the dorsal aorta, resulting in embryo demise at embryonic day 10.5.[22] Furthermore, SRF is important for maintaining normal function of skeletal muscle,[23] liver,[24] pancreas,[25] neurons,[26] as well as lymphocytes and megakaryocytes.[27,28] However, the function of SRF in regulating skeletal development and bone remodeling is unknown.

To determine the role of SRF in regulating osteoblast differentiation and bone development in vivo, we generated osteoblast-specific SRF knockout mice by breeding SRF floxed mice[22] with the transgenic mice carrying the osteocalcin (OC) promoter-driven Cre recombinase.[29–31] Osteoblast-specific deletion of SRF did not affect embryonic bone development but decreased bone mass of adult mice. Deletion of SRF in osteoblasts reduced osteoblast differentiation and mineralization in vitro, which was mediated by reduced expression and secretion of IGF-1 from osteoblasts and impaired IGF-1–induced Akt signals, as well as decreased Runx2 transactivity. These studies have demonstrated a novel function of SRF and SRF-regulated signals in osteoblast differentiation and bone formation.

Materials and Methods

Generation of osteoblast-specific SRF-deficient mice

The floxed SRF mice ($SRF^{f/f}$) were generated as previously reported.[22] To generate osteoblast-specific SRF-deficient mice ($SRF^{\Delta/\Delta}$), $SRF^{f/f}$ mice were crossed with osteocalcin-Cre (OC-Cre) transgenic mice[29] obtained from Dr Thomas Clemens (The Johns Hopkins University, Baltimore, MD, USA). The mice were maintained at the University of Alabama at Birmingham using Institutional Animal Care and Use Committee–approved procedures. Genotyping was carried out as previously described.[22,29]

Dual-energy X-ray absorptiometry (DXA) and microCT (μCT) analyses

DXA and μCT analyses were performed by the Small Animal Bone Phenotyping Core of the Center for Metabolic Bone Disease at the University of Alabama at Birmingham. Bone mineral density (BMD), bone mineral content, and other body composition were assessed by DXA (GE-Lunar PIXImus, version 1.45; GE-Lunar, Madison, WI, USA) in vivo periodically as described previously.[32] For μCT analysis, intact femur from each mouse was scanned using a high-resolution μCT imaging system (μCT40; SCANCO Medical, Wayne, PA, USA). Various bone parameters,[33] including bone volume and trabecular thickness, number, and space, were evaluated.

Bone histomorphometry analysis

Bone histomorphometry analysis was performed by the Histomorphometry and Molecular Analyses Core of Center for Metabolic Bone Disease at the University of Alabama at Birmingham. In brief, femurs were removed from 14-week-old mice and fixed in 10% formalin, decalcified in EDTA, and embedded in plastic polymers. Longitudinal sections (5 μm thick) were stained with Goldner's modified Trichrome staining. Histomorphometric parameters, including bone volume and trabecular thickness, number, and space, were determined with Bioquant image analysis software (R & M Biometrics, Nashville, TN, USA) as previously described.[32] All parameters comply with the recommendations of the Histomorphometry Nomenclature Committee of the American Society of Bone and Mineral Research.[34]

In vitro osteoblast proliferation and differentiation

Primary mouse calvarial cells were isolated from newborn $SRF^{\Delta/\Delta}$ mice or $SRF^{f/f}$ mice by collagenase P digestion and cultured in growth media containing Minimum Essential Medium (Mediatech, Manassas, VA, USA) with 10% fetal bovine serum (Invitrogen, Grand Island, NY, USA). SRF knockout cells (SRF KO) were generated in vitro by deleting SRF in the $SRF^{f/f}$ cells using lentivirus-mediated Cre recombinase (lenti-Cre). Lentivirus-mediated GFP (lenti-GFP) was used as a control (Control). Cell proliferation was assessed by BrdU incorporation in 96-well plates using BrdU kit (EMD Millipore, San Diego, CA, USA). Osteoblast differentiation was induced in osteogenic media containing growth media supplemented with 50 μg/mL ascorbic acid (Sigma, St. Louis, MO, USA) and 10 mM β-glycerol phosphate (MP Biomedicals, Santa Ana, CA, USA).[35] Alizarin Red staining was used to determine mineralization. Samples were also collected to determine RNA and protein expression. The effect of IGF-1 on osteoblast formation was analyzed by addition of IGF-1 (13 nM, GroPep, Thebarton, Australia) in the culture media.[36]

Measurement of IGF-1

IGF-1 levels in the mouse sera or conditioned media of cell cultures were determined using a commercial ELISA kit (PeproTech, Rocky Hill, NJ, USA). For assaying serum IGF-1, mice were bled through retro-orbital vein, and serum samples were collected between 9:00 a.m. and 10 a.m. on a fed state. For assaying IGF-1 in the condition media, cells were placed in a 24-well plate and culture supernatants were collected. IGF-1 was measured using the ELISA kit.

Western blotting analysis

Calvarial cell extracts were prepared and protein concentrations were measured as described previously.[37] Western blotting analyses were performed with the use of specific antibodies for SRF, Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Akt, phospho-Akt, ERK, phospho-ERK (Cell Signaling, Danvers, MA, USA), and Runx2 (MBL, Woburn, MA, USA) and detected with a Western blotting chemiluminescence detection kit (EMD Millipore, Billerica, MA, USA). Mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Fitzgerald (Acton, MA, USA).

