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ABSTRACT

Marrow stromal cells (MSCs) include stem cells capable of forming all mesenchymal tissues, including bone. However, before MSCs can be successfully used in regeneration procedures, methods must be developed to stimulate their differentiation selectively to osteoblasts. Runx2, a bone-specific transcription factor, is known to stimulate osteoblast differentiation. In the present study, we tested the hypothesis that Runx2 gene therapy can be used to heal a critical-sized defect in mouse calvaria. Runx2-engineered MSCs displayed enhanced osteogenic potential and osteoblast-specific gene expression *in vitro* and *in vivo*. Runx2-expressing cells also dramatically enhanced the healing of critical-sized calvarial defects and increased both bone volume fraction and bone mineral density. These studies provide a novel route for enhancing osteogenesis that may have future therapeutic applications for craniofacial bone regeneration.

KEY WORDS: adenovirus, bone, regeneration, gene therapy, Runx2, stem cells.

Healing Cranial Defects with AdRunx2-transduced Marrow Stromal Cells

INTRODUCTION

Tissue engineering combined with gene therapy represents a promising direction for bone-regenerative medicine (Bruder and Fox, 1999). The use of adult stem cells as a platform for regeneration has received particular attention. These cells are found in several tissues, including marrow, skeletal muscle, and adipose tissue (Asahara *et al.*, 2000). Marrow stromal cells (MSCs) contain pluripotent stem cells capable of differentiating into all the major mesenchymal cell types (Bianco *et al.*, 2001). MSCs from animal and human sources have been successfully used to heal cranial and long-bone defects (reviewed in Krebsbach *et al.*, 1999). However, considerable variability in regenerative outcomes has been observed between and among studies. The use of MSCs for repairing bone defects may be limited by the low frequency of osteoprogenitors in marrow and their loss during repeated passaging, as well as by an age-related decline in the numbers of these cells (Oreffo *et al.*, 1998; Muschler *et al.*, 2001; Byers and Garcia, 2004; Derubeis and Cancedda, 2004). To overcome these limitations, investigators must develop methods to increase the osteogenic activity of MSCs.

One strategy for achieving this goal is to use gene-therapy-based expression of factors capable of increasing MSC osteogenic potential. For example, bone morphogenic protein (BMP) expression vectors were successfully introduced into MSCs to increase their osteogenic activity (Turgeman *et al.*, 2001; Park *et al.*, 2003; Tsuda *et al.*, 2003). However, diffusion of BMPs away from the implant results in an essentially uncontrolled osteogenic response. As an alternative to BMP gene therapy, we used the bone-specific transcription factor, Runx2, to direct MSC differentiation into osteoblasts. Exogenous expression of Runx2 in mesenchymal cells or primary MSCs stimulated osteoblast gene expression and mineralization *in vitro* and increased bone formation after *in vivo* subcutaneous implantation (Yang *et al.*, 2003; Zhao *et al.*, 2005b). However, the potential of this approach to regenerate bone in an orthotopic site was not evaluated. In the present study, we tested the hypothesis that Runx2 gene therapy can be used to heal a critical-sized cranial defect.

MATERIALS & METHODS

Isolation and Transduction of MSCs

MSCs were harvested from bones of C57BL6 mice as described previously (Krebsbach *et al.*, 1998). Cells were plated at a density of 50,000/cm² for use in cell culture studies. An adenoviral vector encoding the type II Runx2 isoform (AdRunx2) was generated as described previously (Yang *et al.*, 2003). AdLacZ was purchased from the University of Michigan Vector Core. MSCs were transduced with adenovirus and cultured as previously described (Zhao *et al.*, 2005b).

