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In Vitro and In Vivo Osteoblastic Differentiation of BMP-2- and Runx2-Engineered Skeletal Myoblasts

Charles A. Gersbach,[1,3] Robert E. Guldberg,[2,3] and Andrés J. García[2,3]*

[1]Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, Georgia 30332
[2]Woodruff School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, Georgia 30332
[3]Georgia Tech/Emory Center for the Engineering of Living Tissues, Georgia Institute of Technology, Atlanta, Georgia 30332

Abstract Genetic engineering with osteogenic factors is a promising approach for cell-based therapeutics and orthopedic regeneration. However, the relative efficacy of different strategies for inducing osteoblastic differentiation remains unclear and is further complicated by varied delivery vehicles, cell types, and evaluation criteria. In order to elucidate the effects of distinct gene-based strategies, we quantitatively evaluated osteoblastic differentiation and mineralization of primary skeletal myoblasts overexpressing either the BMP-2 growth factor or Runx2 transcription factor. Retroviral delivery of BMP-2 or Runx2 stimulated differentiation into an osteoblastic phenotype, as demonstrated by the induction of osteogenic gene expression, alkaline phosphatase activity, and matrix mineralization in monolayer culture and on collagen scaffolds both in vitro and in an intramuscular site in vivo. In general, BMP-2 stimulated osteoblastic markers faster and to a greater extent than Runx2, although we also identified experimental conditions under which these two factors produced similar effects. Additionally, Runx2-engineered cells did not utilize paracrine signaling via secreted osteogenic factors, in contrast to cells overexpressing BMP-2, as demonstrated by conditioned media studies and activation of Smad signaling. These results emphasize the complexity of gene therapy-based orthopedic therapeutics as an integrated relationship of differentiation state, construct maturation, and paracrine signaling of osteogenic cells. This study is significant in evaluating proposed therapeutic systems and defining a successful strategy for integrating gene medicine and orthopedic regeneration. J. Cell. Biochem. 100: 1324–1336, 2007.

Key words: bone tissue engineering; transcription factor; gene therapy; skeletal myoblasts; orthopedic regeneration; retrovirus; ectopic implantation

Orthopedic substitutes are required for 2.2 million surgeries each year [Vaccaro, 2002]. Bone tissue engineering has emerged as a promising approach to address the limitations of autogenous bone grafts, allografts, and synthetic implants [Perry, 1999; Bucholz, 2002; Finkemeier, 2002; Vaccaro, 2002]. This strategy incorporates cells, a biodegradable scaffold, and bioactive factors into an implantable construct [Bruder and Fox, 1999; Doll et al., 2001]. However, a suitable autologous osteogenic cell source has been difficult to identify due to availability [Bruder and Fox, 1999], dedifferentiation following in vitro expansion [Bruder et al., 1997; Shi et al., 2002; Simonsen et al., 2002], and an age-related loss of phenotype [Mueller and Glowacki, 2001]. Therefore, genetic engineering of readily accessible non-osteoblastic cell types and progenitor cells, such as skeletal myoblasts, fibroblasts, and bone marrow-derived cells, has emerged as a promising method to induce osteoblastic differentiation and address these cell-sourcing limitations. The vast majority of these approaches utilize osteogenic growth factors, such as bone morphogenetic proteins (BMPs) or osteoblastic

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*Correspondence to: Andrés J. García, Petit Institute for Bioengineering and Bioscience, 315 Ferst Drive, Room 2314, Atlanta, GA 30332-0363.
E-mail: andres.garcia@me.gatech.edu

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transcription factors, including Runx2 [Baltzer and Lieberman, 2004; Franceschi et al., 2004]. However, the relative efficacy of these different approaches to induce osteoblastic differentiation remains unclear and is further complicated by varied delivery vehicles, cell types, and evaluation criteria.

BMP-2 is the most widely used growth factor to stimulate osteoblastic differentiation [Yoon and Boden, 2002]. Soluble BMP-2 protein acts in autocrine and paracrine fashions by binding extracellularly to transmembrane receptors and triggering an intracellular signaling cascade mediated by Smad proteins [Schmitt et al., 1999]. Activated Smads translocate to the nucleus and upregulate a cascade of osteoblastic transcription factors including Dlx5 [Lee et al., 2003] and Runx2 [Nishimura et al., 2002]. Runx2 then coordinates osteoblastic gene expression, including osteocalcin, bone sialoprotein, osteopontin, and alkaline phosphatase [Lian and Stein, 2003]. Runx2 expression is required for osteoblastic differentiation [Ducy et al., 1997] and endochondral ossification [Komori et al., 1997]. BMP-2 signaling also functions via several Smad-independent pathways, including protein kinase C and D and MAPKs [Derynck and Zhang, 2003].

Strategies that engineer cells to express a BMP-2 transgene have been successful in stimulating osteoblastic differentiation and ectopic and orthotopic bone formation in vivo [Baltzer and Lieberman, 2004]. However, concerns regarding release kinetics, dosage, and target-cell specificity of this secreted growth factor still remain [Franceschi et al., 2004]. Furthermore, the unregulated secretion of BMPs has been implicated in tumor growth [Ide et al., 1997; Pouliot et al., 2003; Yang et al., 2005] and bone overproduction [Moutsatsos et al., 2001; Peng et al., 2005], where bone growth exceeds the defect site. More recently, forced expression of Runx2, an intracellular effector, has been shown to induce osteoblastic differentiation [Ducy et al., 1997], matrix mineralization [Byers et al., 2002], and in vivo bone formation [Yang et al., 2003]. These results are consistent across pluripotent cell lines [Yang et al., 2003] and primary cell types including fibroblasts [Hirata et al., 2003; Phillips et al., 2006a], skeletal myoblasts [Gersbach et al., 2004a], and bone marrow stromal cells [Byers et al., 2004; Zheng et al., 2004; Zhao et al., 2005b].

