Critical role of the extracellular signal–regulated kinase–MAPK pathway in osteoblast differentiation and skeletal development

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The extracellular signal–regulated kinase (ERK)–mitogen-activated protein kinase (MAPK) pathway provides a major link between the cell surface and nucleus to control proliferation and differentiation. However, its in vivo role in skeletal development is unknown. A transgenic approach was used to establish a role for this pathway in bone. MAPK stimulation achieved by selective expression of constitutively active MAPK/ERK1 (MEK-SP) in osteoblasts accelerated in vitro differentiation of calvarial cells, as well as in vivo bone development, whereas dominant-negative MEK1 was inhibitory. The involvement of the RUNX2 transcription factor in this response was established in two ways: (a) RUNX2 phosphorylation and transcriptional activity were elevated in calvarial osteoblasts from TgMek-sp mice and reduced in cells from TgMek-dn mice, and (b) crossing TgMek-sp mice with *Runx2+/−* animals partially rescued the hypomorphic clavicles and undemineralized calvaria associated with *Runx2* haploinsufficiency, whereas TgMek-dn*; Runx2+/−* mice had a more severe skeletal phenotype. This work establishes an important in vivo function for the ERK–MAPK pathway in bone that involves stimulation of RUNX2 phosphorylation and transcriptional activity.

Introduction

In addition to its participation in growth factor regulation of proliferation and apoptosis, the extracellular signal–regulated kinase (ERK)–MAPK pathway has important functions in the differentiation of postmitotic cells. For example, in *Xenopus laevis* embryos, inhibition of ERK activation prevents animal caps from differentiating into mesenchymal tissue (Umbhauer et al., 1995), whereas mice harboring deletions in ERK2 exhibit severe defects in primary mesenchyme formation without major changes in cell proliferation or apoptosis (Yao et al., 2003). In addition, this pathway can regulate the activity of several lineage-specific transcription factors, including MyoD (muscle; Zetser et al., 2001), Sox9 (cartilage; Murakami et al., 2000, 2004), and PPARγ (adipose tissue; Adams et al., 1997). In bone, the ERK–MAPK pathway is a major conduit for conveying information about the extracellular environment to the nucleus, and has been implicated in the response of bone to a variety of signals, including hormone/growth factor stimulation (Hurley et al., 1996; Xiao et al., 2002b; Chen et al., 2004), extracellular matrix–integrin binding (Takeuchi et al., 1997; Xiao et al., 2002a), and mechanical loading (You et al., 2001).

Despite intensive investigation, the physiological role of the MAPK pathway in osteoblasts remains controversial, with some studies supporting a stimulatory role in osteoblast differentiation and others proposing that this pathway is inhibitory (for review see Schindeler and Little, 2006). For example, activation of α2β1 integrins by the type I collagen matrix of bone was reported to stimulate in vitro osteoblast differentiation via focal adhesion kinase activation of MAPK (Takeuchi et al., 1997; Xiao et al., 1998). Also, short-term pharmacological inhibition of MAPK signaling blocked osteoblast-specific gene expression in mature osteoblasts, whereas a constitutively active form of the MAPK intermediate, MEK1, was stimulatory (Xiao et al., 2002a). In related studies, we showed that RUNX2, which is an essential transcription factor for bone formation (Ducy et al., 1997), is required for cells to respond to MAPK in vitro. Both phosphorylation and transcriptional activity of RUNX2

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Abbreviations used in ths paper: ALP, alkaline phosphatase; BSP, bone sialoprotein; CCD, cleidocranial dysplasia; E, embryonic day; ERK, extracellular signal–regulated kinase; GAP, GTPase-activating protein; MEK, MAPK/ERK; MEK-DN, dominant-negative MEK1; mOG, mouse osteocalcin gene; OCN, osteocalcin; OSE, osteoblast-specific element.

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were stimulated by the ERK–MAPK pathway, suggesting that this factor is a MAPK substrate and an important mediator of the MAPK response (Xiao et al., 2000; Franceschi et al., 2003). Also, treatment of MC3T3-E1 cells or primary cultures of marrow stromal cells with FGF2 induced RUNX2 phosphorylation and osteocalcin expression by a process requiring MAPK activation (Xiao et al., 2002b). In contrast, other in vitro studies reached an opposite conclusion, showing that the ERK–MAPK pathway can antagonize osteoblast functions. Thus, epidermal growth factor stimulation of ERK prevented SMAD1 activation by bone morphogenetic proteins in epithelial cells (Kretzschmar et al., 1997). This inhibition was explained by ERK-dependent phosphorylation of a distinct site in the linker region of SMAD1 that led to the exclusion of this molecule from the nucleus (Kretzschmar et al., 1999). Also, chronic treatment of osteoblast cultures with MAPK inhibitors was reported to actually stimulate osteoblast differentiation, whereas ERK activation was inhibitory (Higuchi et al., 2002; Nakayama et al., 2003).

The conflicting results of in vitro studies emphasize the need to address the role of the ERK–MAPK pathway in osteoblast function in vivo. To this end, a unique transgenic strategy was developed involving selective expression of constitutively active and dominant-negative forms of the MAPK intermediate MEK1 in osteoblasts using the osteocalcin promoter. With this approach, ERK–MAPK activation was found to stimulate osteoblast differentiation and skeletal development through a pathway involving RUNX2.

Results

Generation of transgenic mice

Transgenic mice were developed using a 647-bp mouse osteocalcin (*Ocn*) gene 2 (mOG2) promoter to drive osteoblast-specific expression of constitutively active (MEK-SP) or dominant-negative (MEK-DN) forms of the MAPK intermediate MEK1 (Wu et al., 1996; Sugimoto et al., 1998). *Ocn* transcription is low in proliferating osteoprogenitor cells, and it does not become elevated until the later stages of differentiation, when cells are largely postmitotic (Owen et al., 1990; Xiao et al., 1997). By using a promoter that is selectively expressed in postmitotic cells, we reasoned that it should be possible to target MAPK functions related to osteoblast differentiation, rather than earlier functions associated with cell proliferation. A schematic representation of the transgene construct is shown in Fig. 1 a. This mOG2 promoter region contains sufficient information to selectively express a lac Z reporter in osteoblasts with no detectable expression in cartilage or joints (Frendo et al., 1998). A total of five transgenic founders were obtained for each construct. Of these, three lines having approximately equivalent transgene expression were retained for further study. Mice were viable and bred normally. As shown in Fig. 1 (b and c), strong transgene expression was detected in skeletal tissues, first appearing at E14.5 and persisting in newborn animals. No transgene expression was seen in soft tissues, such as muscle, brain, or liver (unpublished data). Similar expression levels and tissue distribution were seen with all lines examined. For this reason, subsequent experiments used lines SP221 and DN288, with certain experiments repeated with SP413 and DN315. In situ hybridization conducted in newborn mice showed clear localization of the transgene to osteoblasts on endosteal and select trabecular surfaces of long bones and the conspicuous absence of expression in growth plates (Fig. 1 d). Similar localization to osteoblast layers of the calvarium was also observed (unpublished data).

