile cytokine signaling pathways in metazoan biology-the transforming growth factor-β (TGFβ) pathway. Only five of the mammalian Smads-Smad1, Smad2, Smad3, Smad5, and Smad8-act as substrates for the TGF-β family of receptors; these are commonly referred to as receptor-regulated Smads, or RS-mads. Smads 1, 5, and 8 serve principally as substrates for the BMP and anti-Muellerian receptors, and Smads 2 and 3 for the TGFβ, activin, and Nodal receptors. ASK1 (Apoptosis signal-regulating kinase 1) also known as mitogen-activated protein kinase kinase kinase 5 (MAP3K5) is a member of MAP kinase kinase kinase family and as such a part of mitogen-activated protein kinase pathway. MKK3 a dual-specificity protein kinase of the STE7 family. Activates p38 MAP kinase by phosphorylating a Thr and a Tyr residue in the activation loop. It is activated by cytokines and environmental stress *in vivo*.

Conclusions

We results demonstrated that TGF-beta and p38 MAPK pathway regulation of early osteoblast genes in rat ADSCs imply that the mechanisms of osteoblastic differentiation should be generalized with great caution.

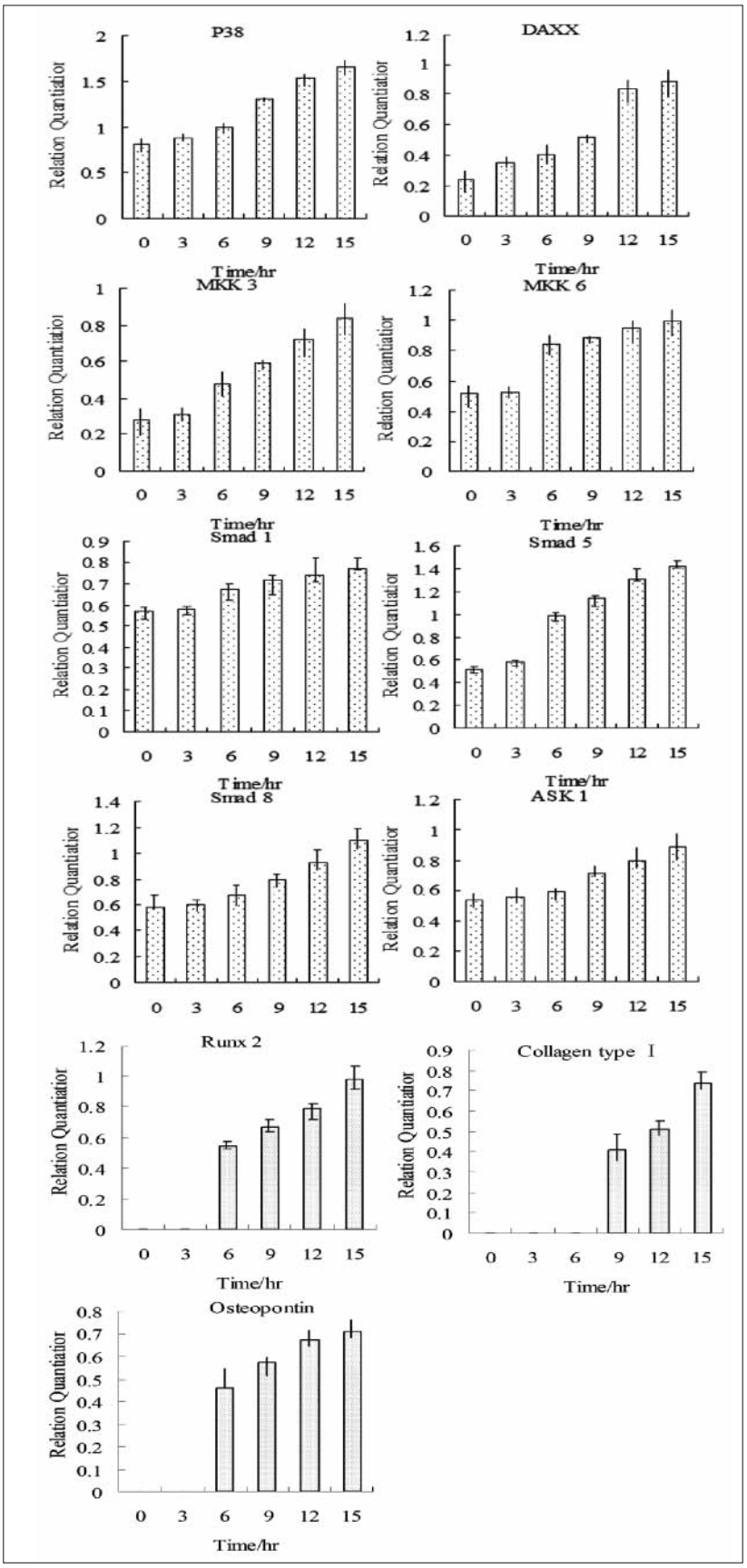

Figure 5. Real time PCR analyses of TGF beta and p38 MAKP signals pathway members and osteoblast markers in osteogenic differentiation. The result indicated that after osteoblast induction the specific genes, including TGF beta signals pathway members (Smad 1, Smad 5 and Smad 8), p38 MAKP signals pathway members (DAXX, ASK1, MKK3, MKK6 and p38) and osteoblast markers (Runx 2, collage type I and osteopontin) were detected, and gene expression level showed a time-lapse increase.

Conflict of Interest

None to declare.

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