Real-time PCR analysis

To determine gene expression, total RNA was extracted per manufacturer's instructions (Invitrogen) and treated with RNase-free DNase I. The reverse transcription reaction was performed on 1 μg of RNA using the First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA). Real-time PCR was performed and analyzed using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) in a Bio-Rad CFX96-Cycler as previously described.[38]

Electrophoretic mobility shift assay (EMSA)

EMSA was performed to determine the Runx2 DNA binding activity. Nuclear extracts were extracted and analyzed using ^{32}P-labeled probe containing Runx2-binding oligonucleotide from the *Bglap2* promoter (underline, Runx 2 consensus): 5′-CAGAAACCAACCACAGGACCCAAACCACAGCCTCCAC-3′ as we previously described.[32] For supershift assays, nuclear extracts were preincubated with anti-Runx2 antibody or control mouse IgG for 20 minutes before the addition of the labeled probe. For competition experiments, an excessive amount of unlabeled probe (100-fold) was added to binding reactions.

Transfection and luciferase assays

To analyze Runx2 transactivity, calvarial cells were plated on 24-well plates at a density of 3×10^4 cells/cm^2 and transfected with 0.2 μg of the pGL3-6 × OSE2 luciferase plasmid plus 0.1 μg of pCMV-β-galactosidase (β-gal) expression plasmid (control for transfection efficiency) using FuGene HD tranfection reagent (Roche, Indianapolis, IN, USA). Cells were then incubated for 72 hours and harvested to determine luciferase and β-gal activity. The luciferase reporter activity was normalized by β-gal activity (Promega, San Luis Obispo, CA, USA). To determine the effects of SRF on Runx2 transactivity, a lentivirus-carrying SRF expression construct was used, and lenti-GFP was used as a control. Overexpression of SRF was determined by Western blotting analysis.

Statistical analysis

Statistical analysis was performed as previously described.[32] Data shown are means ± standard deviation (SD). Differences between two groups were identified by Student's *t* tests. For multiple groups, one-way analysis of variance and Student-Newman-Keuls tests were used to identify differences. Significance was defined as $p < 0.05$.

Results

Osteoblast-specific deletion of SRF reduces bone mass in adult mice

Osteoblast-specific SRF-deficient mice were generated by breeding SRF floxed mice with Cre transgenic mice in which the expression of Cre recombinase is driven by the promoter of osteocalcin (OC). The specificity and efficiency of OC-Cre-mediated recombination in osteoblasts has been verified previously.[29] The OC-Cre:SRF$^{f/f}$ mice (SRF$^{\Delta/\Delta}$) were viable and fertile, with no apparent growth defects at birth.

To determine the effect of SRF deletion in osteoblasts on postnatal bone development, overall BMD was determined by whole-body DXA analysis of mice at the ages of 4, 6, 9, and 12 weeks. No significant difference in BMD was identified between SRF$^{f/f}$ and SRF$^{\Delta/\Delta}$ littermates at weeks 4 and 6. However, SRF$^{\Delta/\Delta}$ mice exhibited significantly reduced BMD at the ages of 9 and 12 weeks in both sexes (Fig. 1*A*). MicroCT analysis of the femurs confirmed that bone density was markedly decreased in SRF$^{\Delta/\Delta}$ mice (Fig. 1*B*). Quantitative analyses further demonstrated significant decrease in trabecular bone mass, indicated by the ratio of bone volume (BV) to tissue volume (TV), whereas cortical bone was not affected significantly in SRF$^{\Delta/\Delta}$ mice (Table 1). Consistently, decreases in trabecular numbers and thickness and increases in trabecular space were evident in the SRF$^{\Delta/\Delta}$ mice (Table 1). In addition, histomorphometrical analysis of the femurs revealed a marked decrease in trabecular bone density in SRF$^{\Delta/\Delta}$ mice (Fig. 1*C*, a1–a4). The thickness of the growth plate did not show any apparent changes in the SRF$^{\Delta/\Delta}$ mice, suggesting the osteoblast-specific deletion did not affect chondrocyte differentiation (Fig. 1*C*, b1–b4).

SRF deletion confers decreased osteoblast differentiation

The cellular phenotype for SRF deletion was examined by ex vivo differentiation of calvarial osteoblasts from newborn SRF$^{\Delta/\Delta}$ mice. As shown in Fig. 2*A*, OC-Cre-driven SRF deletion did not occur efficiently in the calvarial cells from SRF$^{\Delta/\Delta}$ mice until week 3, at which time the cells are at the postproliferation stage to the mineralization stage during in vitro osteogenesis process. At week 4, the SRF$^{\Delta/\Delta}$ cells had reduced mineral deposition compared with the SRF$^{f/f}$ cells (Fig. 2*B*). Therefore, SRF deletion by OC-Cre occurred at the late stage of osteogenesis, and the SRF deletion at this stage reduced osteoblast mineralization.

To further determine the effect of SRF on osteoblast differentiation and function, primary calvarial cells were isolated from SRF$^{f/f}$ mice, and SRF was excised by lenti-Cre (SRF KO). Western blotting analysis confirmed effective deletion of SRF in the SRF KO cells compared with that in control cells infected with lenti-GFP (Fig. 2*C*). In vitro osteogenic differentiation and matrix mineralization were markedly reduced in the SRF KO cells, whereas the control cells (lenti-GFP) exhibited a normal differentiation profile (Fig. 2*D*). Of note, SRF KO conferred a greater inhibition on osteoblast differentiation and mineralization compared with the SRF$^{\Delta/\Delta}$ cells, which may be owing to the fact that ex vivo deletion of SRF in the SRF$^{\Delta/\Delta}$ cells only occurred after 3 weeks in osteogenic media. In fact, deletion of SRF by lenti-Cre in SRF$^{f/f}$ calvarial cells significantly decreased cell proliferation by 30% ($n = 3$, $p < 0.05$), whereas calvarial cells from SRF$^{\Delta/\Delta}$ mice exhibited a similar proliferation rate as those from control SRF$^{f/f}$ mice ($n = 3$, not significant), suggesting additional effects of SRF on proliferation at an earlier stage.