Transgenic modification of MAPK activity alters in vitro osteoblast differentiation

Initial studies examined the effects of transgene expression on the in vitro growth and the differentiation of calvarial osteoblasts. Cells were isolated from 4-wk-old wild-type, TgMek-sp, and -dn mice and grown under differentiating conditions. Transgene expression was first detected after 7 d in culture, and continued to increase throughout the experiment (Fig. 2 a). Western blotting with a specific anti–phospho-ERK antibody was used to verify that transgenes altered ERK–MAPK signaling (Fig. 2 b).

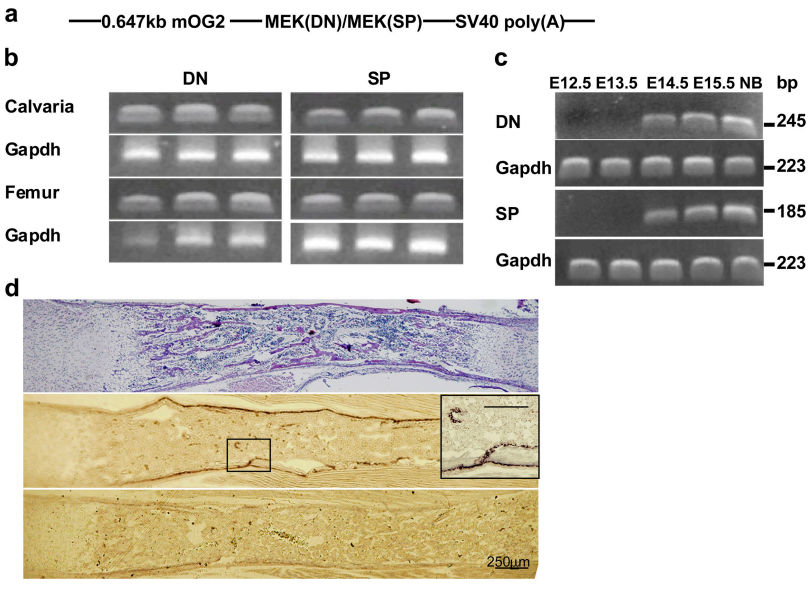

Figure 1. **Development of transgenic mouse lines overexpressing constitutively active and dominant-negative MEK1 in osteoblasts.** (a) Schematic representation of a transgene construct. A −647 to +13 bp fragment of the murine mOG2 promoter was used to drive expression of constitutively active (MEK-SP) and dominant-negative MEK1 mutants in osteoblasts. (b) Tissue distribution of transgene expression. Total RNA was isolated from the indicated tissues of 4-wk-old mice, and transgene expression measured by RT-PCR. The lines used, from left to right, were as follows: DN288, DN388, DN315, SP413, SP221, and SP211. GAPDH mRNA levels are shown as a control for RNA loading. Transgene expression was not detected in muscle, brain, or liver (not depicted). (c) Transgene expression time course during embryonic development. Results with SP221 and DN288 are shown. (d) Distribution of transgene expression in long bone. In situ hybridization was used to localize transgene mRNA to endosteal and select trabecular surfaces of a newborn TgMek-dn femur (line DN288). (top) Hematoxylin and eosin–stained section; middle, in situ hybridization with digoxigenin-labeled sense probe; bottom, antisense probe. Inset, 2× magnification of boxed area. Bar, 250 μm.

TgMek-sp cells had phospho-ERK levels that were nearly twice those of their wild-type littermates, whereas phospho-ERK in TgMek-dn cells was reduced by 50%. In contrast, total ERK levels were not affected by transgene expression. To confirm that the increased phospho-ERK levels in TgMek-sp cells are explained by constitutive activation of MEK, cells were treated with the Raf inhibitor ZM336372, which blocks the MAPK pathway upstream of MEK1/2 (Hall-Jackson et al., 1999). As expected, this inhibitor dramatically reduced phospho-ERK levels in cells from wild-type and TgMek-dn mice, while having no effect on levels in TgMek-sp cells.

As a component in growth factor signaling, the ERK–MAPK pathway can stimulate cell proliferation (Stokoe et al., 1994). However, growth curves for wild-type, TgMek-sp, and TgMek-dn cells were identical, with all groups growing to the same saturation density (Fig. 2 c). This is likely explained by the delayed expression of transgenes from the mOG2 promoter that does not become active until culture day 7, when cells are close to confluence and largely postmitotic (compare Figs. 2, a and c). This late transgene expression facilitated the interpretation of subsequent experiments by removing proliferation and cell density as possible confounding variables.

In contrast to the results of proliferation studies, Mek-dn and -sp transgenes dramatically altered calvarial cell differentiation and gene expression (Fig. 2, d–j). Differentiation of TgMek-dn cells was reduced relative to wild type, as measured by von Kossa staining of mineralized nodules at day 14 (Fig. 2 d), alkaline phosphatase activity (Fig. 2 e), calcium accumulation as mineral (Fig. 2 f) and expression of OCN, bone sialoprotein (BSP), and alkaline phosphatase (ALP) mRNAs (Fig. 2, g–i). Time course studies revealed that the earliest effects of transgenes were seen at day 10, immediately after initial transgene expression was detected at day 7 (Fig. 2 a). Runx2 mRNA was not affected at day 7, but was reduced by 40% on day 10 and 14. Opposite results were obtained with TgMek-sp cells that accumulated twice the mineral of wild-type cells at d 14 and had higher levels of alkaline phosphatase and osteoblast marker mRNAs. Runx2 mRNA levels were only slightly stimulated on days 10 and 14.