These studies have established ex vivo gene therapy with BMP-2 and Runx2 as promising strategies for orthopedic regeneration. However, the relative potency and differential effects of these growth factor and transcription factor-based strategies in inducing osteoblastic differentiation are unclear. In particular, the diverse use of transient and sustained gene-delivery vehicles, transformed and primary cell types, and methods for assessing osteoblastic differentiation causes comparisons between studies to be difficult. Therefore, this study focuses on analyzing osteoblastic differentiation of a clinically relevant cell source engineered to express BMP-2 and/or Runx2 from a constitutive retroviral vector to obtain sustained expression of the transgene. Primary skeletal myoblasts were used as a model non-osteoblastic autologous cell source available in large quantities by a muscle biopsy and compatible with in vitro expansion [Rando and Blau, 1994] and ex vivo gene therapy protocols [Springer and Blau, 1997]. Skeletal myoblasts engineered to express BMP-2 and/or Runx2 were assessed for osteoblastic differentiation in both monolayer and tissue-engineered constructs in vitro, as well as in an ectopic implantation site in syngeneic mice in vivo. Engineered cells were further characterized for their ability to induce osteoblastic differentiation through paracrine signaling.

MATERIALS AND METHODS

Cell Culture

Primary skeletal myoblasts were isolated from the tibialis anterior muscles of adult male Balb/c mice and cultured in selective growth media (Ham's F10, 20% fetal bovine serum, 5 ng/ml bFGF (Promega, Madison, WI), 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate), yielding cultures that were greater than 99% myogenic by desmin staining [Rando and Blau, 1994]. Cells were cultured on tissue culture plastic dishes coated with 0.01% type I collagen (Vitrogen, Palo Alto, CA) in a humidified 5% CO_2 atmosphere at 37°C. Cell culture media and antibiotics were obtained from Invitrogen (Carlsbad, CA), fetal bovine serum was purchased from Hyclone (Logan, UT), and all other cell culture supplements and reagents were acquired from Sigma (St. Louis, MO).

Retroviral Vectors

The pTJ66 retroviral vector [Murphy et al., 2002] and BMP-2 cDNA were generously provided by T.J. Murphy and Scott D. Boden, respectively. The oligonucleotides 5′- AGGCCTAGACTGACAATTGGTATCGATGGCCTACA-3′ and 3′- ACATCCGGATCTGACTGTTAACCATAGCTACCGGA-5′ were annealed together to create an internal MfeI restriction site (underlined) with overhangs compatible with the SfiI cloning site of pTJ66. The product was ligated into SfiI-digested pTJ66 vector. Finally, the human BMP-2 cDNA was digested from the host vector with EcoRI restriction enzyme, ligated into the MfeI restriction site of pTJ66, and verified by sequencing the ligation points. The pTJ66-Runx2 vector has been described previously [Byers et al., 2002].

Retroviral Transduction

Retroviral stocks were produced by transient transfection of helper virus-free ΦNX amphotropic producer cells with plasmid DNA [Byers et al., 2002]. Primary myoblasts were cultured up to 12 passages and plated on 0.01% collagen-coated, tissue culture polystyrene at 2×10^4 cells/cm^2, 24 h prior to retroviral transduction as described previously [Gersbach et al., 2004a]. Cells were transduced again 16 h later to increase transduction efficiency, and retroviral supernatant was replaced with differentiation media (αMEM, 10% fetal bovine serum, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 50 μg/ml L-ascorbic acid, 3 mM sodium β-glycerophosphate, and 10 nM dexamethasone). Cell culture media were replaced every 3 days until terminal assay, unless noted otherwise.

Osteogenic Differentiation

Quantitative RT-PCR (qRT-PCR), Western blotting, alkaline phosphatase biochemical activity, calcium content analysis, and von Kossa staining were performed as described previously [Gersbach et al., 2004a, 2006]. Runx2 (Accession # NM_009820) primer sequences were 5′-GGCCTTCAAGGTTGTAGCCC-3′ (forward) and 5′-CCCGGCCATGACGGTA-3′ (reverse); BMP-2 (Accession # NM_001200) primer sequences were 5′-ATTGTGGCTCCCCCGG-3′ (forward) and 5′-TCAGCCAGAGGAAAAGGGC-3′ (reverse); VEGF-A (Accession # NM_009505) primers were 5′-CATCTTCAAGCCGTCCTGTGT-3′ (forward) and 5′-CAGGGCTTCATCGTTACAGCA-3′ (reverse). Other primer sequences have been reported [Gersbach et al., 2004a]. Secreted BMP-2 protein levels were measured in bulk media samples using an ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Anti-phospho-Smad1/5/8 and anti-Smad1/5 were purchased from Chemicon (Temecula, CA) and Upstate Biotechnology (Charlottesville, VA), respectively.

Scaffold Seeding and Analysis

Fibrous collagen disks (5 mm × 1.5 mm, Kensey Nash, Exton, PA) were incubated overnight in 10% FBS in phosphate buffered saline (PBS). At 2 days post-transduction, 250,000 cells in 10 μl of growth media were seeded onto each side of the disk in a non-tissue, culture-treated 24-well plate, for a total of 500,000 cells/construct. Four hours later, scaffolds were immersed in 2 ml of growth media. After 24 h, growth media were changed to 2 ml of differentiation media. Media were replaced every 3 days until terminal assay. FT-IR spectroscopy and DNA content were assessed as described previously [Gersbach et al., 2004b].

Intramuscular Implantation

Scaffolds seeded with engineered or unmodified myoblasts were cultured for 1 or 28 days in vitro and implanted into the hind limbs of syngeneic 6-week-old immunocompetent male Balb/c mice (Jackson Labs, Bar Harbor, ME), in accordance with an IACUC-approved protocol. A longitudinal 1-cm incision was made in the skin overlying the gastrocnemius muscle, and the muscle was exposed. Scaffolds were inserted into the muscles of both legs through an atraumatic longitudinal split in the direction of the fibers. The muscle was closed over the implants (without suturing) and the skin closed with wound clips. Twenty-eight days after implantation mice were euthanized by CO_2 inhalation and lower legs were harvested, skinned, and fixed in 10% neutral buffered formalin.