SRF deletion reduces IGF-1 expression in primary calvarial cells

Because osteoblast-specific SRF deficiency was found to affect only adult mice in both sexes, we characterized the role of the growth factor IGF-1, one of the critical determinants of longitudinal bone growth, skeletal maturation, and acquisition of bone mass during puberty and thereafter.[8,39,40] SRF has been found to directly regulate IGF-1 expression in liver cells.[24] No significant differences of circulating IGF-1 levels were observed in sera from SRF$^{f/f}$ and SRF$^{\Delta/\Delta}$ mice (Fig. 3*A*). By contrast, IGF-1 expression and secretion were markedly reduced in the SRF KO osteoblasts (Fig. 3*B*). Quantitative real-time PCR analysis demonstrated a dramatic decrease in IGF-1 mRNA expression in the SRF KO osteoblasts compared with that in the control cells (Fig. 3*C*, left). The expression of the receptor for IGF-1 (IGF-1R) was similar in both control and SRF KO cells (Fig. 3*C*, right).

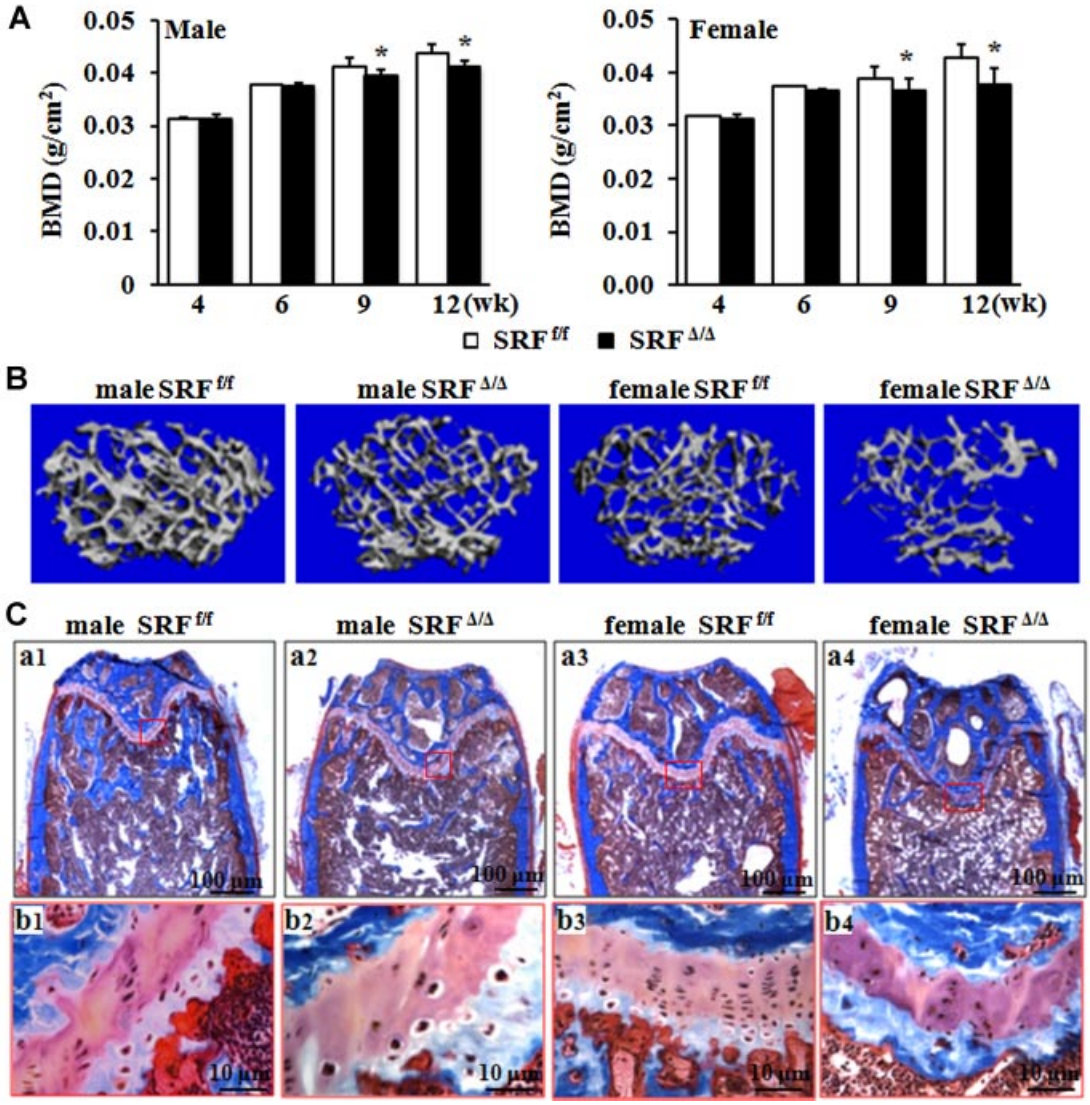

Fig. 1. Decreased bone densitometry in $SRF^{\Delta/\Delta}$ adult mice. (*A*) Bone mineral density (BMD) was assessed at 4, 6, 9, and 12 weeks old in male and female mice. (*B*) Representative three-dimensional images of metaphyseal bone generated from μCT analysis. Representative images from five (male) or four (female) age-matched littermates are shown. $^{*}p < 0.05$. (*C*) Nondecalcified histological sections of distal femur in 13-week-old littermates. The sections of the distal femur (a1–a4, 30× magnification) and growth plate (b1–b4, 480× magnification) were stained with Goldner's Trichrome staining.