RUNX2 phosphorylation and transcriptional activity

The *Ocn, Bsp, Akp2* (ALP), and *Runx2* genes shown to be regulated by Mek-sp and -dn in Fig. 2, g–h are all known to be

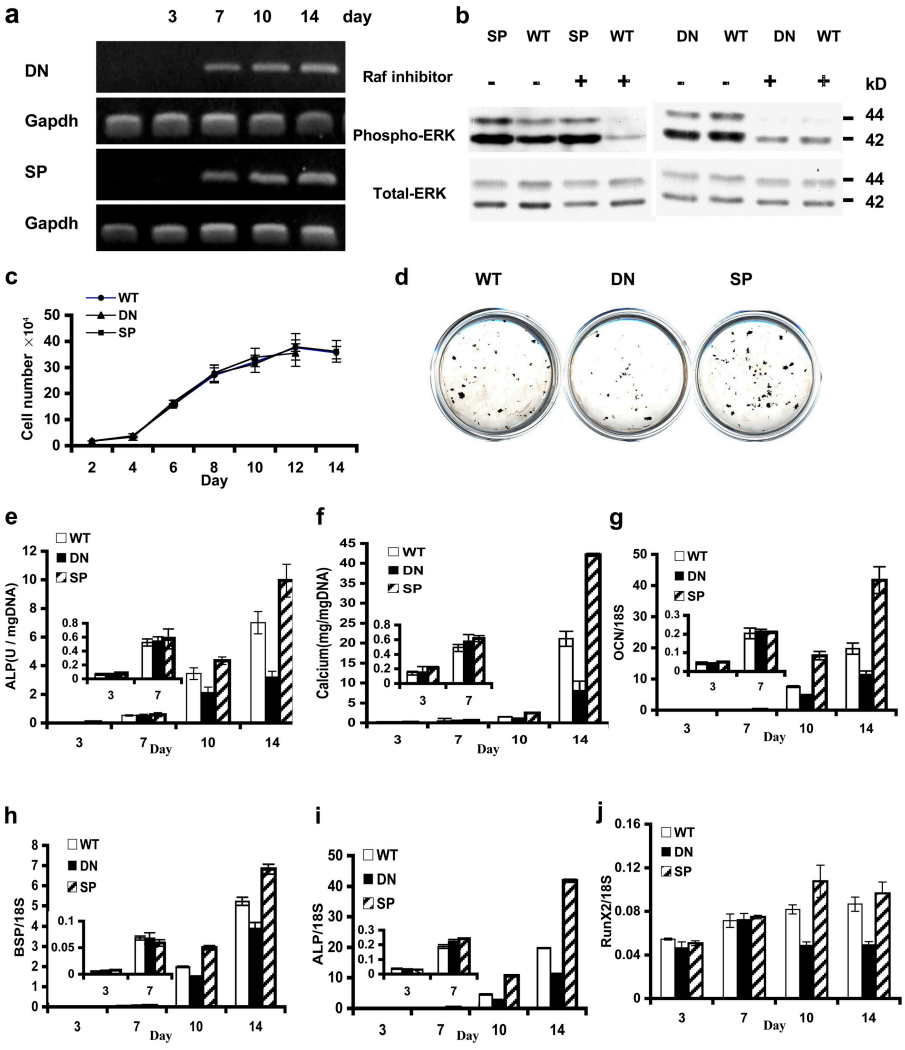

Figure 2. **Altered osteoblast differentiation in calvarial cells from TgMek-dn and -sp mice.** Cells were isolated from calvaria of newborn wild-type and transgenic animals. (a) Time course of transgene expression. Cells were plated and grown in differentiating medium for the indicated times before measurement of transgene mRNA by RT-PCR. (b) Mek-dn and -sp transgene expression alters ERK phosphorylation. Cells were grown as in a and harvested after 10 d for measurement of total and phospho-ERK by Western blotting. The indicated groups were treated with the Raf inhibitor ZM336372 2 h before harvest. (c) Transgene expression does not alter cell growth. (d–j) MEK-DN inhibits, whereas MEK-SP stimulates, osteoblast differentiation. The following differentiation markers were measured: mineralized nodules in 14-d cultures (d), alkaline phosphatase activity (e), total cell layer–associated, acid-extractable calcium (f), and OCN, BSP, ALP, and Runx2 mRNA levels (g–j; all measured by real-time RT-PCR). Values are the mean ± the SD of triplicate independent samples.

directly or indirectly controlled by RUNX2. For *Ocn, Bsp*, and *Runx2* itself, this regulation involves direct binding of RUNX2 to regulatory elements in the proximal promoter region (Ducy et al., 1997; Tou et al., 2003; Roca et al., 2005). In previous cell culture studies, we showed that MAPK activation by either transient transfection of the MC3T3-E1 osteoblast cell line with a MEK-SP expression vector or activation of endogenous ERK phosphorylation by treatment with FGF2 stimulated phosphorylation and transcriptional activity of RUNX2 (Xiao et al., 2000, 2002b).

Two types of experiments were conducted to determine if RUNX2 phosphorylation and transcriptional activity is modified in cells from TgMek-sp and -dn mice, and if changes in phosphorylation could explain differences in osteoblast differentiation. In the first experiment, cells were grown under differentiating conditions for 7 d and metabolically labeled with [^{32}P]orthophosphate, and then total RUNX2 was immunoprecipitated from cell extracts using a specific polyclonal antibody. A replicate culture was labeled with [^{35}S]Met/Cys and processed in the same way to normalize ^{32}P incorporation to total RUNX2. As shown in Fig. 3 (a and b), steady-state RUNX2 phosphorylation was increased twofold in TgMek-sp cells versus wild-type controls, while phosphorylation in TgMek-dn cells was reduced by 50%. In the second study, cells were transfected with 1.3-kb mOG2-luc or 6OSE2-luc reporter genes, and luciferase activity was measured after 3 and 7 d. A previous work showed that both of these promoter constructs are highly responsive to RUNX2 (Ducy et al., 1997). The 1.3-mOG2 fragments, like 0.647-kb mOG2, contains all known elements necessary for osteoblast-specific expression of Ocn, including RUNX2 binding sites (osteoblast-specific element 2 [OSE2]) at −608 and 137, whereas 6OSE2-luc contains six copies of the OSE2 site in front of a minimal mOG2 promoter (from −47 to 13 bp; Ducy and Karsenty, 1995; Yang et al., 2004). After 7 d in culture, luciferase activity from both reporter genes was higher in TgMek-sp cells than wild-type cells, whereas activity in TgMek-dn cells was reduced (Fig. 3, c and d). In contrast, no differences in activity were seen at day 3, which is before Mek-sp and -dn transgenes become active.

