AAV Based *Ex Vivo* Gene Therapy in Rabbit Adipose-derived Mesenchymal Stem/Progenitor Cells for Osteogenesis

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Abstract: To explore a new source of adult derived stem cells for musculoskeletal tissue repair and regeneration, we isolated rabbit adipose-derived mesenchymal stem/progenitor cells (rAMSCs) from adult rabbit adipose tissue. *In vitro*, the isolated cells were shown to possess the potential to differentiate into cells of mesenchymal lineages that include osteoblasts. The isolated cells were transduced with AAV2 vectors encoding eGFP, human BMP-2, and human TGF-β1 genes prior to implantation into hind limb muscles of SCID mice to assess osteogenesis *in vivo*. The results showed that rAMSCs were more efficiently transduced with GFP by AAV2 than by AAV6. The AAV2-BMP2- and AAV-TGF-β1-transduced rAMSCs showed efficient expression of BMP-2 and TGF-β1 proteins *in vitro*. When the combination of AAV2-BMP2- and AAV-TGF-β1-transduced rAMSCs were implanted into the thigh muscles of SCID mice, the cells osteogenically differentiated toward new bone formation at the implanted sites 15 days after implantation. This study demonstrates that rabbit adipose tissue contains cells with the potential to give rise to osteoblasts, and that the combination of AAV2-BMP-2- and AAV2-TGF-β1-transduced rAMSCs may provide a novel strategy to enhance new bone formation *in vivo* in less than 3 weeks. In addition, the data suggest that the AAV2 vector is suitable for genetic engineering of rAMSCs.

Keywords: AAV, rAMSCs, BMP-2, TGF-β1, osteogenesis.

INTRODUCTION

A major barrier to developing effective treatments aimed at reversing bone degeneration in musculoskeletal diseases is the low cellularity and vascularity of the affected tissues. Although current medical treatments effectively reduce pain, they are unable to adequately heal the associated defects. Recently developed gene and cell therapies hold promise for overcoming these obstacles [1,2].

Recently, viral vector-based genetic engineering of stem cells has emerged as a promising method to address the need for sustained and robust cellular differentiation and extracellular matrix production for musculoskeletal repair [1-3]. The transduction of these cells with potent therapeutic genes such as morphogenetic proteins and growth factors can further enhance their therapeutic capability prior to implantation in skeletal defects. Unlike other viral vectors, recombinant adeno-associated viruses (rAAV) have recently emerged as a particularly promising delivery vehicle for *in vivo* gene therapy because of their broad cell tropism and less immune response [4]. In addition, their genomes remain episomal form within the nucleus of nondividing cells, which allows long-term gene expression *in vivo* without potential mutagenesis. Furthermore, rAAV2 vectors carrying bone morphogenetic protein genes have shown great potential for improving new bone formation in *ex vivo* gene therapy by genetic modification of mesenchymal stem cells prior to implantation [5,6].

Cell therapies using adult derived mesenchymal stem cells have attracted much attention because these cells can be harvested from a variety of sources including bone marrow, muscle, and fat [7-10]. Adipose tissue as a source of mesenchymal stem cells is particularly attractive because of the ease of its isolation and its abundance in the body. Most studies on adipose-derived mesenchymal stem cells have concentrated on cells isolated from human adipose tissue [11-13]. There is a paucity of information, however, regarding adipose-derived mesenchymal stem cells (AMSCs) from other species. Since the rabbit is commonly used as a model for musculoskeletal degenerative diseases, which are often associated with bone healing and cartilage repair problems [14,15], it would be beneficial to determine the differentiation potential of rabbit AMSCs (rAMSCs) for

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use in evaluating musculoskeletal tissue repair using the rabbit model system.

Therefore, to assess the potential of the AAV vector carrying genes for morphogenetic proteins and growth factors for *ex vivo* therapy of bone formation via rabbit adipose tissue, we evaluated whether the combination of rAMSCs transduced with bone morphogenetic protein 2 (BMP-2) and transforming growth factor β1 (TGF-β1) genes by the rAAV2 vectors are able to enhance osteogenesis and ectopic bone formation in muscle.