IGF-1 enhances osteogenesis in SRF-deficient cells

To determine whether decreased IGF-1 expression in the SRF KO cells contributed to the decreased mineralization, we tested whether recombinant IGF-1 could rescue the phenotype. As determined by Alizarin red staining (Fig. 4*A*, *B*), addition of IGF-1 (13nM) in the osteogenic media during in vitro osteogenesis markedly increased mineralization of the control cells. In addition, IGF-1 also restored mineralization of the SRF KO cells.

SRF deletion impairs IGF-1-activated signaling in primary calvarial cells

To determine whether SRF KO may affect IGF-1–induced signaling pathways, we characterized the two major signaling pathways that are activated by IGF-1, the protein kinase B (Akt) and extracellular signal-regulated kinase (ERK) signaling pathways.[41] IGF-1–induced activation and phosphorylation of Akt and ERK were evident in the control cells (Fig. 4*C*). The SRF KO did not affect IGF-1–induced ERK phosphorylation, whereas IGF-1–induced Akt phosphorylation was reduced. In addition, overexpression of SRF enhanced IGF-1–induced Akt phosphorylation (Fig. 4*D*). Therefore, SRF deletion in osteoblasts reduced IGF-1 expression and secretion by osteoblasts and inhibited IGF-1–activated Akt, which may contribute to the decreased differentiation and mineralization of osteoblasts in vitro.

SRF deletion inhibits Runx2 expression and Runx2 transactivity during osteogenesis

Runx2 is a key regulator for osteogenesis. Its expression and activity are regulated by many growth factors and signaling pathways, including IGF-1 and Akt.[42,43] Accordingly, we determined the effect of SRF KO on the Runx2 expression and activity. The expression of Runx2 was induced in the control cells

Table 1. MicroCT Analysis of Bone Parameters in $SRF^{f/f}$ and $SRF^{\Delta/\Delta}$ Mice

		$SRF^{f/f}$	$SRF^{\Delta/\Delta}$
Trabecular bone			
BV/TV	Male	0.149 ± 0.057	0.069 ± 0.025^a
	Female	0.065 ± 0.010	0.025 ± 0.018^b
Tb.N (mm^{-1})	Male	4.476 ± 0.960	3.037 ± 0.405^a
	Female	3.078 ± 0.750	1.924 ± 0.421^a
Tb.Th (mm)	Male	0.045 ± 0.007	0.031 ± 0.003^a
	Female	0.027 ± 0.005	0.025 ± 0.004
Tb.Sp (mm)	Male	0.215 ± 0.061	0.315 ± 0.025^b
	Female	0.319 ± 0.064	0.481 ± 0.119^a
Cortical bone			
BV/TV	Male	0.368 ± 0.062	0.301 ± 0.047^{NS}
	Female	0.365 ± 0.052	0.324 ± 0.024^{NS}

$n = 5$ for male and $n = 4$ for female/group.

BV/TV = bone volume per tissue volume; Tb.N = trabecular number; Tb.Th = trabecular thickness; Tb.Sp = trabecular space; NS = not significant.

[a] $p < 0.05$.

[b] $p < 0.01$.

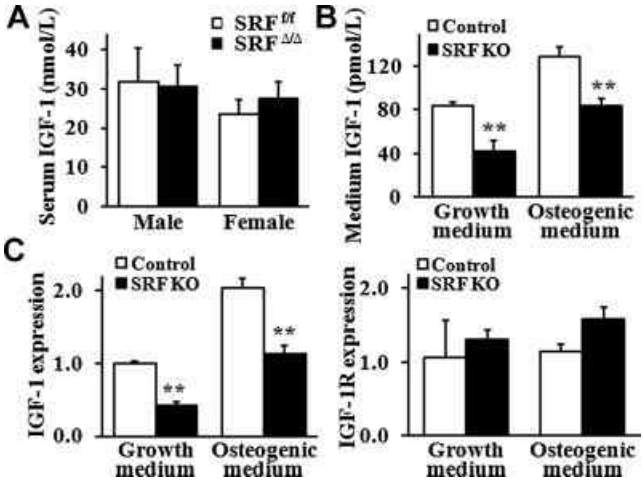

Fig. 3. Decreased IGF-1 expression in SRF-deficient osteoblasts. (*A*) Serum IGF-1 levels in $SRF^{f/f}$ and $SRF^{\Delta/\Delta}$ mice at 12 weeks of age ($n = 5$); (*B*) IGF-1 levels in culture supernatant of SRF KO and control osteoblasts. (*C*) Quantification of mRNA expression of IGF-1 and IGF-1R in SRF KO and control osteoblasts cultured in growth or differentiation medium for 1 week. Data shown are means ± SD from three independent experiments performed in triplicate. $^{**}p < 0.01$.

that were cultured in osteogenic media for 10 days (Fig. 5*A*). By contrast, the induction of the Runx2 expression was significantly reduced in the SRF KO cells. Addition of IGF-1 in the culture media was able to enhance Runx2 expression in both control cells and SRF KO cells cultured either in growth or osteogenic medium (Fig. 5*A*).