These results cannot be explained by differences in total RUNX2 levels or overall cell differentiation that were unaffected by transgenic status at the 7-d time point (Fig. 2, e–j). Rather, the ability of RUNX2 to stimulate OSE2-dependent transcriptional activity was increased. Collectively, these studies show that modest perturbations in ERK–MAPK activity via transgenic expression of MEK-SP or -DN in postmitotic osteoblasts dramatically affect in vitro RUNX2 phosphorylation, RUNX2-dependent transcriptional activity, and osteoblast differentiation. Changes in RUNX2 activity preceded differentiation changes, which is consistent with the concept that Mek-dependent regulation of this transcription factor accounts, at least in part, for subsequent changes in differentiation.

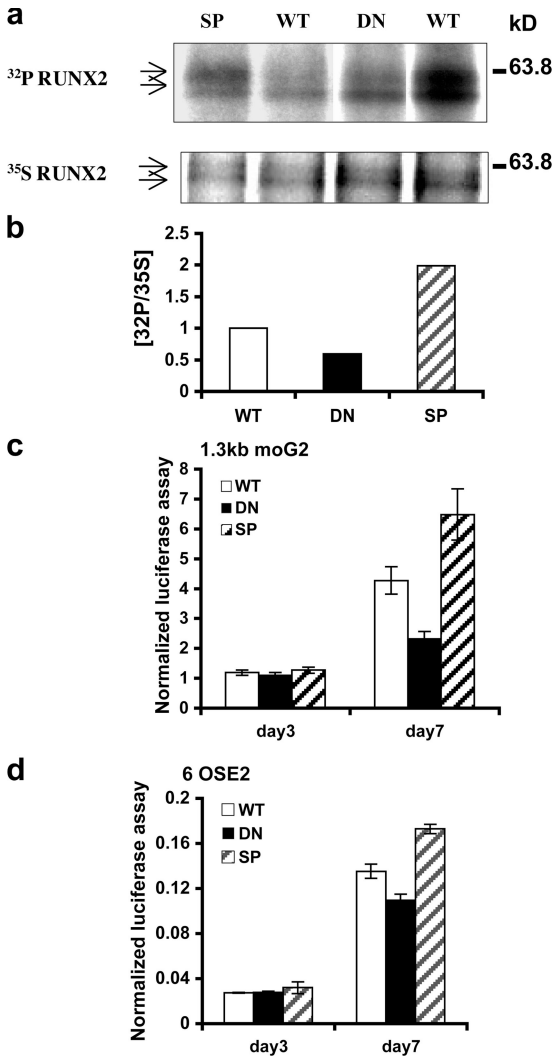

Figure 3. **Changes in RUNX2 phosphorylation and transcriptional activity in osteoblasts from TgMek-dn and -sp mice.** (a and b) Regulation of RUNX2 phosphorylation. Calvarial cells were grown under differentiating conditions for 7 d before metabolic labeling with [^{32}P]orthophosphate or [^{35}S]methionine/cysteine (to normalize for total RUNX2) and immunoprecipitation with an anti-Runx2 antibody. Each IP reaction contained 500 μg total protein. (b) Normalized ^{32}P incorporation into RUNX2. (c and d) Runx2-dependent transcriptional activity. Cells were transfected with p1.3mOG2-luc (c) or p6OSE2mOG2-luc (d) plasmids and grown under differentiating conditions for the times indicated before measurement of luciferase activity. Values are the means ± the SD of triplicate independent samples.

TgMek-sp and -dn alter skeletal development

Consistent with results obtained in osteoblast cultures, skeletal development was accelerated in TgMek-sp and delayed in TgMek-dn embryos (Fig. 4). This was reflected by differences in skeletal size, body weight, and mineralization. Skeletons from TgMek-sp embryos were significantly larger (based on femur lengths at embryonic day (E) 15.5 [Fig. 4, c and f] and body weight [Fig. 4, a and d]) than wild-type littermates, and weights and embryo size were reduced in TgMek-dn mice. Moreover, differences in calvarial mineralization were apparent with mineralized area of calvarial preparations at E15.5 being reduced by 20% in TgMek-dn and stimulated by an equivalent amount in TgMek-sp embryos (Fig. 4, b and e).

Striking differences in skeletal maturation of long bones were also seen when histological sections of E15.5 embryos were examined (Fig. 5 a). At this age, the initial stages of endochondral bone formation have normally already taken place,