Micro-Computed Tomography

Mineralization of cell/scaffold constructs was quantified by high-resolution X-ray micro-computed tomography (micro-CT) using a Scanco Medical VivaCT 40 imaging system (Bassersdorf, Switzerland). Specimens were scanned at

21 μm voxel resolution and evaluated at a threshold corresponding to a linear attenuation of 1.92 cm^{-1}, filter width of 1.2, and filter support of 2.0. The reconstructed and thresholded 3-D images were evaluated using direct distance transformation methods to calculate mineralized matrix volume within each construct [Hildebrand et al., 1999].

Histological Analysis

Formalin-fixed samples were decalcified with 5% formic acid prior to paraffin embedding for hematoxylin–eosin or Masson's trichrome staining. Sections (5 μm) were deparaffinized and stained as indicated.

Data Analysis

Data are reported as mean ± standard error of the mean (SEM), and statistical comparisons using SYSTAT 8.0 were based on an analysis of variance (ANOVA) and Tukey's test for pairwise comparisons, with a P-value < 0.05 considered significant.

RESULTS

Retroviral Overexpression

Primary skeletal myoblasts were cultured up to 12 passages and transduced with empty vector, Runx2, and/or BMP-2 retroviral supernatant. Combined Runx2 and BMP-2 treatment was performed by mixing equal volumes of retroviral supernatant. The pTJ66 retroviral vector uses the promoter activity of the 5′ LTR for transgene expression, followed by an internal ribosomal entry site and a zeocin-resistance-enhanced green fluorescent protein (eGFP) fusion protein, allowing for non-invasive analysis of transduction efficiency [Byers et al., 2002]. High levels of eGFP transgene expression were confirmed for all treatments by flow cytometry (Fig. 1A). Transgene expression was further analyzed by qRT-PCR for Runx2 and BMP-2 mRNA levels (Fig. 1B). Runx2 and BMP-2 expression were significantly upregulated by treatment with Runx2 and BMP-2 retrovirus, respectively. Importantly, dilution of retroviral supernatant in the combined treatment did not affect transgene mRNA levels relative to

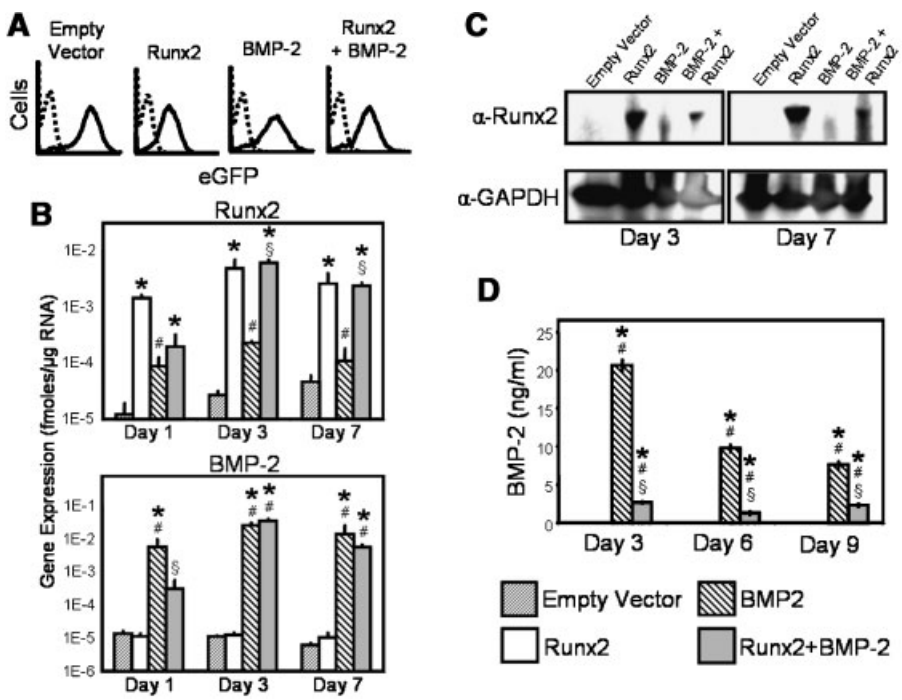


Fig. 1. Retroviral expression of Runx2 and BMP-2 transgenes. **A**: Transduction was confirmed by flow cytometry. **B**: Significant levels of Runx2 ($P < 1E-8$) and BMP-2 ($P < 9E-12$) mRNA were detected by qRT-PCR in samples transduced with the respective virus (mean + SEM, n = 3). Elevated protein levels were detected by (**C**) Western blotting for Runx2 and (**D**) ELISA of the culture media for BMP-2 (mean ± SEM, n = 3, $P < 5E-12$). * versus empty vector, # versus Runx2, § versus BMP-2.

undiluted virus stocks. Protein levels were assessed by Western blot for Runx2 (Fig. 1C) and ELISA of the bulk media for BMP-2 (Fig. 1D). Neither Runx2 nor BMP-2 protein was detectable in control myoblasts. Retroviral delivery of Runx2 significantly enhanced protein levels at 3 and 7 days. Lower levels of Runx2 protein were also detected in samples treated with BMP-2. Runx2 overexpression alone did not induce BMP-2 expression. However, BMP-2 protein in the bulk media was significantly reduced in the combined treatment relative to BMP-2 overexpression alone, despite equivalent BMP-2 mRNA levels. Additionally, secreted amounts of BMP-2 protein decreased over time despite no significant changes in mRNA expression. These observations may be explained by increased internalization or incorporation of soluble BMP-2 into the extracellular matrix assembled by Runx2- or BMP-2-engineered cells, or the upregulation of BMP-2-binding agonists, such as noggin. Nevertheless, these results confirm successful retroviral overexpression of substantial and sustained levels of Runx2 and BMP-2 transgenes in primary skeletal myoblasts.