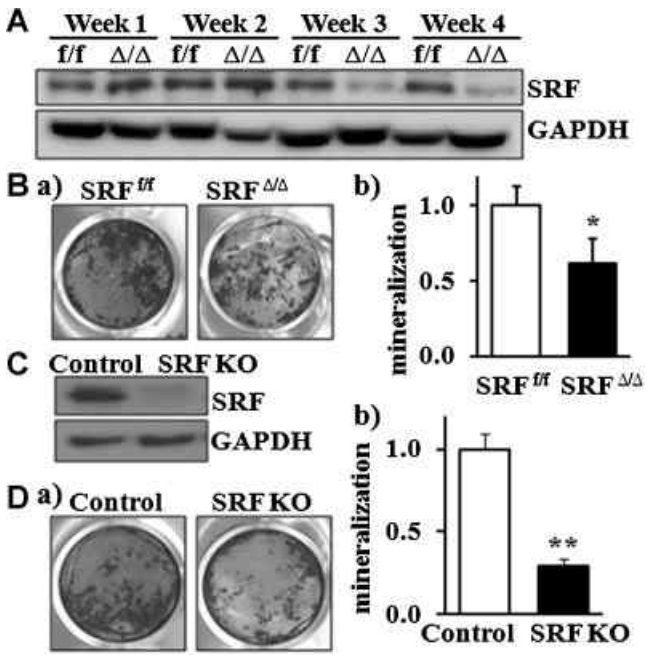

Fig. 2. Reduced osteoblast differentiation in SRF-deficient calvarial cells. (*A*) Western blotting analysis of SRF in calvarial cells from $SRF^{f/f}$ mice and $SRF^{\Delta/\Delta}$ mice cultured in osteogenic medium for up to 4 weeks. (*B*) a, Alizarin Red staining of mineralization at 4 weeks. b, Quantification of mineralized area in a. (*C*) Western blotting analysis of SRF in calvarial cells from $SRF^{f/f}$ mice that were infected with lenti-Cre (SRF KO) or lenti-GFP (control). (*D*) The control and SRF KO cells were cultured in osteogenic media for 4 weeks. a, Alizarin Red staining of mineralization. b, Quantification of mineralization shown in a. $^{*}p < 0.05$; $^{**}p < 0.01$.

The effect of SRF deletion on Runx2 DNA binding activity was determined by EMSA using a probe containing Runx2 DNA binding consensus. The specificity and efficiency of the probe for Runx2 DNA binding activity was validated with nuclear extracts from control cells (Fig. 5*B*a). Osteogenic media induced Runx2 DNA binding activity in the control cells (Fig. 5*B*b). In contrast, the activity was inhibited in the SRF KO cells. IGF-1 treatment enhanced the activity in both control and SRF KO cells (Fig. 5*B*). To further determine whether the altered Runx2 expression level was responsible for the effects of IGF-1 and SRF on the Runx2 DNA binding activity, we determined the activity in the Runx2-overexpressing cells. Overexpression of Runx2 in control and SRF KO cells was confirmed by Western blot analysis (Fig. 5*C*). Short treatment of IGF-1 for 30 minutes did not affect Runx2 expression (Fig. 5*C*) or Runx2 DNA binding activity (Fig. 5*D*). The Runx2 DNA binding activity appears to be similar in both control and SRF KO cells, indicating that SRF may not directly affect Runx2 DNA binding ability (Fig. 5*D*).

In contrast, despite Runx2 overexpression (Fig. 6*A*), the SRF KO cells exhibited decreased Runx2 transactivity (Fig. 6*B*), suggesting a role for SRF in regulating Runx2 activity as a transcription factor. To further determine the effect of SRF on Runx2 transactivity, SRF was overexpressed in control and SRF KO calvarial cells. Lentivirus-mediated overexpression of SRF was confirmed by Western blotting analysis (Fig. 6*C*). Runx2 transactivity was significantly decreased in the SRF KO calvarial cells as determined by the luciferase reporter assay (Fig. 6*D*, first set). Overexpression of SRF alone enhanced Runx2 transactivity in the control cells (Fig. 6*D*, white boxes). More important, the SRF overexpression restored Runx2 transactivity in the SRF KO cells (Fig. 6*D*, black boxes), supporting a role for SRF in regulating Runx2 transactivity in calvarial cells.