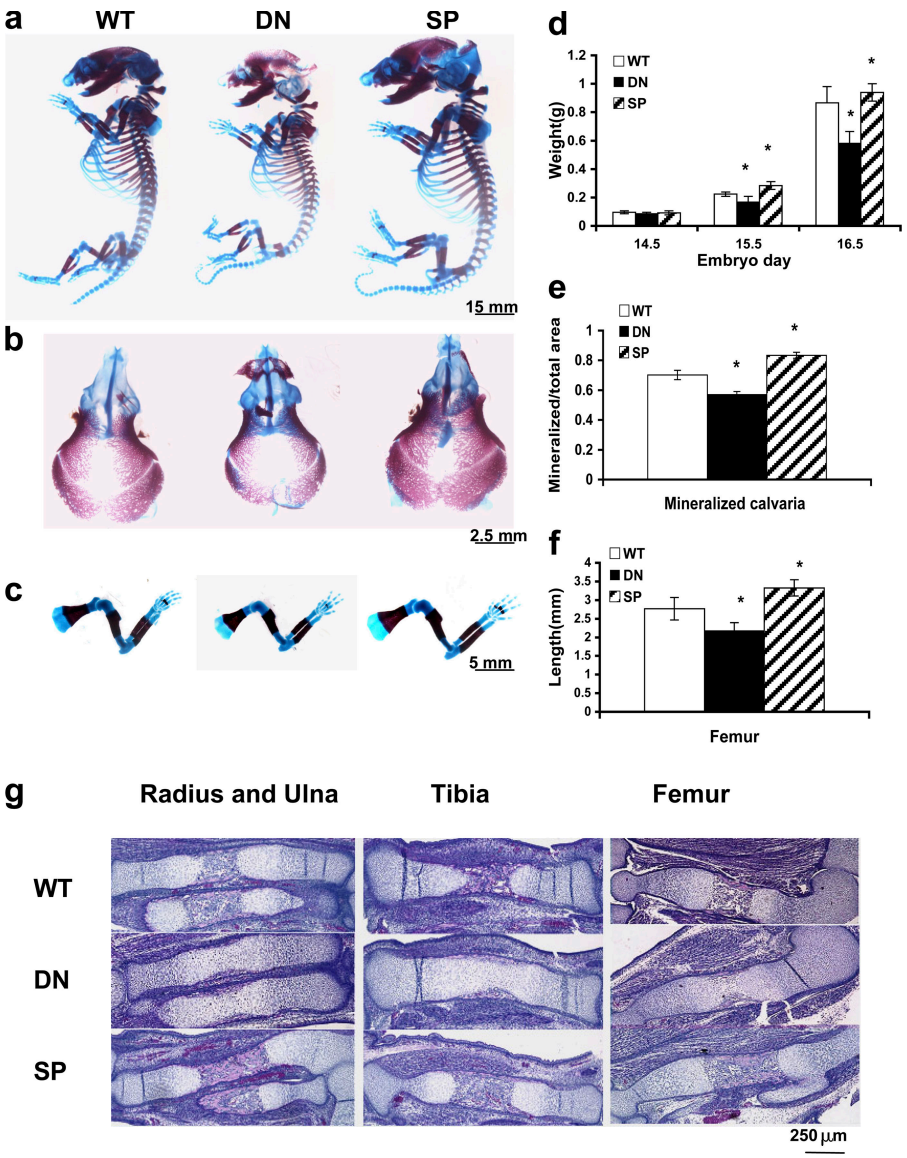

Figure 4. **Altered skeletal development in TgMek-dn and -sp mice.** (a) Whole mounts of E15.5 skeletons stained with alcian blue and alizarin red. (d) Effects of transgene expression on embryo weights. (b and e) Cranial bones showing differences in mineralization (b) and quantification of mineralized area (expressed as the percentage of total calvarial area; e). (c and f) Hindlimbs showing differences in the size of bones with transgene expression (c) and quantification of femur lengths (f). (g) Histology of long bones from wild-type, TgMek-dn, and -sp mice. Note delay in bony collar and trabecular bone in TgMek-dn embryos. Statistical analysis values are expressed as the mean ± the SD. $n = 8$ mice/group. *, significantly different from wild type at $P < 0.01$.

with vascular invasion and metaphyseal bone formation already well underway (Komori et al., 1997). However, this process was significantly delayed in TgMek-dn embryos. Only early bone collar formation was apparent, with cartilage still occupying the inner metaphyseal regions of all long bones examined. In contrast, TgMek-sp embryos exhibited accelerated trabecular bone formation, with the metaphyseal region already having expanded toward the diaphyses. These results indicate that ERK–MAPK activity is important for the early stages of intramembraneous and endochondral bone development.

Interaction between Mek-sp and -dn transgenes and Runx2

The cell culture studies shown in Fig. 3, as well as previous work from this laboratory (Xiao et al., 2000), suggest that RUNX2 is an important target for regulation by the ERK–MAPK pathway in osteoblasts. To test this concept in vivo, we examined genetic interactions between *Runx2* and Mek-sp or -dn transgenes. *Runx2 +/−* mice are known to phenocopy many aspects of the human genetic disease cleidocranial dysplasia (CCD), including the characteristic clavicular hypoplasia and open fontanelles of the cranium with lesser involvement of other skeletal sites (Otto et al., 1997). Because clavicles and cranial bones are particularly sensitive to the effects of *Runx2* haploinsufficiency, we predicted that these two tissues should be preferentially sensitive to transgenic modification of the ERK–MAPK pathway under conditions where RUNX2 is limiting.

TgMek-sp or -dn mice were crossed with *Runx2 +/−* mice, and skeletal phenotypes were determined using whole-mount skeletal preparations (Fig. 5). Fig. 5 (a–d) show the analysis of E18.5 animals from TgMek-sp *x Runx2 +/−* breedings, and Fig. 5 (e–h) show results of the TgMek-dn *x Runx2 +/−* cross. *Runx2 +/−* mice exhibited the expected clavicular hypertrophy and calvarial hypomineralization of CCD, with a resultant reduction of clavicle cross-sectional area of 75 (Fig. 5, c and g), and 28, and 18%, respectively, in mineralized areas of calvaria (Fig. 5, d and h). In contrast, femur length was not significantly affected (Fig. 5, b and f).

