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Transcriptional Regulation of Osteoblasts

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Abstract
The differentiation of osteoblasts from mesenchymal precursors requires a series of cell fate decisions controlled by a hierarchy of transcription factors. These include RUNX2, Osterix (OSX), ATF4 and a large number of nuclear coregulators. During bone development, initial RUNX2 expression coincides with the formation of mesenchymal condensations and precedes the branching of chondrogenic and osteogenic lineages. Given its central role in bone development, it is not surprising that RUNX2 is subject to a variety of controls. These include posttranslational modification, especially phosphorylation, and interactions with accessory nuclear factors. Specific examples of RUNX2 regulation to be reviewed include phosphorylation by the ERK/MAP kinase pathway and interactions with DLX5. RUNX2 is regulated via phosphorylation of critical serine residues in the proline/serine/threonine domain. In vivo, the transgenic expression of constitutively active MAP kinase in osteoblasts accelerated skeletal development, while a dominant-negative MAPK retarded development in a RUNX2-dependent manner. DLX5-RUNX2 complexes can be detected in osteoblasts and this interaction plays a critical role in maintaining osteoblast-specific expression of the bone sialoprotein gene. These studies allow us to begin understanding the complex mechanisms necessary to fine-tune bone formation as mesenchymal progenitors progress down the osteoblast lineage.

Introduction

Endochondral bone formation is controlled by a hierarchy of transcription factors that are expressed in a defined temporal sequence (fig. 1). RUNX2 is expressed very early in skeletal development, first appearing with the formation of mesenchymal condensations in areas destined to become bone and persisting through subsequent stages of bone formation [Ducy et al., 1997]. Several other transcription factors function together with RUNX2 to move cells down chondrocyte or osteoblast lineages. For osteoblasts, this is accomplished by Osterix (OSX), which commits osteochondroprogenitor cells to the osteoblast lineage [Nakashima et al., 2002]. Subsequently, another factor, ATF4, controls the transcriptional activity of mature osteoblasts [Yang et al., 2004]. RUNX2 also participates in the chondrogenic lineage. However, at early stages, it is likely suppressed by the chondrocyte-specific factors Sox 8/9 [Zhou et al., 2006]. Evidence for this model largely comes from genetic studies showing bone phenotypes of decreasing severity as *Runx2, Osx* or *Atf4* are knocked out. Thus, skeletal development in *Runx2*-deficient embryos fails to progress beyond the cartilage anlage stage [Komori et al., 1997; Otto et al., 1997]. These embryos exhibit no sign of cartilage hypertrophy, bony collar devel-

Abbreviation used in this paper

ECM	extracellular matrix

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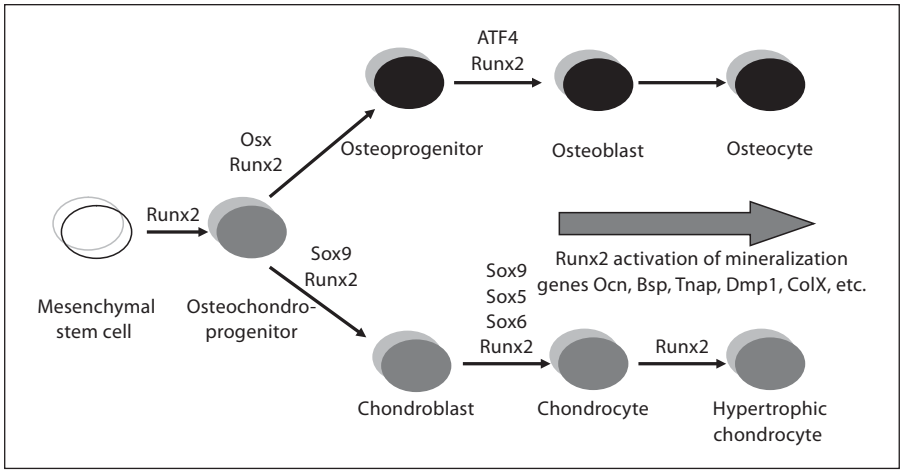


Fig. 1. Transcription factor control of skeletal lineages. Major transcription factors that, based on genetic studies, are involved in osteoblast and chondrocyte differentiation are included in this chart. Also shown is the sequential nature of transcription factor expression with RUNX2 persisting throughout osteoblast and chondrocyte lineages [Franceschi et al., 2007].

opment, vascular invasion or marrow formation. In contrast, $Osx^{-/-}$ embryos have hypertrophic cartilage, early collar formation and normal levels of RUNX2, suggesting that this factor is downstream of RUNX2 [Nakashima et al., 2002]. $ATF4^{-/-}$ mice form bone, but at reduced rates, consistent with this factor playing a regulatory role in bone formation [Yang et al., 2004].