Osteoblastic Differentiation In Vitro

Gene expression was analyzed at 1, 3, and 7 days post-transduction for osteoblastic matrix proteins (osteocalcin and bone sialoprotein), transcription factors (Osterix and Dlx5), mineralization-associated enzymes (alkaline phosphatase), and angiogenic factors (VEGF-A) (Fig. 2). Osteocalcin, bone sialoprotein, Osterix, and alkaline phosphatase were all upregulated by either Runx2 or BMP-2 at 7 days. Additionally, BMP-2 induced gene expression faster than Runx2 as demonstrated by expression profiles at 1 and 3 days. However, with the exception of bone sialoprotein, all genes showed similar levels in response to Runx2 and BMP-2 treatment after 7 days. Bone sialoprotein was upregulated to a greater extent by BMP-2 relative to Runx2 treatment. Interestingly, the combined treatment of Runx2 and BMP-2 resulted in synergistic effects (note logarithmic scale) on expression of osteocalcin, bone sialoprotein, Dlx5, and VEGF-A at later time points.

Functional osteoblastic differentiation was assessed by alkaline phosphatase biochemical activity and matrix mineralization in monolayer culture. Alkaline phosphatase activity was undetectable in control myoblasts, but significantly induced in Runx2- and BMP-2-overexpressing cells (Fig. 3A). At 14 days post-transduction, alkaline phosphatase activity was equivalent in cells with Runx2, BMP-2, and combined treatments. Matrix mineralization was assessed by calcium content (Fig. 3B) and von Kossa staining for phosphate deposits (Fig. 3C). There were no mineralized regions detected in control samples. Interestingly, Runx2-treated cells showed higher levels of

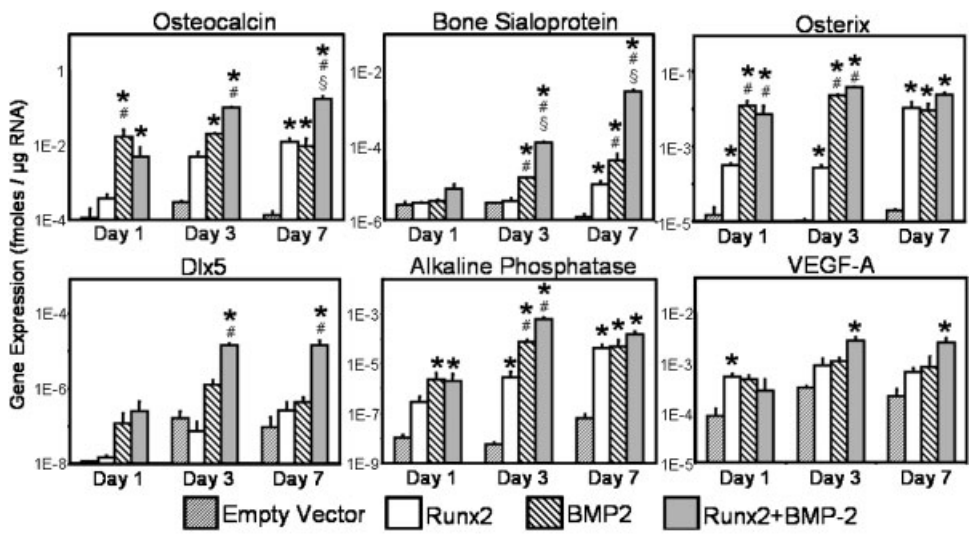


Fig. 2. Runx2- and BMP-2-induced gene expression was assessed by qRT-PCR analysis of osteoblastic markers including osteocalcin ($P < 9E\text{-}10$), bone sialoprotein ($P < 7E\text{-}12$), Osterix ($P < 8E\text{-}12$), Dlx5 ($P < 2E\text{-}6$), alkaline phosphatase ($P < 9E\text{-}12$), and VEGF-A ($P < 2E\text{-}5$) (mean + SEM, n = 3). * versus empty vector, # versus Runx2, § versus BMP-2.