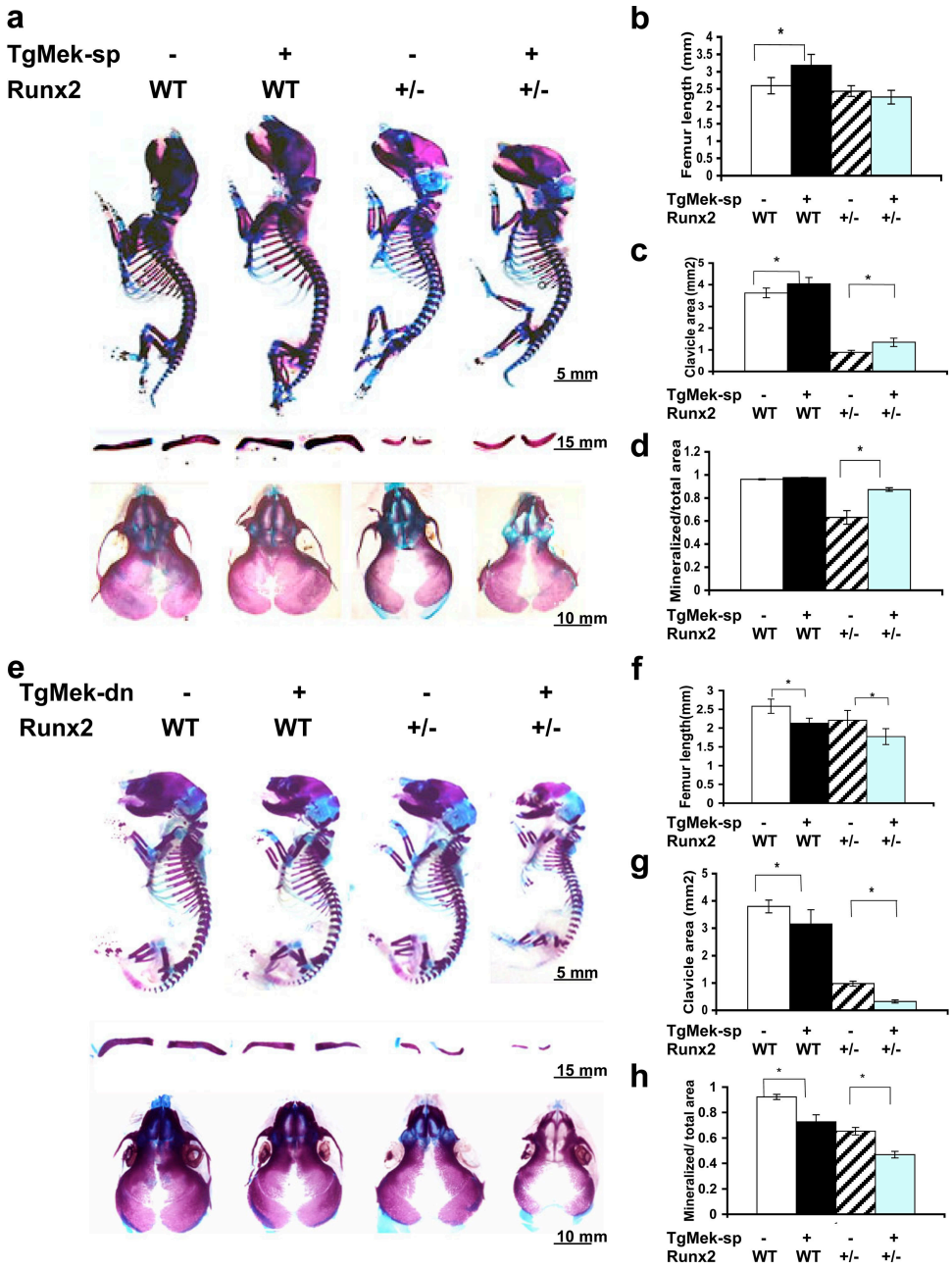

Figure 5. **Genetic interactions between Mek-dn and -sp transgenes and *Runx2*.** TgMek-dn or -sp mice were crossed with Runx2+/− mice to generate the genotypes indicated. (a–d) Partial rescue of CCD phenotype in *Runx2*+/− mice with Mek-sp. (a) Skeletal whole mounts of newborn mice stained with alcian blue and alizarin red (top), isolated clavicles (middle), and crania (bottom). (b–d) measurements of femur length (b), clavicle areas (c), and mineralized area of calvaria (expressed as a fraction of total calvarial area). (e–h) Increased severity of CCD phenotype with Mek-dn. Groups are as in a–d. Statistical analysis values are expressed as the means ± the SD. $n = 8$ mice/group. Comparisons are indicated by bars. *, significantly different at $P < 0.01$.

The Mek-sp transgene was able to partially rescue both the clavicular hypertrophy and calvarial hypomineralization of *Runx*+/− mice. Transgene expression increased the cross-sectional area of clavicles by 53% in *Runx2*+/− animals, although it only increased this parameter by 12% in wild-type mice (Fig. 5, c). Restoration of calvarial mineralization was also observed with TgMek-sp, increasing this parameter in *Runx2*+/− animals to 90% of the wild-type control (a 23% increase relative to *Runx2*+/− mice; Fig. 5, d). In contrast, Mek-sp had no effect on femur length in *Runx2*+/− mice, but it did increase this parameter by 18% in wild-type littermates (Fig. 5, b).

Results with the TgMek-dn *x Runx2*+/− cross were even more striking. First, after four consecutive breedings, we failed to obtain viable newborn pups with the TgMek-dn;*Runx2*+/− genotype. This is in contrast to the TgMek-sp;*Runx2*+/− genotype that was present in the expected Mendelian ratio. When embryos were harvested by Caesarean section at E18.5, TgMek-dn;*Runx2*+/− embryos were obtained in the expected ratios, indicating that this genotype cannot survive the birth

process. Embryos were smaller and exhibited selective worsening of CCD-associated features. Specifically, Mek-dn reduced clavicular cross-sectional area by 67% in *Runx+/−* embryos, but only reduced this value by 17% in *Runx+/+* mice (Fig. 5, g). Also, Mek-dn reduced calvarial mineralization by 31% in *Runx2+/−* embryos, but only reduced this value by 15% in wild type (Fig. 5, h). Collectively, these results show that in *Runx2+/−* mice, the clavicles, and calvaria are selectively sensitive to manipulation of ERK–MAPK activity in osteoblasts, which is consistent with the concept that RUNX2 is an in vivo target of this pathway.

Discussion

In this study, we show that the ERK–MAPK pathway can positively regulate bone development in vivo through a mechanism involving RUNX2. Phosphorylation and transcriptional activity of RUNX2 were stimulated in calvarial osteoblasts from Tg Mek-sp mice and inhibited in TgMek-dn cells. Furthermore, *Runx2* haploinsufficiency selectively enhanced the effects of Mek-sp and -dn transgenes on clavicles and calvaria, two tissues known to be preferentially sensitive to *Runx2* gene dosage. On the basis of this work, we conclude that ERK–MAPK signaling is critical for in vivo osteoblast activity, and propose that this response is explained, at least in part, by phosphorylation and activation of RUNX2.

These results are consistent with previous cell culture studies from this and other laboratories. For example, we reported that MAPK activation, either by transfection of osteoblasts with constitutively active MEK1 or treatment with FGF2, rapidly increased RUNX2 phosphorylation and transcriptional activity through a process that was blocked by MAPK inhibition, whereas acute inhibition of MAPK in differentiated cells blocked osteoblast-specific gene expression (Xiao et al., 2000, 2002a,b). In related studies, Lai et al. (2001) showed that expression of a dominant-negative ERK1 blocked differentiation of human osteoblasts. Similarly, differentiation of human marrow stromal cells to osteoblasts is accompanied by sustained ERK1/2 phosphorylation; pharmacological MAPK inhibition or dominant-negative ERK blocked osteoblast formation in this system and stimulated adipogenesis (Jaiswal et al., 2000). Also, several reports support our hypothesis that MAPK actions in osteoblasts are, at least in part, mediated by RUNX2. During osteoblast differentiation of human marrow stromal cells, RUNX2 type II levels remain relatively unchanged, but binding to OSE2 DNA increased, as did RUNX2 phosphorylation (Shui et al., 2003). Also, mechanical loading of osteoblasts, which is known to be mediated, in part, through integrin activation, induces MAPK (Pavalko et al., 1998; Schmidt et al., 1998); loading of periodontal ligament cells (which are osteoprogenitor-like cells associated with tooth cementum) increased RUNX2 phosphorylation and binding to OSE2 DNA via an ERK–MAPK-dependent process (Ziros et al., 2002). In osteoblast-like prostate cancer cells, differentiation is accompanied by ERK1/2 activation, increased RUNX2-OSE2 binding, and *Ocn* expression, responses that are all blocked by MAPK inhibition (Zayzafoon et al., 2004). Lastly, IGF-1, which activates P13K and, subsequently, the ERK–MAPK pathway, stimulates RUNX2-OSE2 binding and phosphorylation in vascular endothelial cells (Qiao et al., 2004), as well as differentiation of marrow stromal cells (Celil and Campbell, 2005; Celil et al., 2005).