MATERIALS AND METHODS

Experimental Animals

All experiments involving animals were approved by the University of Pittsburgh Animal Care & Use Committee. Adult New Zealand white rabbits used for stem cell isolation were purchased from Myrtle's Rabbitry Inc. (Thompsons Station, TN, US) and SCID mice (NOD.CB17 PRKDA SCID/J) used for implantation were purchased from Jackson Laboratories (Bar Harbor, ME, USA).

Cell Isolation and Culture

According to methods described previously [13,16], the adipose tissue was harvested from 3- month-old New Zealand white rabbits in the neck region and was washed extensively in phosphate buffered saline (PBS) containing 1% P/S (v/v). The tissues were minced in 1x PBS, digested with collagenase type I (0.075% w/v) (Sigma St Louis MO) at 37°C for 30 minutes, then neutralized in DMEM, containing 10% fetal bovine serum (FBS). The digests were centrifuged at 1200 rpm for 10 minutes to obtain high-density stromal vascular pellets. The pellets were resuspended in a red blood cell lysis buffer containing 155 mM NH_4Cl in 10 mM Tris-HCl buffer, pH 7.5 (Sigma Chemical Co, St Louis MO) at room temperature for 10 minutes to lyse contaminating red blood cells. The cells were collected by centrifugation and filtered through a 100 μm nylon mesh to remove cellular debris. The recovered cells were incubated in DMEM supplemented with 10% FBS and 1% P/S (v/v) in T-25 flasks at 37°C in 5% CO_2. After 24 hours, the cells were washed in DMEM to remove non-adherent cells. The adherent cells were maintained in DMEM with 10% FBS and 1% P/S and without other supplements to prevent spontaneous differentiation [10]. When the cells reached confluence, they were trypsinized, and aliquots of the cells were cryopreserved in liquid nitrogen. The rest of the cells were expanded in culture for further studies. Human adipose-derived mesenchymal stem/progenitor cells (hAMSCs) were also harvested from patients undergoing liposuction by using same method just described.

rAMSCs Osteogenic Differentiation

The rAMSCs were cultured in osteogenic medium (OM) [17], which consisted of DMEM supplemented with 10% FBS, 0.1μM dexamethasone, 50 μM ascorbate-2-phosphate, 10 mM β-glycerophosphate, and 1% P/S (v/v) until they reached 80% confluence. The cells were cultured in the presence of BMP-2 at concentrations of 0, 12.5, 25, 50, 100, 200, and 400 ng/ml (Sigma Chemical Co, St Louis, MO). The medium was replaced every day for 4 days and supplemented with the same concentrations of BMP-2 as above. After 4 days, the cells were washed, lysed by freezing at -80 ºC, and pelleted by centrifugation; the supernatant was used for the determination of alkaline phosphatase (ALP) activity following methods previously described [18]. HIG-82 cells (gif t from Christopher H. Evans) [19], a rabbit synoviocyte cell line served as negative control. All the experiments were performed in triplicate on three different rAMSC preparations. Calcification was assessed by Von Kossa staining as follows: rAMSCs were cultured in 6-well plates in osteogenic medium containing 200 ng/ml of BMP-2. Medium containing osteogenic factors was changed every 4 days. The control cells were cultured in DMEM without osteogenic factors. After 3 weeks of culture, the medium was aspirated from the experimental and control wells and the cell sheets were washed with PBS (pH 7.4), and fixed for 10 minutes at 4 °C in 0.3 M cacodylic buffer (pH 7.4) (Fisher Scientific, Pittsburgh, PA) containing 2.0% paraformaldehyde. The cell layers were rinsed with 0.1 M cacodylic buffer, and 1.0 ml of filtered 3% silver nitrate was added (Eastman Kodak Company, Rochester, NY). Plates were exposed to ultraviolet light in the dark (Fotodyne Foto/Prep I UV source) for 1 hour and then washed several times in Milli-Q water. Mineralization nodules were photographed and cells were stored in PBS at 4ºC.