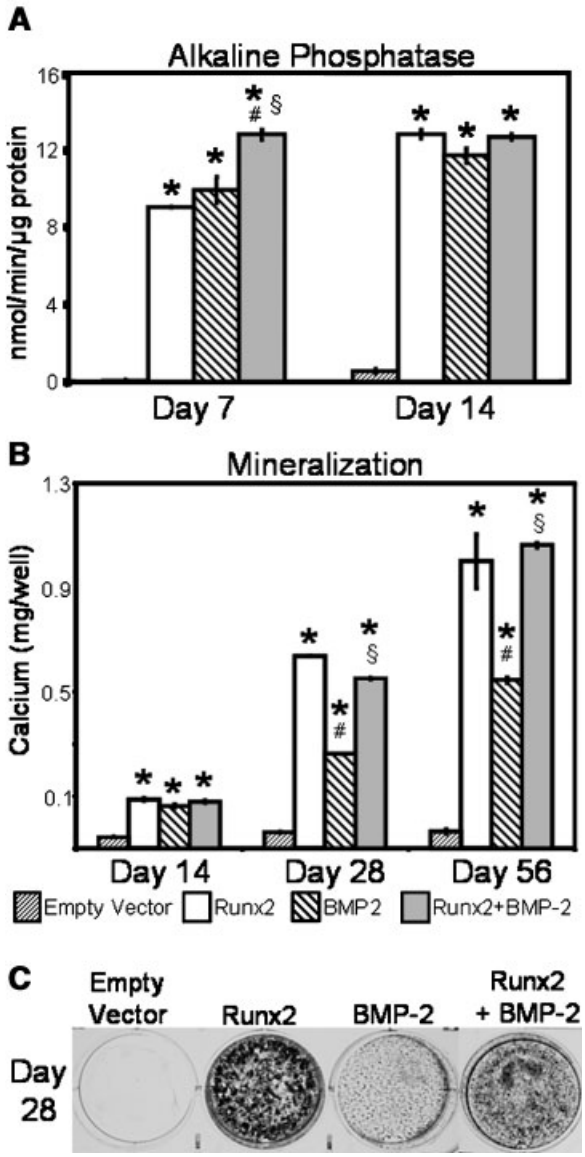


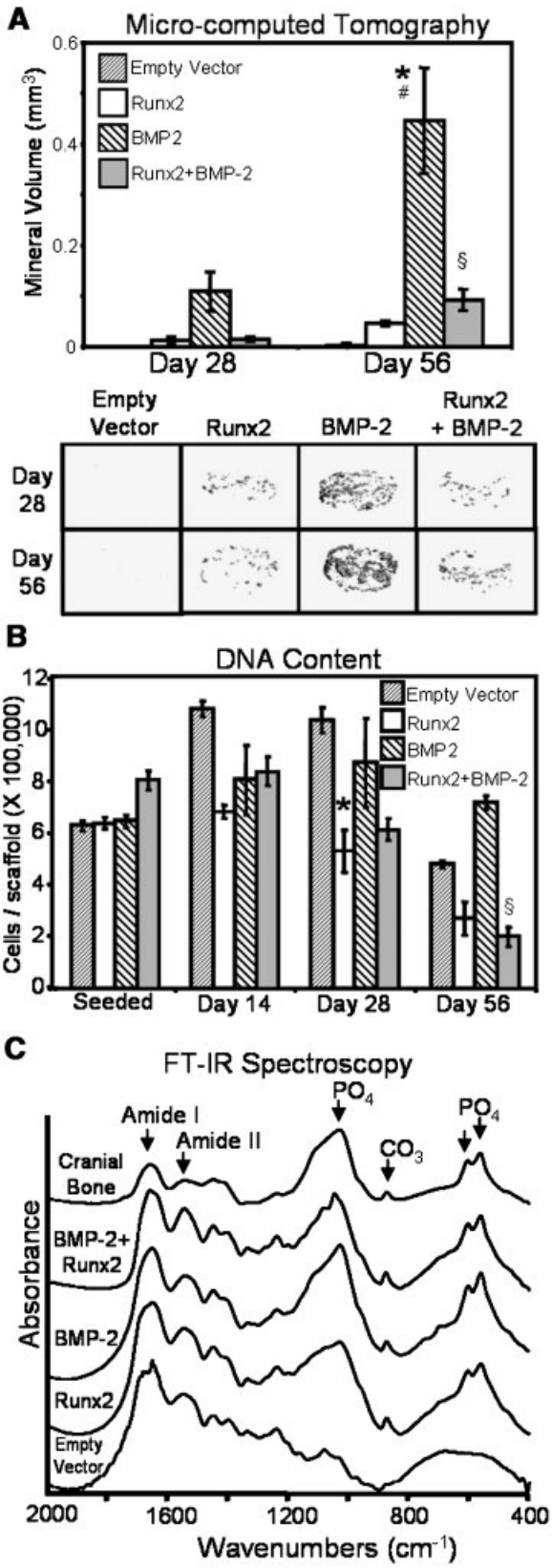
Fig. 3. Osteoblastic differentiation of Runx2- and BMP-2-engineered myoblasts was analyzed by (**A**) alkaline phosphatase biochemical activity ($P < 7E\text{-}12$, mean ± SEM, n = 3), (**B**) calcium content ($P < 6E\text{-}12$, mean ± SEM, n = 3), and (**C**) von Kossa staining for mineralization. * versus empty vector, # versus Runx2, § versus BMP-2.

mineralization than BMP-2-treated monolayer cultures for up to 8 weeks, despite similar levels of osteogenic gene expression and alkaline phosphatase activity at late time points.

Matrix Mineralization In Vivo

Cells were seeded onto fibrous collagen disks to assess osteoblastic differentiation in a 3-D, tissue-engineered environment. Mineralization on these scaffolds was assessed by micro-CT (Fig. 4A). In contrast to 2-D monolayer cultures, BMP-2-expressing cells exhibited significantly higher levels of mineralization compared to Runx2 cells in the cell-scaffold construct. The trends in calcium content of these constructs were similar to the levels of mineralization measured by micro-CT (data not shown). Importantly, the increased amount of mineralization by BMP-2 cells correlated to higher cell numbers in the scaffold (Fig. 4B). Constructs containing cells treated with both Runx2 and BMP-2 showed decreased cell numbers compared to BMP-2-engineered cells, similar to constructs seeded with cells expressing only Runx2. The in vitro mineral deposits were confirmed to be a carbonate-containing biological hydroxyapatite by FT-IR spectroscopy, similar to that of cranial bone (Fig. 4C). Constructs containing control myoblasts exhibited bands corresponding to a mineral-free extracellular matrix.

The cell-seeded collagen scaffolds were implanted intramuscularly into the hind limbs of immunocompetent syngeneic mice to evaluate the mineralization capacity of the genetically engineered cells in vivo (Fig. 5). Constructs were implanted 24 h after cell seeding (Fig. 5A) or after 4 weeks of in vitro pre-culture (Fig. 5B–E), since we have previously shown enhanced mineralization in vivo following in vitro construct development [Byers et al., 2004]. Mice were euthanized at 4 weeks post-implantation and hind limbs were analyzed for ectopic mineralization by micro-CT and histology. Collagen/cell constructs that were implanted without pre-culture showed moderate levels of mineral deposits for empty vector or Runx2-engineered cells. These mineral levels were not statistically different from mineral levels for cell-free constructs and scaffolds containing non-engineered myoblasts (Fig. 5A). Constructs with cells expressing BMP-2 alone or BMP-2 and Runx2 showed significantly greater amounts of ectopic mineralization (Fig. 5A). In contrast, constructs which matured in vitro prior to implantation showed significant and equivalent amounts of mineral for Runx2, BMP-2, and dual-treated cells relative to background levels with control cells (Fig. 5B). Hematoxylin and eosin staining (Fig. 5D) showed greater cellularity in pre-cultured mineralized implants relative to empty vector controls, despite greater cell numbers on control scaffolds at implantation. This result is likely



due to minimal proliferation of differentiated myocytes in control samples, in vivo proliferation of implanted osteogenic cells, and/or recruitment of native osteoprogenitors by implanted cells engineered to express osteogenic factors. Masson's trichrome staining (Fig. 5E) also showed lower cell numbers in control samples (purple nuclei), as well as acellular residual collagen scaffold (blue and red). Runx2, BMP-2, and dual-treated samples showed significant matrix remodeling and/or deposition with collagen staining (blue), aligned and interwoven in highly cellularized regions, resembling early intramembranous bone formation. Histological staining of implants consisting of scaffold only or scaffold with non-engineered cells was similar to empty vector samples for all analyses, suggesting no adverse response to retrovirally transduced cells.