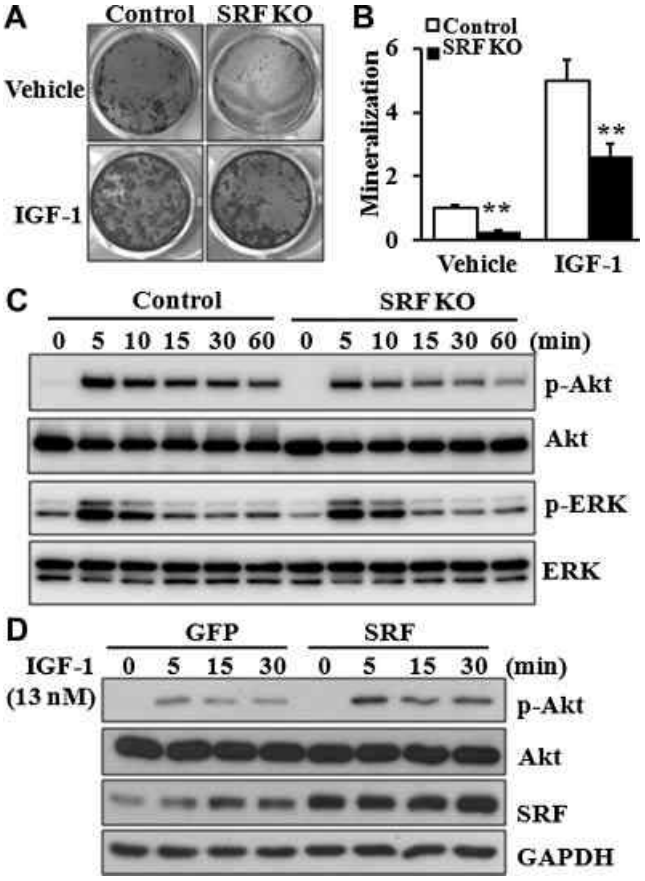

Fig. 4. IGF-1 activated signaling in SRF-deficient osteoblasts. (*A*) IGF-1 enhances mineralization of osteoblast cells. SRF KO or control cells were cultured in differentiation medium with or without IGF-1 (13 nM). Mineralization was assessed by Alizarin Red staining at 3 weeks of culture. (*B*) Quantification of mineralized area in *A*. Data shown are means ± SD, results from three independent experiments are shown. $^{**}p < 0.01$. (*C*) IGF-1–induced activation of Akt and ERK was determined by Western blotting analysis of phosphorylated Akt and ERK. SRF KO or control primary osteoblasts were serum-starved for 24 hours and exposed to IGF-1 (13 nM) for the indicated time. Total Akt and ERK were determined and used as loading control. Representative blots from three independent experiments are shown. (*D*) Effects of SRF overexpression on IGF-1-activated Akt. Primary calvarial cells that overexpressed SRF (SRF) or GFP (GFP) were serum-starved for 24 hours and exposed to IGF-1 (13 nM) for the indicated time. Western blotting analysis was performed to determine SRF expression and activation of Akt. The expression of GAPDH was used as a loading control.

Discussion

Control of osteoblast differentiation and matrix mineralization is an intricate process requiring the interplay of cell signaling, transcriptional, and posttranscriptional regulatory events. In this study, we demonstrated that SRF is an important modulator for osteoblast mineralization and bone homeostasis. We found that osteoblast-specific deletion of SRF did not affect embryonic bone formation and chondrocyte maturation but decreased postnatal bone development of adult mice. The impact of osteoblast-specific deletion of SRF on the bone structure was similar in both male and female mice (Fig. 1), indicating that sex hormones are not involved. Furthermore, reduced bone mass of the osteoblast-specific SRF deletion mice was evident after week 9, suggesting the potential roles of growth factors.

We found that the SRF deletion significantly reduced the expression of IGF-1 in osteoblasts (Fig. 3*C*). Such an observation is consistent with those in skeletal muscle cells and liver cells in which SRF deficiency downregulates IGF-1.[24,44] IGF-1 is an abundant growth factor in bone, and osteoblasts are the main source for skeletal IGF-1 production.[45] The IGF-1/IGF-1R system is important in bone metabolism through a combination of endocrine, autocrine, and paracrine modes of action.[29,46] Especially during puberty, IGF-1 is indispensable for peak increases of BMDs via both growth hormone–dependent and –independent mechanisms. Targeted IGF-1 overexpression in osteoblasts increased trabecular bone volume at puberty.[47] In contrast, the development of peak BMD in the puberty was completely blocked in the IGF-1–deficient mice.[39] Similarly, we observed reduced bone mass in the osteoblast-specific SRF-deficient mice at the age of 9 weeks and thereafter (Fig. 1*A*). The timing of the bone phenotype in the osteoblast-specific SRF-deficient mice is strikingly similar to that of the IGF-1–deficient mice,[39] indicating that IGF-1 may mediate the effect of SRF on bone development. In addition, osteoblast-specific SRF deficiency resulted in bone loss prominently in the trabecular bone, but not in the cortical bones (Table 1). This is consistent with previous demonstration that local IGF-1 derived from osteoblast cells appears to play a significant role in the maintenance of trabecular bone.[47,48] We also found that the SRF deletion significantly reduced the expression of IGF-1 in osteoblasts but not in serum, suggesting the defect in locally expressed IGF by osteoblasts contributes to the decreased bone mass in the SRF-deficient mice after puberty (Fig. 3*A*). This also explains why cortical bones were not affected in the osteoblast-specific SRF-deficient mice because circulating IGF-1, mainly derived from the liver, contributes primarily to cortical bone integrity.[8] Therefore, decreased local IGF-1 expression by the SRF-deficient osteoblasts regulates trabecular bone loss in the $SRF^{\Delta/\Delta}$ mice. The in vitro osteogenesis studies further support an important role of IGF-1 in promoting osteoblast differentiation. Exogenous addition of IGF-1 enhanced osteoblast differentiation in normal control cells and partially restored osteoblast mineralization in the SRF deletion cells (Fig. 4*A*, *B*). In addition, SRF deletion did not affect osteoblast mineralization induced by bone morphogenic protein 2 (data not shown), which plays an important role in osteoblast differentiation, mineralization, and bone formation during early skeletal development.[49] Therefore, osteoblast-derived IGF-1 plays a critical and selective role in mediating the osteogenic function of SRF.

Ex vivo culture of calvarial cells from $SRF^{\Delta/\Delta}$ mice confirmed that SRF was decreased only when cells were cultured in osteogenic medium for 3 weeks or longer (Fig. 2*A*), at which time point cells were at a late stage of differentiation and mineralization.[50] In contrast, in vitro deletion of SRF in calvarial cells at an early stage resulted in more severe defects in osteoblast differentiation and mineralization (Fig. 2*B*, *D*), which may be attributed to decreased proliferation of the SRF KO cells. The additional role of SRF in regulating osteogenesis at the early proliferative stage is consistent with the function of SRF in