This work is to be contrasted with other in vitro studies that reported antagonism between the ERK–MAPK pathway and BMPs in epithelial cells (Kretzschmar et al., 1997, 1999) and inhibitory effects of this pathway on osteoblast differentiation (Higuchi et al., 2002; Nakayama et al., 2003; Schindeler and Little, 2006). Possible explanations for these disparate results may be related to differences in the magnitude and duration of MAPK activation and/or stage of cell maturation that are both known to affect MAPK responsiveness. For example, treatment of PC12 neuronal progenitor cells with EGF transiently stimulated ERK1/2 phosphorylation and cell proliferation, whereas NGF led to sustained ERK activation and neuronal differentiation, a result explained by the formation of transient versus long-lived complexes of MAPK-activating factors (Kao et al., 2001). Similarly, in studies with MC3T3-E1 preosteoblasts, EGF suppressed BMP-dependent Smad 1 activation, whereas sustained ERK–MAPK activation by extracellular matrix synthesis or constitutively active Ras stimulated BMP activity (Suzawa et al., 2002). In contrast, the aforementioned studies, indicating an inhibitory role for MAPK in osteoblast differentiation, generally either treated cells with MAPK inhibitors for extended times or stably transfected cells with expression vectors that produced high levels of constitutively active or dominant-negative MAPK intermediates during all stages of growth and differentiation.

To examine the in vivo actions of the ERK–MAPK pathway in osteoblasts, this study used the mOG2 promoter to drive expression of constitutively active/dominant-negative MEK1 in developing mice. Because this promoter is only active in mature osteoblasts that are largely postmitotic (Owen et al., 1990), we were able to discriminate between effects of MAPK on cell proliferation versus differentiation. Furthermore, transgenic modification led to sustained, but quite modest, changes in ERK1/2 phosphorylation (∼50% increased/decreased levels). Using this approach, ERK–MAPK activation was shown to stimulate osteoblast differentiation of calvarial osteoblasts from transgenic animals and to accelerate bone accumulation in developing embryos without affecting cell proliferation. In contrast, dominant-negative MAPK inhibition slowed osteoblast differentiation in cell culture and delayed bone development. In vivo effects of transgenes were most striking in calvaria and long bones. Of particular interest, long bones of TgMek-dn mice displayed delayed bone collar formation, whereas TgMek-sp was associated with an increase in metaphyseal bone without any obvious effects on cartilage. This is consistent with an in situ analysis of transgene expression that was unable to detect transgene expression in growth plates (Fig. 1).

In agreement with our in vitro studies, we also obtained evidence that MAPK regulates RUNX2 transcriptional activity. Calvarial osteoblasts from transgenic mice exhibited the predicted changes in RUNX2 phosphorylation and transcriptional activity, with these parameters being stimulated in TgMek-sp cells and inhibited with Mek-dn. Of note, changes in RUNX2

were coincident with initial activation of Mek-sp and -dn transgenes and preceded any changes in osteoblast differentiation markers or RUNX2 mRNA that were all equivalent in wild-type, TgMek-sp, and -dn groups at the 7-d time point examined (Figs. 2 and 3). Thus, it is unlikely that changes in RUNX2 phosphorylation/activity are secondary to changes in osteoblast differentiation induced by Mek-sp/dn transgenes. Studies that examined genetic interactions between Runx2 and Mek-sp and -dn transgenes provided further evidence for in vivo regulation of RUNX2 by MAPK (Fig. 5). Because clavicles and calvaria are preferentially sensitive to Runx2 haploinsufficiency, they are good markers for changes in RUNX2 transcriptional activity. The observation that TgMek-sp is able to rescue the CCD phenotype of *Runx2+/−* embryos, whereas TgMek-dn exacerbated clavicular and calvarial defects provides compelling in vivo evidence for our hypothesis that the ERK–MAPK pathway regulates RUNX2.

Studies in other differentiating systems, such as muscle, fat, and cartilage, suggest that the ERK–MAPK pathway, acting through tissue-specific transcription factors, may generally control progenitor/stem cell lineage decisions in mesenchymal tissues. For example, in *X. laevis* muscle differentiation, MAPK increases levels of XMyoD, possibly by decreasing protein turnover (Zetser et al., 2001). In fat, phosphorylation of PPARγ by ERK1 inhibits transcriptional activity of this factor to block adipogenesis (Adams et al., 1997). Lastly, MAPK activation by FGFs, constitutively active FGFR3, or MEK1 mutants in cartilage increases levels of Sox9 to keep chondrocytes in a pre-hypertrophic state (Murakami et al., 2000, 2004).

Studies in cartilage are of particular interest in that they used the *Col2a1* promoter to express the same constitutively active MEK1 mutant used in this study. In contrast to our results with osteoblasts, *Col2a1*-mediated expression led to a chondrodysplasia phenotype that is characterized by shortened limbs and inhibition of hypertrophy without affecting chondrocyte proliferation. The observed limb shortening was explained by a reduction in the size of chondrocytes in the hypertrophic zone. Because the ERK–MAPK pathway is known to activate *Sox9* (Murakami et al., 2000), the authors postulated that this transcription factor prevents hypertrophy, which normally requires *Sox9* down-regulation. In contrast, our use of the mOG2 promoter to express constitutively active MEK1 resulted in expression in osteoblasts rather than cartilage, acceleration of bone development and skeletal mineralization, and no obvious changes in cartilage. As was the case for stimulation of *Sox9* in cartilage, we find that ERK–MAPK can activate RUNX2-dependent transcriptional activity in osteoblasts.

Although our studies focused on RUNX2 as the primary mediator of MAPK signals, other transcription factors may also participate in this response, functioning together with RUNX2 to control gene expression. Of primary interest, ERK is known to phosphorylate and activate a second kinase, RSK2, in the cytoplasm of many cells (Fisher and Blenis, 1996). RSK2 phosphorylates ATF4, a transcription factor that, together with RUNX2, controls the osteoblast-specific expression of the osteocalcin gene (Yang et al., 2004). Mice deficient in either RSK2 or ATF4 have similar bone phenotypes characterized by delayed cranial and long-bone development (Yang et al., 2004). Furthermore, RUNX2 and ATF4 physically and functionally interact in osteoblasts, although it is not known whether phosphorylation of RUNX2 and/or ATF4 affects this interaction (Xiao et al., 2005). While this work was under review, Elefteriou et al. (2006) reported that the high bone-mass phenotype of mice harboring an osteoblast-specific deletion of the Ras GTPase-activating protein (GAP), Nf1, involves activation of the ERK–MAPK pathway. Although these authors attributed the phenotype of *Nf1−/−* mice exclusively to activation of ATF4 via RSK2, the involvement of Runx2 was not directly examined. To address the possible involvement of ATF4 in TgMek-sp and -dn mice, we transfected calvarial osteoblasts with an ATF4 reporter-luciferase construct containing oligomerized copies of the ATF4-responsive enhancer OSE1 (Ducy and Karsenty, 1995; Yang et al., 2004). Unlike results with the RUNX2 reporters (Fig. 3), this construct was not affected by transgene activity (not depicted). Based on this result, the involvement of ATF4 in the osteogenic response seen with transgenic ERK–MAPK activation appears to be secondary to that of RUNX2. However, this issue still needs to be examined in greater detail.