Paracrine Signaling

BMP-2-expressing cells are known to stimulate osteoblastic differentiation of neighboring cells through paracrine signaling by secreted BMP-2 growth factor [Gazit et al., 1999; Krebsbach et al., 2000]. This effect is greatly responsible for the considerable success of BMP-2-based gene therapy strategies [Gazit et al., 1999]. However, unregulated secretion of soluble factors limits the ability to achieve targeted effects and may be responsible for abnormal bone formation [Moutsatsos et al., 2001; Peng et al., 2005] and tumorigenesis [Ide et al., 1997; Pouliot et al., 2003; Yang et al., 2005]. It is unclear whether cells overexpressing Runx2, an intracellular effector, also transmit osteogenic signals through secretion of cytokines or growth factors. Therefore, we transduced cells with the indicated virus, exchanged media after 2 days, and harvested conditioned media at 4 days and every other day thereafter. The conditioned media were immediately filtered through a 0.1-μm syringe filter and transferred to non-engineered myoblasts. This process was repeated every 2 days for 8 days, at which point both donor cells and cells receiving conditioned media (recipient

Fig. 4. Engineered cells were seeded onto fibrous collagen scaffolds and analyzed for mineralization by (**A**) micro-CT ($P < 1E\text{-}8$, mean ± SEM, n = 6) and (**B**) DNA content ($P < 8E\text{-}6$, mean ± SEM, n = 3). * versus empty vector, # versus Runx2, § versus BMP-2. **C**: In vitro mineralization was confirmed to be a biological hydroxyapatite by FT-IR spectroscopy.