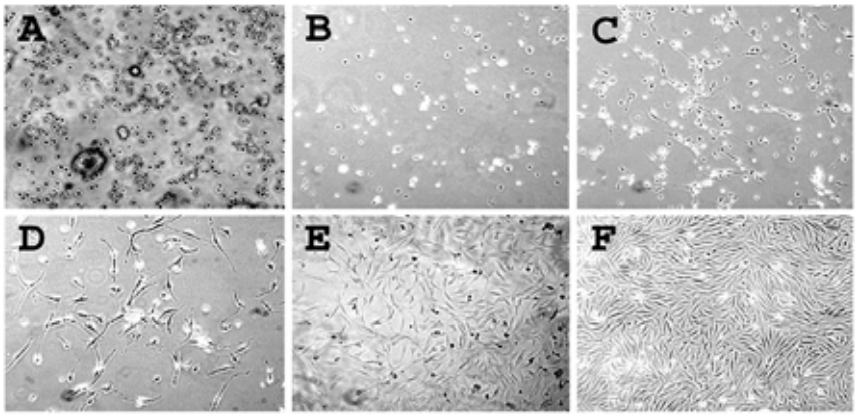


Fig. (1). Morphological change of rAMSCs over a six-day culture period. (**A**) Morphological appearance on day 1 of plating. (**B**) Day 2, most of the cells were attached. (**C**, **D**, **E**) Cells began to expand rapidly in culture from day 3 to 5. (**F**) Day 6, cells reached confluence.