Animal Experiments

All procedures were approved by the University Committee on the Use and Care

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Table. Real-time PCR Primers and Probes

Genes	TaqMan [®] probe (FAM-labeled)	Primer Sequence/Assay ID
OCN	AGCGGCCCTGAGTCTGACAAAGCC	F: GACCTCACAGATGCCAAGCCC R: CCCTCTGCTTGGACATGA
Runx2 ^a	AAGCTTCTTTGGGATCCGAGCACC	Mm00501578-m1
BSP ^a	GGTTCCAGTCCAGGGAGGCAGTGA	Mm00492555-m1
ALP ^a	ACGAGTGCCTGCAGGATCGGAACGT	Mm00475831-m1
GAPDH ^a	TGAACGGATTGGCCGTATTGGGCG	Mm99999915-g1

^a Assays-on-Demand™ gene expression products.

of Animals and were in compliance with State and Federal laws. MSCs were transduced with adenovirus at a titer of 300 plaque-forming units/cell. After 24 hrs, cells were trypsinized and adsorbed to a gelatin sponge (Gelfoam, Upjohn, Kalamazo, MI, USA). For ectopic bone formation, transplants were subcutaneously implanted into six-week-old C57BL6 mice as previously described (Zhao *et al.*, 2005b). For the calvarial defect model, eight-week-old male mice were anesthetized with ketamine and xylazine. After exposure of the dorsal cranium, a 5-mm-diameter defect was created by means of a high-speed drill with a trephine bur. Gelfoam scaffold seeded with transduced cells was implanted into the defect, and samples were harvested after 7 wks.

RNA Analysis

Osteoblast marker mRNA expression was measured by quantitative real-time PCR (QRT-PCR). The following mRNA sequences were measured: Runx2, osteocalcin (OCN), bone sialoprotein (BSP), and alkaline phosphatase (ALP), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Total RNA was extracted and mRNA levels quantified as previously described (Zhao *et al.*, 2005a). The Table describes the primers and QRT-PCR probes used.

Radiography, Histology, and Micro-CT Analysis

Radiographic analysis was performed with the use of a microradiographic apparatus (Faxitron X-ray Corporation, Wheeling, IL, USA). For histology, implants were fixed in 4% paraformaldehyde for paraffin embedding and sectioning (8- μ m sections). Slides of adjacent sections were stained with hematoxylin and eosin for determination of tissue morphology.

Calvaria were scanned by micro-computed tomography (mCT MS8X-130; EVS Corp., London, ON, Canada). Specimens were viewed with a scanning direction parallel to the coronal aspect of calvaria. High-resolution scanning, with an in-plane pixel size and slice thickness of 24 μ m, was performed. To cover the entire thickness of the calvarial bone, we set the number of slices at 400. We used GEMS MicroView[®] software (Gems Corp., London, ON, Canada) to make three-dimensional (3-D) reconstructions from scans. We used a threshold value of 750 to obtain the 3-D image.

Statistical Analysis

We performed statistical analyses using Student's *t* test. Experimental data are reported as means \pm SD. Sample sizes are indicated in Fig. legends.

RESULTS

Comparison of AdRunx2 Induction of Osteoblast Gene Expression *in vitro* and *in vivo*

We previously characterized actions of AdRunx2 on osteoblast gene expression and mineralization of murine MSCs *in vitro* and showed that AdRunx2 could increase *in vivo* osteogenic activity after subcutaneous implantation (Zhao *et al.*, 2005b). However, we did not examine gene expression *in vivo*. Because cells are exposed to very different environments in cell culture and *in vivo*, we compared the time-course of osteoblast marker gene expression under these two conditions.

MSCs were transduced with AdRunx2 or AdLacZ (control vector) and either grown in cell culture or subcutaneously implanted into C57BL6 mice. In both groups, cells/implants were harvested at the indicated times for measurement of mRNAs by QRT-PCR. In cell culture, Ad-Runx2-transduced MSCs expressed higher levels of Runx2 mRNA than did controls (Fig. 1A). Runx2 mRNA levels were highest one day after transduction (90-fold induction). Although Runx2 expression decreased with time, it remained elevated relative to controls for the duration of the experiment. Ad-Runx2 transduction also increased expression of ALP, BSP, and OCN mRNAs, with ALP mRNA being expressed at early times, followed by BSP and OCN (Fig. 1B). ALP expression was higher in Runx2 MSCs for up to 6 days, and then rapidly declined to values comparable with those of the AdLacZ control. For BSP and OCN, a slower, but similar, induction profile was observed, such that values were equivalent to those of controls after 9 days (Figs. 1C, 1D).