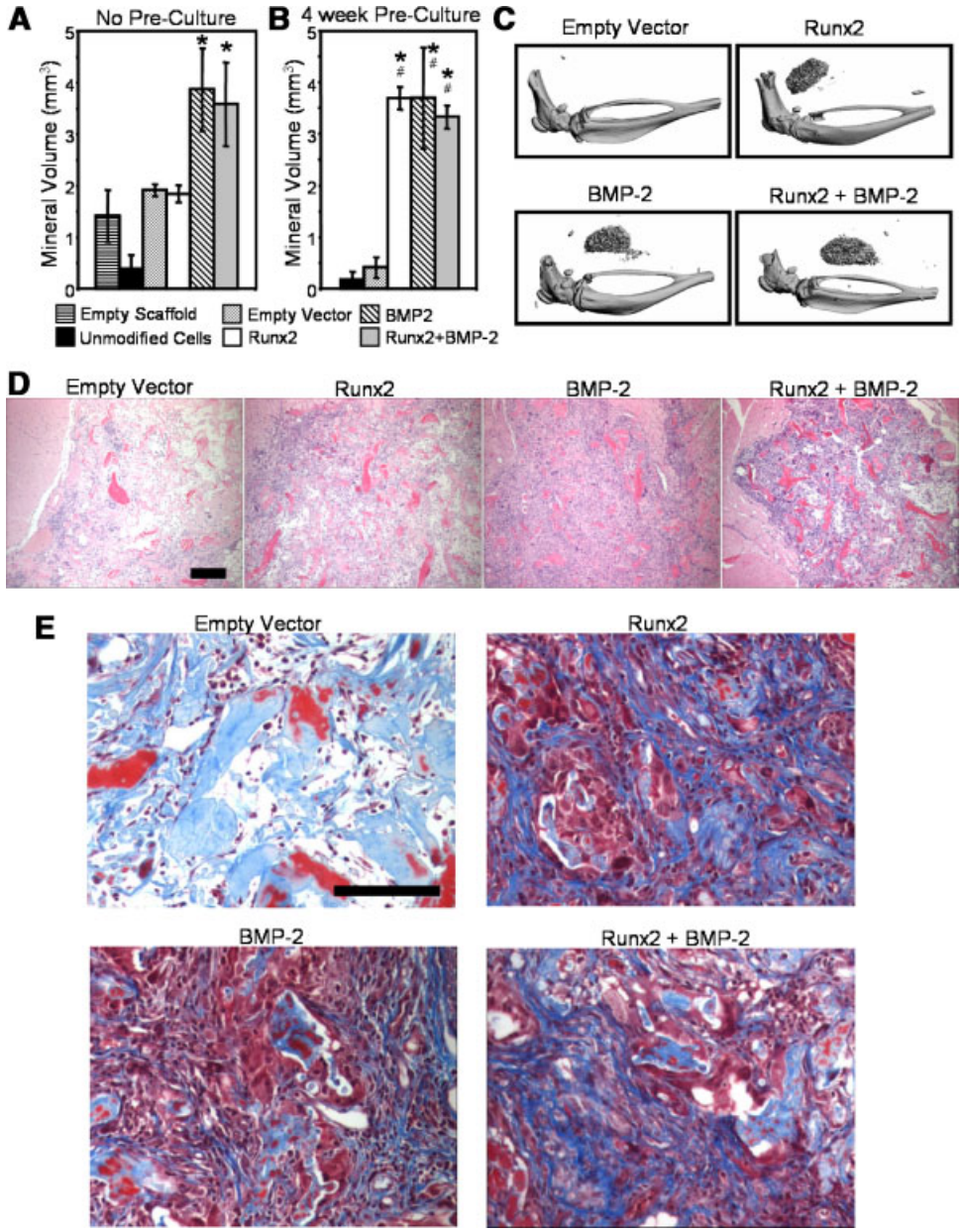


Fig. 5. Cell-seeded collagen scaffolds were implanted intramuscularly in the hind limbs of immunocompetent syngeneic mice at (**A**) at 1-day post-seeding or (**B**) after 28 days of in vitro pre-culture in differentiation media. Animals were euthanized at 4 weeks post-implantation and formalin-fixed hind limbs were analyzed for ectopic mineralization by micro-CT ($P < 8E\text{-}7$, mean ± SEM, n = 6). * versus empty vector, # versus Runx2. **C**: Images are representative of pre-cultured implants. Pre-cultured samples were also assessed histologically by (**D**) hematoxylin and eosin and (**E**) Masson's trichrome staining. Scale bars indicate 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cells) were harvested for analysis of osteogenic gene expression by qRT-PCR (Fig. 6A). Donor cells, which were treated with the indicated retrovirus, showed similar expression profiles as in Figure 2. Interestingly, conditioned media from any group did not affect the expression of osteocalcin, the most frequently characterized target of BMP-2 and Runx2. However, conditioned media from BMP-2-engineered cells induced both bone sialoprotein and Osterix expression in recipient cells. Conditioned media from Runx2-engineered cells had no statistically significant effects on any gene investigated, although a repeatable trend of increased Osterix expression was observed. Furthermore, co-treatment with Runx2 and

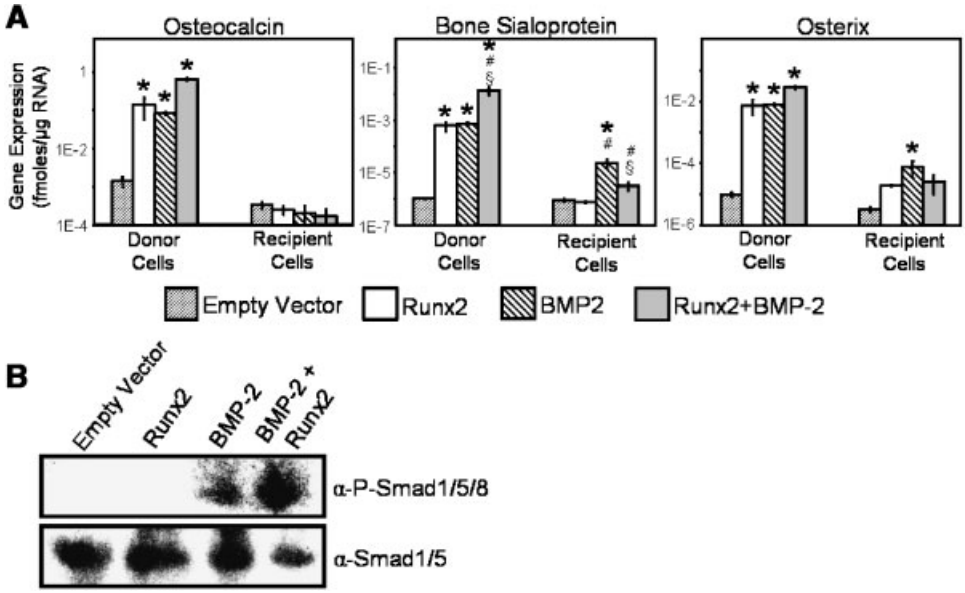


Fig. 6. Paracrine signaling by genetically engineered cells. **A**: Conditioned media studies were performed to analyze the ability of engineered cells to transmit osteogenic signals via soluble growth factors or cytokines. Donor myoblasts were engineered with the indicated retrovirus and differentiation media were exchanged every other day. Starting at 4 days post-transduction, media harvested from donor cells were filtered and added to non-engineered myoblasts (recipient cells) for a total of 8 days. Both donor cells and recipient cells were analyzed by qRT-PCR for osteocalcin ($P < 4E\text{-}9$), bone sialoprotein ($P < 8E\text{-}12$), and Osterix ($P < 2E\text{-}9$) gene expression (mean ± SEM, n = 3). * versus empty vector, # versus Runx2, § versus BMP-2. **B**: Phosphorylation of Smad signaling molecules was analyzed by Western blot. The presence of phosphorylated Smads in BMP-2-engineered cells confirmed activation of BMP signaling pathways. There was no Smad phosphorylation detected in Runx2-treated samples, demonstrating osteoblastic differentiation in the absence of BMP signaling.

BMP-2 showed a decreased effect on bone sialoprotein and Osterix relative to BMP-2 alone, possibly due to the dilution of BMP-2 protein detected in Figure 1D. To further examine the possibility of BMP signaling in Runx2-treated cultures, levels of Smad activation were assessed by Western blot (Fig. 6B). Significant levels of Smad phosphorylation were detected in BMP-2-engineered cultures. However, there was no detectable Smad activation in Runx2-overexpressing cells. Collectively, these results suggest that Runx2-overexpressing myoblasts, in contrast to BMP-2-secreting cells, do not utilize paracrine signaling by soluble factors in stimulating osteoblastic differentiation.

DISCUSSION

Genetic engineering of non-osteoblastic cells with osteogenic factors has emerged as a promising strategy to address cell-sourcing limitations associated with bone tissue engineering. However, the relative efficacy of these different approaches to induce osteoblastic differentiation remains unclear and is further complicated by varied delivery vehicles, cell types, and evaluation criteria. The present study provides a direct and quantitative comparison between transcription factor- and growth factor-based strategies to induce osteoblastic differentiation in vitro and in vivo. We used retroviral delivery to primary skeletal myoblasts in order to compare sustained expression of Runx2 and BMP-2 transgenes in a clinically relevant autologous cell source [Jankowski et al., 2002]. This work supports previous studies which indicate that BMP-2 overexpression may be a more potent osteoinductive strategy [Hirata et al., 2003; Yang et al., 2003]. However, these results also suggest that Runx2 overexpression can be an equally effective approach under the appropriate conditions or when measured at longer time points.