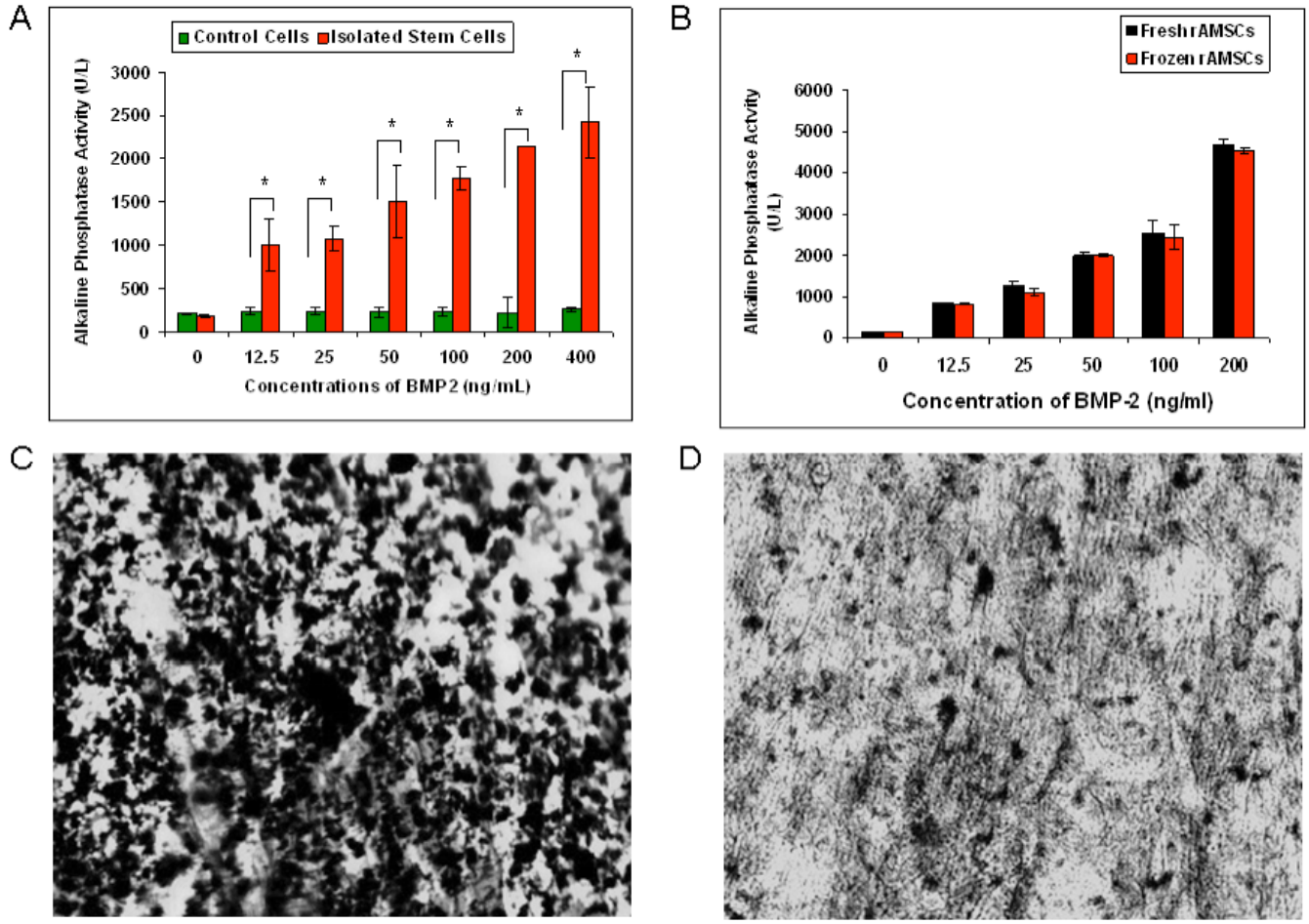


Fig. (2). Osteogenic differentiation of rAMSCs**.** (**A**) rAMSCs respond to increasing doses of BMP-2, but HIG-82 cells did not (n=3) (* $p<0.001$). (**B**) The ALP activities of both freshly isolated and 1- year liquid nitrogen preserved rAMSCs showed the same sensitivity to increasing concentrations of BMP-2 (n=3). Von Kossa staining of rAMSCs displayed bone calcification in rAMSCs cultured in OM + BMP-2 (**C**), but not in rAMSCs with DMEM (**D**).

rAMSCs Transduction

The GFP, human BMP-2, and human TGF-β1 genes were carried by self-complementary AAV vectors [20]. The viral vector stocks of serotypes 2 and 6 of AAV-D(+)-CMV-GFP, serotype 2 of AAV-D(+)-CMV-BMP-2, and serotype 2 of AAV-D(+)-CMV-TGF-β1 were produced and purified according to published protocols [4], and vector titers were approximately 5×10^{12} viral particles per ml. Both AAV2 and AAV6 vectors carrying GFP reporters were tested to determine an appropriate serotype of AAV vector for efficient transduction of rAMSCs [21]. Transductions of AAV-GFP, AAV2-BMP-2 and AAV2-TGF-β1 in rAMSCs were performed m.o.i. $1x10^5$, when the cells were 85% confluent. Forty eight hours after infection, the transduction efficiencies of GFP reporter (by fluorescent microscopy), BMP-2 (by ALP activity) and TGF-β1 (by ELISA) were detected, prior to implantation.