This study establishes an important role for the ERK–MAPK pathway in RUNX2 regulation, osteoblast differentiation, and fetal bone development. Furthermore, the animal models developed will be extremely useful for assessing roles of ERK–MAPK signaling during bone remodeling in adult and ageing mice, as well as for evaluating the possible therapeutic value of MAPK manipulation as a means of regulating bone mass in diseased states.

Materials and methods

Generation of transgenic mice

The plasmid pGL647 contains a region of the mOG2 gene from −647 to +13 bp, which was previously shown to contain sufficient information to drive osteoblast-specific gene expression in vivo (Frendo et al., 1998). Two mutated forms of MEK1 cDNA were individually subcloned into pGL647. MEK(SP) includes S218/222E mutations and deletion of residues 32–51 to produce a constitutively active MEK1 mutant (Sugimoto et al., 1998), whereas the dominant-negative mutant MEK(DN) contains S218/222A mutations (Wu et al., 1996). Plasmids were linearized by digestion with ClaI, and DNA fragments for microinjection were purified using a Nucleospin Extraction kit (CLONTECH Laboratories, Inc.). Transgene constructs are quantified and microinjected into (C57BL/6 X SJL) F2 mouse oocytes (Charles River Laboratories) and surgically transferred to pseudopregnant C57BL/6 dams. The founders were screened by PCR using mouse tail genomic DNA. Transgenic expression in tissues was assessed by RT-PCR using two sets of transgene-specific primers. Primers used for MEK(SP) were 5′-CGGAGACCAACTTGGAGGC-3′ and 5′-CGAATTCGTTGGCCATTTC-3′; primers used for MEK(DN) were 5′-CGGAGACCAACTTGGAGGC-3′ and 5′-CGAAGGCGTTGGCCATGGC-3′. The last six nucleic acids in the ends of downstream primers harbored mutations that were used to discriminate between transgene and endogeneous MEK1 expression. Transgenic founder animals were bred into C57BL/6 mice for at least six generations to obtain a defined genetic background. Previously described heterozygous Runx2+/− mice (Otto et al., 1997) were obtained from P. Ducy (Baylor College of Medicine, Houston, TX). All studies with mice were performed in compliance with the University of Michigan Committee for the Use and Care of Animals.

Histological examination and in situ hybridization

Tissues were fixed in 10% formalin and embedded in paraffin, and 7-μm-thick sections were cut and stained with hematoxylin and eosin. RNA in situ hybridization was done using a digoxigenin-labeled riboprobe specific for SV40 sequences in the transgene (Roche). Sections were viewed with a

microscope (Eclipse 50i; Nikon) using a 10× objective. Images were captured with a digital camera (Stereo TLRC; Leica) using Image-Pro Plus 5.1 (Media Cybernetics) acquisition software without further manipulation.

Whole-mount skeletal preparations

Skeletal morphology was analyzed by alizarin red and alcian blue staining, followed by tissue clarification with KOH, as previously described (Yang et al., 2004). To measure bone length, isolated bones were laid next to a ruler and photographed using a digital camera and stereo dissection scope. Bone area was calculated using ImagePro Plus software.

Cell culture and Western blot analysis

Primary calvarial osteoblasts were isolated from 4-wk-old transgenic mice and littermates, as previously described (Ducy and Karsenty, 1995). Cells were grown in α-MEM/10% FCS until confluent. Cells were then replated at a density of 5×10^3 cells/cm^2 in 35-mm dishes and grown for up to 2 wk in differentiation medium (α-MEM, 10% FCS, 50 μg/ml ascorbic acid, and 5 mM β-glycerol phosphate). Quantitative RT-PCR was performed using an ABI PRISM 7700 Sequence Detection System. Optimized primers and probes for *Ocn*, *Bsp*, *Akp2*, *Runx2*, and *Gapdh* were purchased from Applied Biosystems. Total and phospho-ERK1/2 were measured on Western blots using specific antibodies at a dilution of 1:2,000 (Cell Signaling Technology).

Cell transfections and metabolic labeling

Primary calvaria osteoblasts were isolated from newborn mice and cultured in 75-cm flasks for 3 d until confluent. Cells were then trypsinized and replated on 35-mm dishes at a density of 2.5×10^5 cells/cm^2. After 24 h, cells were transfected with Fugene6 (Roche) according to the manufacturer's instructions. Each transfection contained 0.5 μg of p1.3mOG2-luc or p6OSE2-luc (Ducy and Karsenty, 1995) and 0.05 μg of pRL-SV40 (encodes *Renilla reformis* luciferase to control for transfection efficiency). Cells were then cultured in differentation medium for an additional 6 d before harvesting and assaying for luciferase activity using a Dual Luciferase Assay kit (Promega) on a luminometer (Monolight 2010; BD Biosciences).

For cell labeling, calvarial cells were cultured for 6 d, as described in the previous paragraph, and transferred to phosphate or cysteine and methionine-free medium containing 0.1% dialyzed FCS (Sigma-Aldrich). After an overnight preincubation, cells were labeled for 5 h in the same media containing either 150 μCi/ml of [^{32}P]orthophosphate or [^{35}S]cysteine/methionine (MP Biomedical). Preparation of nuclear extracts and immunoprecipitation of Runx2 were described previously (Xiao et al., 2000). ^{32}P/^{35}S incorporation was measured using an A2024 InstantImager (Packard).

Statistical analysis

Statistical analyses were performed using Instat 3.0 (GraphPAD Software, Inc.). For cell culture studies (Figs. 2 and 3), values are the means ± the SD of triplicate independent samples. In studies with mice, $n = 8$ mice/group. Analysis of variance followed by Tukey-Kramer multiple comparisons test was used to assess significance between groups as indicated.

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