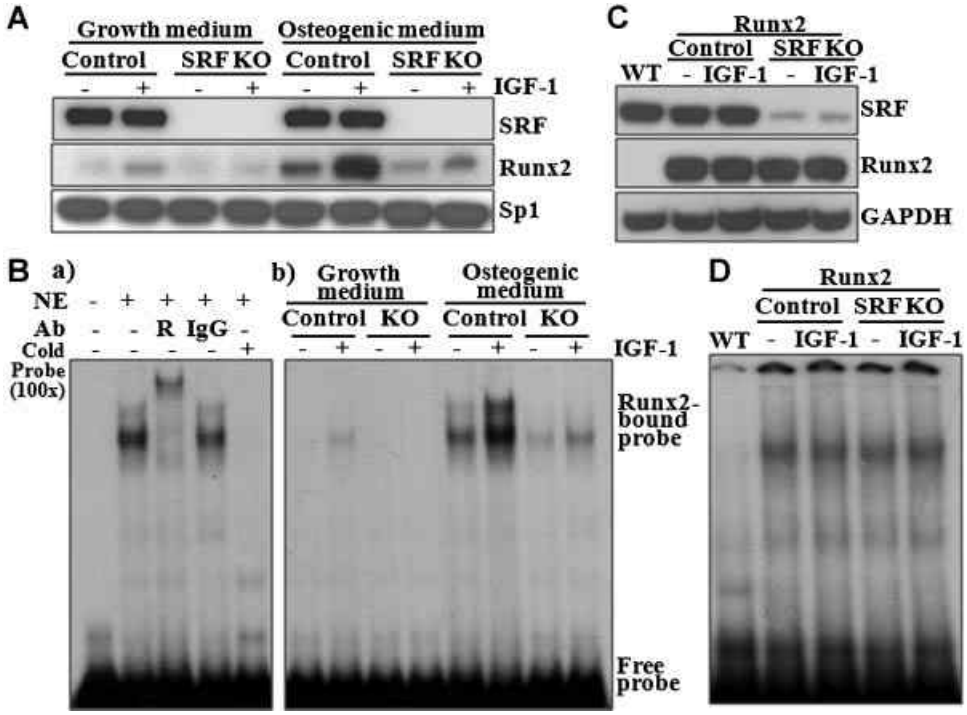

Fig. 5. Effects of IGF-1 and SRF on Runx2 expression and DNA binding activity. (*A*) Western blotting analysis of nuclear expression of SRF and Runx2. SRF KO and control cells were cultured in differentiation media for 10 days, and nuclear extracts were prepared and subjected to Western blotting analysis to determine expression of SRF and Runx2. The expression of Sp1 was used as a loading control. (*B*) EMSA of Runx2 activity. a, Verification of Runx2-specific binding activity by supershift assays with Runx2 antibody and competitive binding assays. NE = nuclear extracts. b, Runx2 DNA binding activity in nuclear extracts from *A*. Representative images from two independent experiments are shown. (*C*) Western blotting analysis of Runx2 and SRF; control or SRF KO calvarial cells that were transfected with pcDNA3-Runx2 plasmids were treated with 13 nM IGF-1 for 30 minutes before sample collection. (*D*) EMSA was performed to determine Runx2 DNA binding for the samples prepared from *C*. Representative images from two independent experiments are shown.

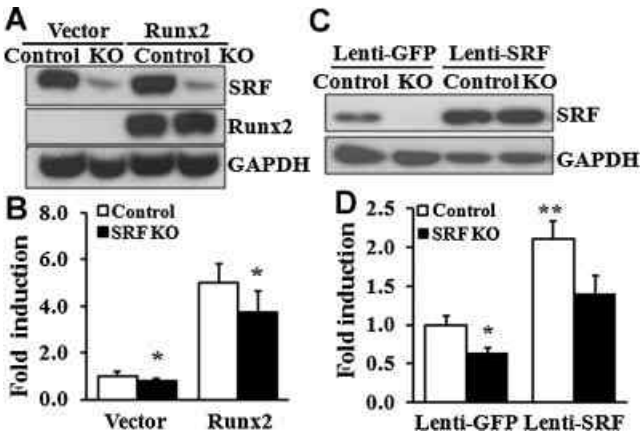

Fig. 6. Effects of SRF on Runx2 transactivity. (*A, B*) SRF deletion on Runx2 transactivity. (*A*) Western blotting analysis of SRF and Runx2. Control and SRF KO cells were cotransfected with pcDNA-Runx2 or empty vector, pGL3-6 × OSE2 and pCMV-β-gal, and cell lysates were prepared and subjected to Western blotting analysis to determine SRF and Runx2 production. (*B*) Luciferase activities were analyzed on cell lysates prepared from *A*. Data shown are relative luciferase activities normalized using the expression of β-gal. $n = 3$, $^{*}p < 0.05$. (*C, D*) SRF overexpression on Runx2 transactivity. (*C*) Western blotting analysis of SRF. Control and SRF KO cells were infected with lenti-GFP virus or lenti-SRF virus, and cell lysates were prepared and subjected to Western blotting analysis. (*D*) Runx2 transactivity. Cells were transiently transfected with pGL3-6 × OSE2 and pCMV-β-gal and were analyzed for luciferase activities. Data shown are relative luciferase activities normalized using the expression of β-gal. $n = 3$, $^{*}p < 0.05$; $^{**}p < 0.01$.

regulating cell growth through the regulation of many growth factors and cell cycle genes.[21]

In addition to regulating IGF-1 expression by osteoblasts, SRF was also found to affect IGF-1–activated signals in osteoblasts. Upon IGF-1 binding, IGF-1R dimerizes and undergoes autophosphorylation, leading to the activation of insulin receptor substrates,[51] which activates two distinct pathways, including the phosphatidylinositol 3 kinase (PI3K)/Akt and ERK signaling pathways.[41] The activation of Akt and ERK signaling pathways is crucial for osteogenic differentiation from uncommitted mesenchymal precursor cells and for maturation of committed osteoblasts.[12,52] SRF deficiency selectively inhibited IGF-1–activated PI3K/Akt but not ERK signaling in calvarial cells (Fig. 4*C*), which was not owing to the effect of SRF on the expression of IGF-1 receptor. On the other hand, overexpression of SRF was found to enhance the IGF-1–induced activation of Akt, further supporting a direct role of SRF on IGF-1–induced activation of Akt signals.