Implantation of the Transduced Cells *In Vivo*

The *in vivo* experiment for implantation of modified rAMSCs was divided into 2 arms: treatment and control. The treatment arm was subdivided into 4 treatment groups: 1) *AAV2-MBP2 group* - 9 mice implanted with $6x10^6$ AAV-BMP-2-transduced rAMSCs, 2) *AAV2-TGF-β1 group* - 9 mice implanted with $6x10^6$ AAV-TGF-β1-transduced rAMSCs, 3) *AAV-BMP2 and AAV-TGF β1 group* - 9 mice implanted with $3x10^6$ AAV-BMP2- transduced rAMSCs and $3x10^6$ AAV-TGF-β1- transduced rAMSCs, 4) *non-transduced rAMSCs* - 9 mice implanted with $6x10^6$ non-transduced rAMSCs. All implantations were performed on the right thigh muscles. Left thigh muscles served as untreated controls. The cells were dropped into sponge scaffolds and incubated in regular medium (DMEM supplemented with 10% FBS and 1% P/S) for 16 hours. The sponge scaffolds of each group were rinsed in DMEM medium without serum, and were then transplanted into the right thigh muscles of nine 8-week-old SCID mice by a small incision after the mice were anesthetized intraperitoneally with 2.5 % Avertin (100µl/10g weight). Fifteen days post-implantation, the mice were euthanized, and thigh muscles were isolated for histological analysis. The muscle tissues were harvested and snap frozen in liquid nitrogen-cooled isopentane for 1 minute, and then were removed into a pre-chilled tube prior to being stored at -80 °C. The muscles were then cryosectioned (10 µm thickness)

at -28 °C in a cryostat microtome (Microm HM 550, Walldorf, Germany). The muscle sections were stained by H&E and alizarin red without prior fixation to assess osteogenesis according to standard protocols [22,23].

Statistical Analyses

The significance of the difference between groups of experiments *in vitro* and *in vivo* was evaluated by analysis of variance followed by a two-tailed Student's t-test. Results were expressed as the mean ± SEM. A p value < 0.05 was considered the cut off for significance.

RESULTS

Morphological Appearance of Cultured rAMSCs

To observe morphological changes of the isolated rAMSCs, cells were cultured and monitored each day after isolation. The day when the cells were isolated is designated as day 1. On day 1, most of the cells were floating with few cells attached to the bottom of the flask (Fig. (**1A**)). By day 2, a number of cells had attached to the bottom of the flask (Fig. (**1B**)), and by day 3, the cells began to expand rapidly (Fig. (**1C**)). By day 4 and 5, the cells exhibited a spindle morphological appearance and continued to expand rapidly in culture (Fig. (**1D**) and (**E**)). By day 6, the cells reached more than 90% confluence (Fig. (**1F**)).

rAMSCs Osteogenesis *In Vitro*

To confirm that the rAMSCs exhibited the potential to differentiate toward an osteogenic lineage, the cells were treated with BMP-2 at different concentrations and were assessed for ALP activity. ALP activity in the rAMSCs increased with increasing concentrations of BMP-2, but ALP activity in the control cells (HIG-82) did not respond to BMP-2, even at high concentrations (Fig. (**2A**), * $p < 0.001$). The analysis of ALP activity also demonstrated that both fresh rAMSCs and those preserved for 1 year had the same strong osteogenic differentiation potential in the presence of BMP-2 (Fig. (**2B**)), indicating 1- year preservation did not affect their osteogenic differentiation ability. Moreover, Von Kossa staining showed bone calcification of rAMSCs at 3 weeks after their culture in OM supplemented with BMP-2 (200 ng/ml (Fig. (**2C**)), but not in rAMSCs cultured in DMEM (Fig. (**2D**)). These data clearly demonstrate that rAMSCs have the potential for osteogenesis in the presence of BMP-2, and *in vitro* osteogenic differentiation is dependent on the dose of BMP-2 added for stimulation.

AAV2 Vector Efficiently Transduced rAMSCs

AAV vectors showed a higher efficiency of GFP expression in rAMSCs than in hAMSCs (Fig. (**3**)). In addition, AAV2 vectors demonstrated higher efficiencies than AAV6 vectors in both rAMSCs and hAMSCs. The results indicate that the AAV2 vector is suitable for *ex vivo* gene therapy in both rabbit and human adipose-derived mesenchymal stem cells due to its unique tropism, as was shown in previous studies in murine and human mesenchymal stem cells [5,6]. The expression levels of both BMP-2 and TGF-β1 in the supernatant of AAV2 vector-transduced rAMSCs were determined prior to implantation