In vivo expression profiles for implanted MSCs are shown in Fig. 2. Overall, patterns were similar to those seen in cell culture, with some important differences. Ad-Runx2 transduction increased Runx2 mRNA 10-fold at the first timepoint examined (1 wk). Levels then declined at 2 and 3 wks, although they still remained elevated relative to those in controls. Similarly, ALP, BSP, and OCN mRNAs were increased by Runx2 after 1 wk (Figs. 2B, 2C, 2D). However, a gradual increase in expression was also observed in control implants, consistent with the known capacity of MSCs to differentiate into osteoblast *in vivo* in the absence of genetic modification (Krebsbach *et al.*, 1998). After 3 wks, ALP mRNA in these cells was actually somewhat higher than that in the Runx2 group, while BSP and OCN mRNAs were equivalent.

AdRunx2-transduced MSCs Stimulate Healing of a Critical-sized Calvarial Defect

To evaluate the ability of AdRunx2-transduced MSCs to promote bone repair, we seeded cells onto a gelatin sponge and implanted them into critical-sized calvarial defects (Krebsbach *et al.*, 1998). After 7 wks, radiographic analysis revealed that AdRunx2 MSCs had completely closed defects (Fig. 3A), while only limited bone healing was observed with AdLacZ-transduced control cells. Previous work showed that AdLacZ and untransduced MSCs have equivalent osteogenic activity

(Zhao *et al.*, 2005b). Histological evaluation confirmed that complete bone bridging was achieved with AdRunx2 MSCs (Fig. 3B). Newly formed bone was continuous with host bone and contained a pronounced marrow space, with little residual gelatin scaffold (Fig. 3C). In contrast, only limited new bone formation was seen at the margins of defects in the control group. We used micro-CT images to evaluate the 3-D structure of the newly formed bone (Fig. 3D). Quantitative measurements demonstrated that the bone volume fraction of a standard cylindrical volume incorporating the defect increased from $41.01 \pm 14.25\%$ in controls to $55.79 \pm 4.98\%$ ($p < 0.05$) in the Runx2-treated group. Bone mineral density also increased from 279.3 ± 81.3 mg/cc to 347.9 ± 27.6 mg/cc ($p < 0.05$).

DISCUSSION

The combination of tissue engineering and stem cell therapy is a promising strategy for the regeneration of bone with well-defined morphologies (Rose and Oreffo, 2002; Warren *et al.*, 2003a). In the present study, we showed that the osteogenic potential of MSCs was enhanced by adenoviral expression of Runx2, and that virally transduced cells can heal a critical-sized cranial defect.

Osteoblast marker genes were strongly induced by AdRunx2-transduction of MSCs, regardless of whether cells were cultivated *in vitro* or implanted into subcutaneous sites. However, clear differences in the magnitude and duration of responses were observed. *In vivo*, control MSCs had a greater tendency to differentiate in the absence of exogenous Runx2. Specifically, ALP, BSP, and OCN mRNAs gradually increased over time, reaching expression levels that were equivalent to or greater than those of AdRunx2-transduced cells after 3 wks. This is consistent with reports of MSCs spontaneously formed bone *in vivo* (Krebsbach *et al.*, 1998). Marker induction *in vitro* was also quite transient, and generally returned to basal levels after 9 to 12 days, while mRNA