Activation of Akt signaling pathways promotes osteoblast differentiation by inducing the expression and activity of several bone-specific genes, including the key osteogenic transcription factor, Runx2.[12,42,43] Runx2 is necessary and sufficient for commitment of mesenchymal progenitors to osteoblast lineage and for the development of functional osteoblasts.[53] In humans, Runx2 haploinsufficiency results in cleidocranial dysplasia, a disease characterized by defective bone formation and supernumerary teeth.[54] Multiple signaling pathways that affect osteoblast function converge on Runx2 signals.[55] Akt signaling

has been reported to enhance DNA binding activity of Runx2, and Runx2 upregulates PI3K subunits, suggesting that Runx2 and Akt may be mutually regulated in osteoblasts.[42] We found that IGF-1 activated Akt signaling (Fig. 4) and enhanced Runx2 DNA binding activity (Fig. 5*B*), which was decreased by the SRF deficiency. In addition, induced Runx2 expression and activity during osteogenesis was inhibited in the SRF-deficient cells, which was associated with inhibition of Runx2 downstream target genes, such as Col1a1, osteocalcin, and Osterix (data not shown). Induction of Runx2 expression contributes to IGF-1–enhanced Runx2 DNA binding activity (Fig. 5*A*, *B*), as short-term IGF-1 treatment in Runx2-overexpressed cells did not affect Runx2 DNA binding activity (Fig. 5*D*). Deletion of SRF decreased Runx2 transactivity (Fig. 6*A*, *B*), without interfering with Runx2 DNA binding ability (Fig. 5*C*, *D*), whereas overexpression of SRF increased Runx2 transactivity in wild-type cells and restored Runx2 transactivity in the SRF KO (Fig. 6*D*). Previous studies have demonstrated that SRF interacts with Runx2 directly in murine C3H10T1/2 multipotential mesenchymal cell line.[56] Therefore, it is likely that SRF may serve as a cotranscription factor or affect Runx2 binding to other cofactors to regulate Runx2 transactivity in osteoblasts.

In summary, we have demonstrated that osteoblast-derived SRF regulates osteoblast mineralization and postnatal bone development of adult mice. Inhibition of IGF-1 expression, decreased IGF-1–activated Akt signaling and Runx2 activity, and reduced Runx2 transactivity by SRF deficiency in osteoblasts contributes differentially to the decreased osteoblast differentiation and function (Fig. 7). Our studies provide novel molecular insights into the function of SRF in regulating bone formation during postnatal skeletal development.

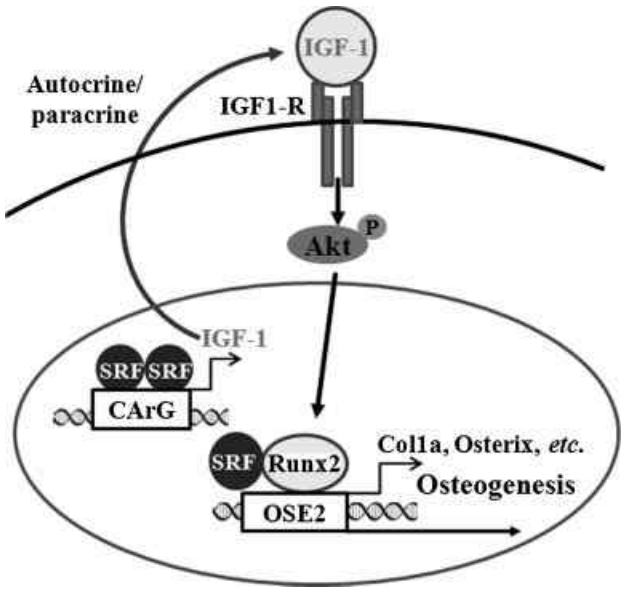

Fig. 7. A schematic model of SRF in regulating osteoblast formation. SRF positively regulates expression of IGF-1, IGF-1–activated Akt signals, and Runx2 DNA binding activity, as well as directly modulates Runx2 transactivity, which together enhances Runx2 transactivity and thus promotes osteoblast differentiation and mineralization.

Disclosures

All authors state that they have no conflicts of interest.

Acknowledgments

The authors thank Jay M McDonald, MD (University of Alabama at Birmingham), for helpful discussion and critical review. We thank the Histomorphometry and Molecular Analyses Core Laboratory and the Small Animal Phenotyping Core of the Center for Metabolic Bone Disease at the University of Alabama at Birmingham, supported by National Institutes of Health Grant P30-AR46031 (Ponnazhagan S, PI). This work was supported in part by grants from the National Institutes of Health AR055339 (YC), HL092215 (YC), and HL-62572 (JMM), and by VA Merit Review Award BX000369 (YC).

Authors' roles: Study design: JC, KY, JMM, HW, and YC. Study conduct and collection: JC, KY, and XM. Data analysis: JC, KY, HW, and YC. Drafting manuscript: JC and KY. Revising manuscript content: JMM, HW, and YC. All authors approved the final version of the manuscript. JC, KY, HW, and YC take responsibility for the integrity of the data analysis.

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