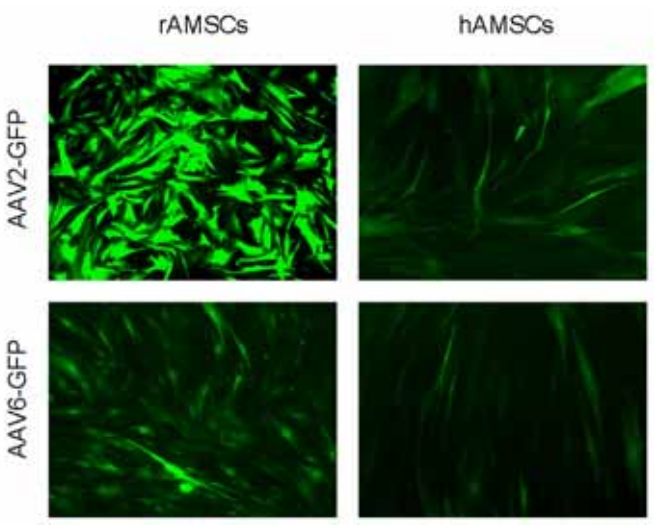
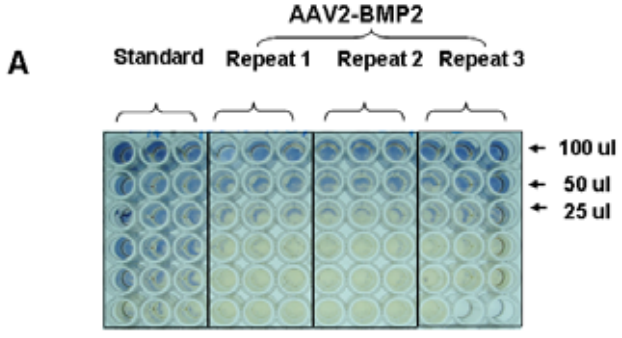


Fig. (3). AAV2 vector efficiently transduced both rAMSCs and hAMSCs. AAV2-GFP showed higher efficiency than AAV6-GFP in both rAMSCs and hAMSCS. However, both serotypes of AAV vectors had higher efficiencies in rAMSCs compared with hAMSCs.

(Fig. (**4**)). Higher volumes of supernatant from AAV2-BMP-2-transduced rAMSCs exhibit higher levels of ALP activity (Fig. (**4A**), blue color), consistent with our observation *in vitro*. Furthermore, the supernatant from AAV2-TGF-β1-transduced rAMSCs showed significantly higher activities of TGF-β1 by ELISA assay both with and without acid activation than did the negative controls (Fig. (**4B**), * $p<0.001$).



B

	TGF-β1 (pg/ml)	TGF-β1 (pg/ml) W/Acid Activation
Negative Control	4.6	1570.2
AAV2-TGF-β1	1818.8 ± 290.5* (n=3)	7035.6 ± 277.2* (n=3)

Fig. (4). BMP2 and TGF-β1 expression in the supernatant of AAV2-transduced rAMSCs. The levels of ALP activity in 96 well-plates 48 hours after infection depended on the supernatant volume (100 ul, 50 ul and 25 ul) of AAV2-BMP2-transduced rAMSCs (blue color) (**A**). TGF-β1 expression evaluated by ELISA was significantly higher in rAMSCs prior to implantation with and without acid activation in the control cells (* $p < 0.001$) (**B**). Note that n=3 for both experiments and controls.