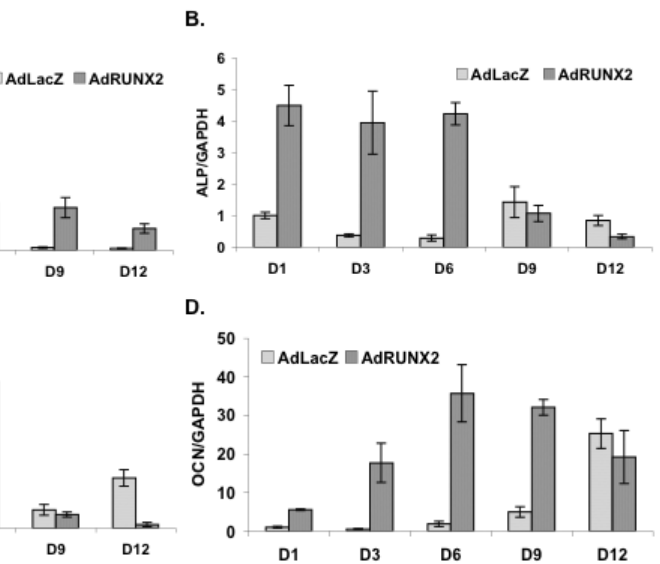


Figure 1. Induction of osteoblast-related mRNAs *in vitro*. MSCs were transduced with Ad-LacZ or Ad-Runx2 at a titer of 300 pfu/cell. Cells were harvested at the times indicated. Total RNA was extracted and analyzed for expression of Runx2 (A), ALP (B), BSP (C), and OCN (D) mRNAs, by means of quantitative real-time PCR, as described in MATERIALS & METHODS. All mRNA levels are expressed relative to levels of GAPDH mRNA, which remained constant throughout the experiment. Data are means \pm SD of 3 independent samples.

levels were more sustained *in vivo*. For example, in cell culture, ALP mRNA was maximally induced at 1 day and declined to control levels after 9 days. *In vivo*, levels also decreased to control levels after 2 wks, but these were still 40 to 50% of the levels seen at 1 wk.

To explore the potential clinical relevance of this gene therapy approach, we evaluated the ability of AdRunx2 MSCs to repair a critical-sized cranial defect. X-ray evaluation of the newly formed bone revealed that Runx2-expressing cells completely repaired calvarial defects. In contrast, only partial

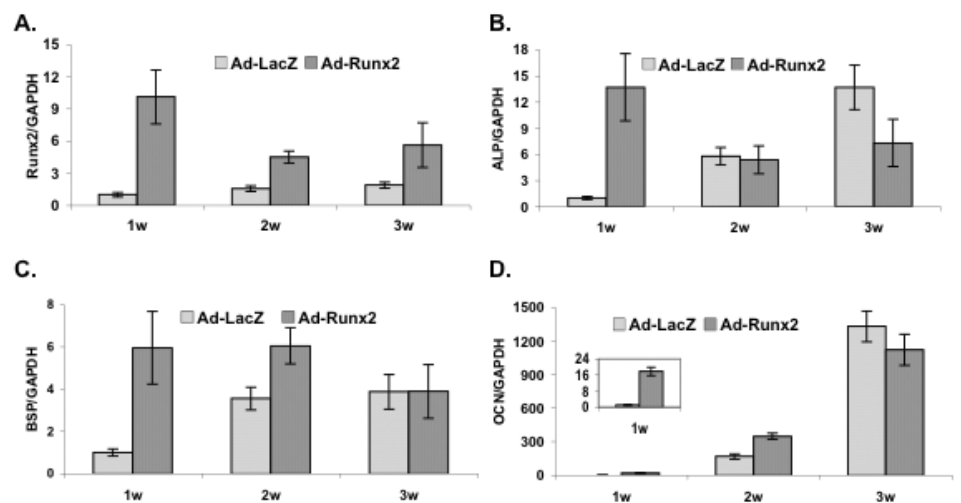


Figure 2. *In vivo* gene expression pattern after MSC implantation. MSCs were transduced with 300 pfu/cell of the indicated adenovirus. Twenty-four hours after transduction, 2×10^6 cells were seeded into gelatin sponges and implanted subcutaneously into immunodeficient mice, as described in MATERIALS & METHODS. Implants were harvested at the times indicated. Total RNA was extracted and analyzed by quantitative real-time PCR (as in Fig. 1). Data are means \pm SD of 3 independent samples.