This study highlights the need for rigorous evaluation of osteoinductive strategies in multiple settings to define an appropriate method to achieve a desired therapeutic outcome. Runx2-overexpressing cells generated greater mineralization in confluent monolayer

cultures relative to BMP-2-treated cells. This is consistent with previous results comparing Runx2 overexpression with treatment of recombinant BMP-2 protein [Gersbach et al., 2004a]. However, BMP-2-engineered cells showed significantly greater levels of mineralization in 3-D collagen scaffolds, correlating with greater cell numbers. Runx2 and BMP-2 are both known to mediate proliferation, cell-cycle progression, and apoptosis of osteoblastic cells [Chen et al., 2001; Pratap et al., 2003]. The greater number of BMP-2-expressing cells on cultured collagen scaffolds could be explained by Runx2-mediated inhibition of proliferation [Pratap et al., 2003], BMP-2-mediated suppression of apoptosis [Chen et al., 2001], or differences in osteoblastic activity in 2- and 3-D cultures [Kale et al., 2000; Huang et al., 2004; Hosseinkhani et al., 2006; Phillips et al., 2006b]. These results underscore the necessity for measuring several parameters of cell activity in multiple environments when assessing cell sources for gene therapy strategies in orthopedic regeneration.

Yang et al. [2003] demonstrated a synergistic effect on osteogenesis with dual treatment of BMP-2 and Runx2 adenoviral vectors in a multipotent mesenchymal cell line. Although we show synergistic effects on osteoblastic gene expression, this effect was not propagated to downstream cellular effects including enzyme activity and mineralization. The inconsistencies between these studies may be the result of differences relating to transient expression in a multipotent cell line versus sustained transgene expression in a lineage-committed primary cell type. We also show diminished detection of secreted BMP-2 in Runx2 co-expressing cultures (Fig. 1D) without a loss of osteoblastic activity. This may be the result of enhanced cellular uptake or BMP-2 incorporation in the extracellular matrix. Further investigation is necessary to determine whether these mechanisms contribute to the observed synergy in dually treated cells. Additionally, we did not observe the development of osteocytes, cartilage, or marrow cavities as reported for Runx2- and BMP-2-engineered cell lines implanted in ectopic sites in immunodeficient mice [Yang et al., 2003]. Previous studies suggest that this may be the result of using primary cells and immunocompetent mice in the present work, and longer implantation times may be necessary for the development of more complex tissues [Musgrave et al., 1999].

Paracrine signaling by BMP-2-expressing cells plays a central role in stimulating osteogenesis [Gazit et al., 1999; Xiao et al., 2002]. This effect can be advantageous for obtaining an adequate degree of osteoblastic differentiation and mineralization. However, the unregulated secretion of potent signaling molecules limits target-cell specificity and risks tumorigenesis [Ide et al., 1997; Pouliot et al., 2003; Yang et al., 2005] or abnormal bone formation [Moutsatsos et al., 2001; Peng et al., 2005]. Choi et al. [2005] have described a mechanism by which Runx2 induces BMP-2 expression, suggesting that Runx2-overexpressing cells may stimulate osteoblastic differentiation via paracrine signaling of secreted factors. Additionally, a recent report suggests that BMP signaling is required for Runx2-stimulated osteoblastic differentiation [Phimphilai et al., 2006]. However, we did not detect secretion of BMP-2 or -4 into the surrounding media by Runx2-engineered cells. Furthermore, there were no detectable levels of Smad activation in these cells, nor was there any significant effect of conditioned media from Runx2-engineered cells on osteogenic gene expression. These results suggest that BMP signaling was not involved in Runx2-stimulated osteoblastic differentiation of primary skeletal myoblasts in this system. The discrepancies between these studies may be the result of cell-type dependent effects in stimulating osteoblastic differentiation [Byers et al., 2002]. In fact, although Runx2-binding sites have been identified in the promoters of the human BMP-2 and -4 genes, deletion of these sites did not alter promoter activity in osteoblastic cells [Helvering et al., 2000].

In addition to BMP-2 and the Runx2 transcription factor, there are several other gene therapy-based strategies under investigation for orthopedic regeneration. Promising results have been achieved by focusing on other members of the BMP family [Krebsbach et al., 2000; Jane et al., 2002], VEGF and RANK-L [Ito et al., 2005], constitutively active BMP receptors [Koefoed et al., 2005], and LIM-mineralization proteins [Pola et al., 2004]. Other studies have demonstrated synergistic effects by incorporating multiple transgenes [Yang et al., 2003; Zhao et al., 2005a] and transgenes supplemented with glucocorticoids [Phillips et al., 2006a] or immunosuppressive agents [Kaihara et al., 2004; Li et al., 2005]. Additionally, the limitations of uncontrolled growth factor secretion

and constitutive transgene expression are also being addressed with tissue-specific promoters [Hou et al., 1999], inducible expression systems [Moutsatsos et al., 2001; Gafni et al., 2004; Peng et al., 2004; Gersbach et al., 2006], and co-expression of regulatory factors [Peng et al., 2005]. Optimization of scaffold parameters is also essential to regulating the osteoblastic activity of these genetically engineered cells [Phillips et al., 2006b].

This study emphasizes the complexity of ex vivo gene therapy as an integrated relationship of cell activity and differentiation state, construct maturation, and paracrine signaling of osteogenic cells. Our results indicate that sustained BMP-2 expression in skeletal myoblasts is a more potent strategy for osteoinduction compared to delivery of the Runx2 transcription factor. However, we also identified conditions in which Runx2 is equally effective as BMP-2 in stimulating osteoblastic differentiation and matrix mineralization. Furthermore, Runx2-based strategies may avoid complications arising from uncontrolled signaling of secreted factors. Collectively, these results underscore the necessity for thorough evaluation of genetically engineered cells to identify the appropriate system for specific clinical needs. This work is significant to evaluating these systems and defining a successful strategy for integrating gene medicine and orthopedic regeneration.

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