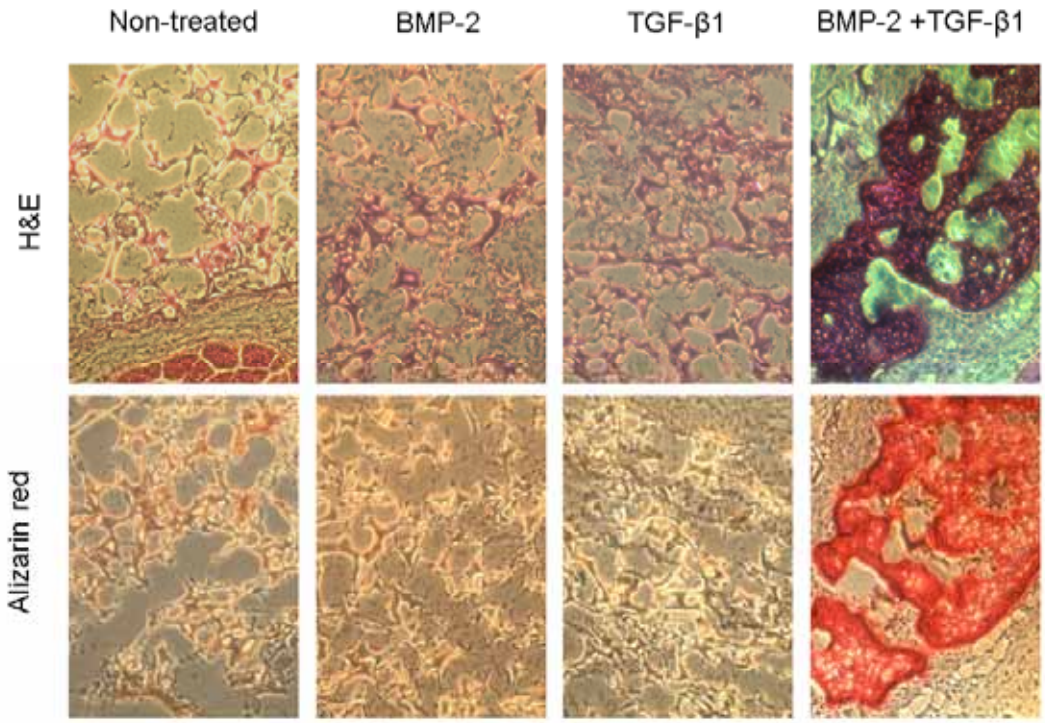


Fig. (5). rAMSCs osteogenesis *in vivo*. The muscle sections were stained with alizarin red and H&E. New osteoblasts and ectopic bone formation were found in the muscles implanted with a combination of AAV-BMP-2- and AAV- TGF-β1-transduced rAMSCs 15 days-post implantation, but not in the muscle implanted with AAV-BMP-2- or AAV- TGF-β1-transduced rAMSCs alone or with un-transduced rAMSCs within the same time period. All panels are shown at 200x magnification.

Osteogenesis of Modified rAMSCs *In Vivo*

To assess osteogenesis of rAMSCs *in vivo*, the AAV vector-transduced rAMSCs and un-transduced rAMSCs were implanted into the thigh muscles of SCID mice. Fifteen days post-implantation, the thigh muscles were isolated for histological analysis. Ectopic bone formation was observed in the muscle of mice implanted with combined AAV-BMP-2- and AAV-TGF-β1-transduced rAMSCs, but not in the muscle of mice implanted with either AAV-BMP-2- or AAV-TGF-β1 transduced rAMSCs alone. Osteoblasts were present in muscle sections stained with alizarin red (Fig. (**5**)). The results demonstrate that rAMSCs transduced by the combination of AAV2-BMP-2 and AAV2-TGF-β1 enhance osteogenic differentiations *in vivo*.

DISCUSSION

The results of the current study indicate that combined osteogenic factor (BMP-2) and growth factor (TGF-β1) genetic modification of rAMSCs is able to enhance stem cell osteogenesis and ectopic bone formation in muscle. These data characterizing rAMSCs from rabbit is an important step in understanding their potential use in the rabbit model for musculoskeletal regenerative medicine. Our study clearly demonstrates that rAMSCs exhibit the potential to differentiate *in vitro* toward osteogenic lineage when they are co-cultured with BMP-2 and that such differentiation tendency is dependent on increasing dosages of BMP-2. Furthermore, we found that both fresh rAMSCs and preserved rAMSCs showed similar strong ALP activities in the presence of BMP-2, indicating the potential clinical utility of rAMSCs from adipose tissues in stem cell therapy for bone repair since they can be stored frozen for at least 1 year without loss of activity.