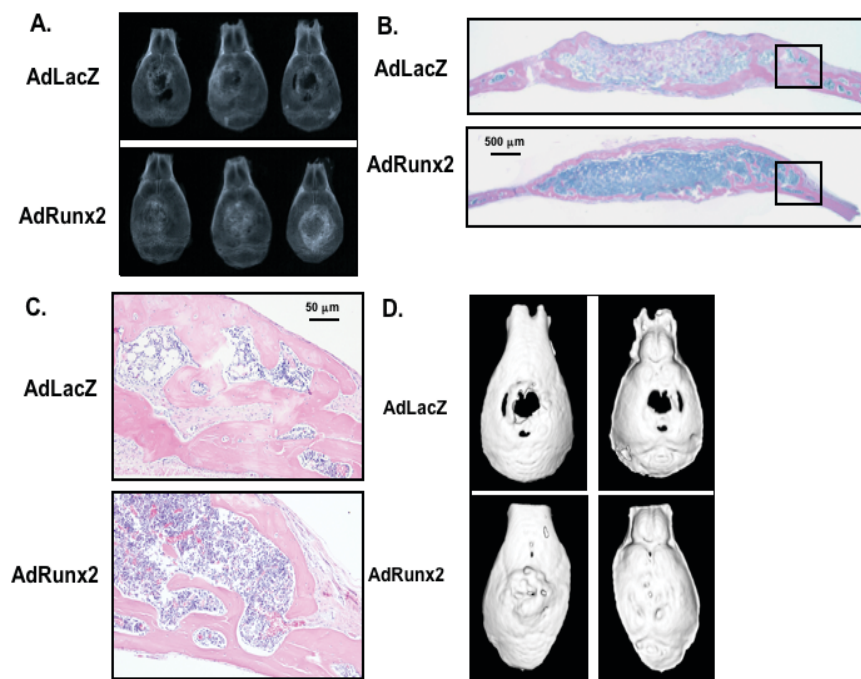


Figure 3. Repair of critical-sized calvarial defects with Runx2 genetically engineered marrow stromal cells. MSCs were transduced with 300 pfu/cell of the indicated adenovirus. After 24 hrs, 5×10^6 cells were seeded on a gelatin sponge and implanted into 5-mm calvarial defects as described in MATERIALS & METHODS. After 7 wks, implants were harvested and analyzed by x-ray (A), histology (B,C), and micro-CT (D). Panel C shows a higher magnification of the rectangular regions shown in B. Representative 3-D reconstruction images with micro-CT in each group are shown in panel D. Bars = (in panel B) 500 μm and (in panel C) 50 μm .

healing was observed with control MSCs. The newly formed bone derived from AdRunx2 cells also had a higher bone mineral density. Histological examination demonstrated that newly formed bone completely covered defects implanted with AdRunx2 MSCs, while only limited new bone formation was seen in the control group. Consistent with these findings, quantitative analysis by micro-CT revealed that bone formed by AdRunx2 MSCs filled a greater fraction of the defect volume and had a higher mineral density than did controls.

Interestingly, in the calvarial model, the newly formed bone contained marrow that was not observed in the previously described subcutaneous model (Zhao *et al.*, 2005b). There are several possible explanations for this result. First, it is known that the dura mater underlying the cranium contains adult stem cells (Warren *et al.*, 2003b). In response to surgical trauma, these cells may migrate into the defect and secrete growth factors (FGFs, BMPs) that are missing in the subcutaneous site. Alternatively, the cranial region is known to have a rich blood supply. The infiltration of new blood vessels into the scaffold could bring more oxygen and growth factors to the center of the scaffold. This would facilitate MSC survival, scaffold resorption, and marrow formation. Last, the thickness of the scaffold may also play a role in marrow formation (Krebsbach *et al.*, 1998).

As pointed out in the INTRODUCTION, considerable variability has been observed in the ability of MSCs to heal cranial defects. In our studies, AdLacZ-transduced control MSCs showed lower activity than was reported in certain

studies where unmodified MSCs were used (Krebsbach *et al.*, 1999). The basis for these differences is not currently known. However, it is unlikely to be explained by suppression of osteogenic activity by AdLacZ, which did not affect the osteogenic activity of MSCs after subcutaneous implantation (Zhao *et al.*, 2005b).

Retroviral vectors have the advantage of stable transgene expression and no immune response. However, potential risks associated with these vectors include insertional mutagenesis (Noguchi, 2003) and possible tumor formation associated with prolonged Runx2 expression (Blyth *et al.*, 2001). Unlike retroviruses, adenovirus transgene expression is quite brief (St George, 2003). In the present study, AdRunx2 MSCs expressed recombinant protein for only approximately 3 wks, with maximal induction after 1 wk. This expression pattern is consistent with the results reported from a previous study that examined adenovirus expression of the Tl-1 isoform of Runx2 (Kojima and Uemura, 2005). Short-term Runx2 expression may be too brief to have major deleterious effects. This hypothesis is strongly supported by the data of Kojima and Uemura (2005), who failed to observe significant changes in oncogene expression after Runx2 overexpression. Short-term

Runx2 expression may also have certain advantages in terms of skeletal gene therapy. We found that transient expression of Runx2 was sufficient to induce complete bridging of a critical-sized calvarial defect. In contrast, sustained transgenic overexpression of Runx2 reportedly leads to osteopenia (Liu *et al.*, 2001; Geoffroy *et al.*, 2002).

Recently, other groups reported on the use of Runx2 gene transfer to enhance the osteogenic potential of MSCs. However, equivocal outcomes were reported. Using a retroviral vector, Byers and co-workers assessed the ability of Runx2 overexpression in rat MSCs to heal a calvarial defect (Byers *et al.*, 2006). After seeding cells in defects using a polycaprolactone scaffold, they observed no significant differences between the group implanted with MSCs expressing Runx2 and those implanted with empty scaffold. Mineral deposition occurred mainly in discrete local areas that mirrored the shape of the scaffold, and no integration with host bone was observed. In contrast, bone formation in our studies occurred mainly at the interface between the host and the implant, leading to complete integration of new and host bone. There are several possible explanations for these disparate results. First, differences between mouse and rat MSCs may contribute to the different healing outcomes (Krebsbach *et al.*, 1997; Osyczka *et al.*, 2004). Second, the biological responses of MSCs may be influenced by the different scaffolds used in these experiments (Boyan *et al.*, 1996). The porous structure of the gelatin scaffold we used is known to facilitate *in vitro* cell seeding and *in vivo* infiltration

by cells from surroundings sites (Takahashi *et al.*, 2005). Additionally, the type I collagen used in gelatin scaffolds is known to induce osteoblast differentiation (Lynch *et al.*, 1995; Xiao *et al.*, 1998). Last, differences in Runx2 transgene expression patterns and durations may contribute to the different outcomes. As mentioned previously, different gene delivery vectors were also used in these studies. The retroviral vector used in the Byers study directs sustained Runx2 expression that may promote osteoclastogenesis and interfere with new bone formation (Geoffroy *et al.*, 2002). Collectively, these results highlight the importance of cell-sourcing, scaffolds, and gene therapy vectors in the regeneration response.

In conclusion, our results show that the lineage of MSCs can be directed toward osteoblasts by genetic manipulation of the Runx2 transcription factor. Furthermore, AdRunx2-transduced MSCs exhibited increased ability to heal a cranial defect when compared with AdLacZ-transduced control MSCs.

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