It has been shown that human adipose-derived mesenchymal stem cells have no capacity to differentiate into an osteoblastic lineage if they are not genetically engineered to overexpress BMP-2, and that human processed lipoaspirate (HPLA) cells genetically modified by adenoviral vector to overexpress BMP-2 can induce bone formation *in vivo* and heal a critically sized femoral defect in an athymic rat [24]. With the availability of several novel serotypes and rational design of AAV capsid mutants, it is currently indeed becoming feasible to achieve efficient transduction of human hematopoietic stem cells (HSC) by AAV vectors [25], allowing long-term transgene expression [26]. Another study also showed that AAV2 vector-mediated gene transfer of TGF-β1 to human bone marrow-derived mesenchymal stem cells (hMSC) improves cartilage repair in athymic rats [27].

In the current study, we investigated whether AAV vectors could deliver the foreign genes to both rabbit and human adipose-derived stem/progenitor cells *in vitro*. Our findings demonstrate that the transduction of AAV vector has a tissue-selective tropism in adipose-derived mesenchymal stem cells and that AAV2 could efficiently transduce both rAMSCs and hAMSCs, indicating that the AAV2 vector is well suited for genetic engineering of these stem cells. Moreover, the efficiency of AAV vectors in rAMSCs is higher than in hAMSCs. Since currently there is no specific cell marker for rabbit adipose-derived stem cells, we could not detect stem cell markers of rAMSCs. Bone morphogenetic proteins (BMPs) such as BMP-2 belong to the transforming growth factor-β (TGF-β) superfamily [28].

The local delivery of TGF-β1 by a hydrolgel carrier appears to have therapeutic potential for enhancing bone formation in rabbit after radiation treatment [29], and the enhancement of bone ingrowth by TGF-β1 in a canine model indicates the mechanism of action of TGF-β1 may include both proliferation of osteoprogenitor cells and production of matrix by committed osteoblasts [30]. In a previous study [31], the beneficial effect of an osteogenic factor (BMP-4) combined with an angiogenic factor (VEGF) using plasmid DNA delivery could enhance human bone marrow stromal cell-driven bone regeneration in SCID mice [31]. However, it is not clear whether the combination of AAV-BMP-2- and AAV-TGF-β1-transduced rAMSCs has the same synergistic effects on osteogenic differentiation. Here, we were focusing on rAAV mediated gene transfer of BMP-2 and TGF-β1 genes into rAMSCs to promote their osteogenesis *in vivo*. The presented data demonstrate that the combination of TGF-β1 and BMP-2 might enhance the differentiation of rAMSC toward osteogenic lineage and accelerate new bone formation 15 days post-implantation. However, new bone formation could not be observed in muscle treated with either AAV-BMP-2- or AAV-TGF-β1-transduced rAMSCs within the same time period, being consistent with previous studies [5,6]. New bone formation *in vivo* promoted by implantation of either AAV2-BMP-7-expressing hAMSCs [5] or AAV2-BMP-2-transduced murine MSCs (mMSCs) [6] usually requires 3-5 weeks. These findings suggest that new bone formation by osteogenic differentiation of mesenchymal stem cells promoted by BMP-2 or BMP-7 needs longer time. However, the combination of BMP-2 and TGF-β1 enhances new bone formation *in vivo* likely through the effects of TGF-β1 on both proliferation of progenitor cells and matrix mineralization during new bone formation [30].

CONCLUSIONS

Mesenchymal stem cells derived from rabbit adipose tissue can be efficiently transduced by the AAV2 vector to express BMP-2 and TGF-β1, enhancing *in vivo* osteogenesis. These transduced cells may be valuable for studying bone regeneration in the rabbit model.

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