RUNX2 mRNA is present throughout bone development beginning at embryonic day 9.5 (E9.5) in the mouse and persisting in active osteoblasts and osteocytes throughout adult life [Ducy et al., 1997]. There is good evidence that Runx2 regulates separate events at different times during skeletal development. As noted above, it has early roles in cartilage hypertrophy, bony collar formation and vascularization, but is also required for terminal differentiation of osteoblasts and osteocytes via induction of genes involved in extracellular matrix (ECM) formation and mineralization. These findings suggest that other factors and signals must be able to modify Runx2 activity according to stage of development without necessarily altering levels of Runx2 protein. Figure 2 illustrates how RUNX2 activity can be regulated without altering Runx2 protein levels. In this study, differentiation of MC3T3-E1 preosteoblast was induced by growth in ascorbic acid-containing medium, thereby allowing cells to secrete a collagenous ECM. As ECM accumulates, there is a dramatic induction of osteoblast marker genes such as bone sialoprotein (Bsp) and Ocn, but, surprisingly, RUNX2 mRNA and protein levels remain relatively constant.

There are at least 3 possible ways RUNX2 transcriptional activity could be regulated in the absence of changes in RUNX2 protein levels. First, covalent modification could alter RUNX2 transcriptional activity. Second, levels of cofactors or ability of cofactors to interact with RUNX2 could be regulated. Lastly, Runx2 protein levels could be controlled by proteosome-mediated degradation. All 3 types of regulation have been previously described, although we will focus on the first 2.

Posttranslational Modification

The first example involves a pathway incorporating integrin-mediated activation of the ERK/MAP kinase pathway that results in phosphorylation and stimulation of RUNX2 transcriptional activity. Integrins provide a direct link between cells and ECM to convey information about mechanical loads and ECM stiffness experienced by bone to control differentiation [You et al., 2001; Engler et al., 2006]. Integrins are also important signal transduction molecules in their own right that activate Ras-ERK and p38 MAP kinase, calcium channels and mechanosensors [Hynes, 2002].

Osteoblast differentiation requires synthesis of a collagen-containing ECM. As is shown in figure 2, bone sialoprotein and osteocalcin are only expressed in cells grown under conditions where they can synthesize a collagenous matrix [Franceschi, 1992]. Using a combination of chemical inhibitors and genetic approaches, we previously established that the response of osteoblasts to ECM is mediated by $\alpha 2\beta 1$ integrins and the ERK/MAP kinase pathway [Xiao et al., 1997, 1998, 2000].

The role of RUNX2 in MAPK responsiveness was established in studies with the Ocn gene. MAPK activation via transfection of cells with constitutively active MEK1

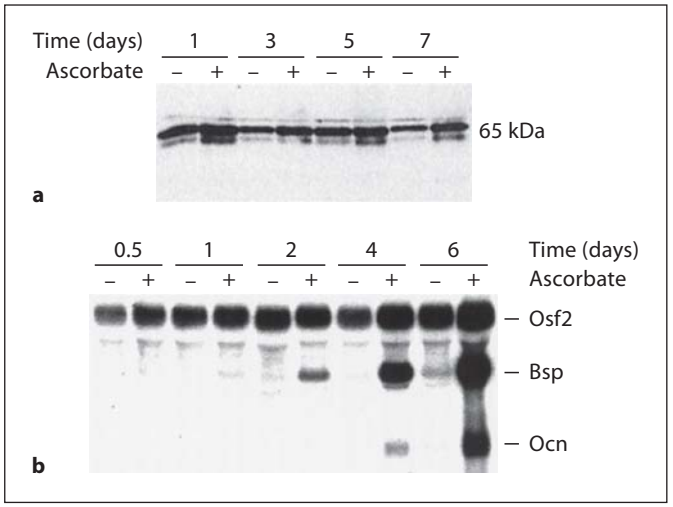
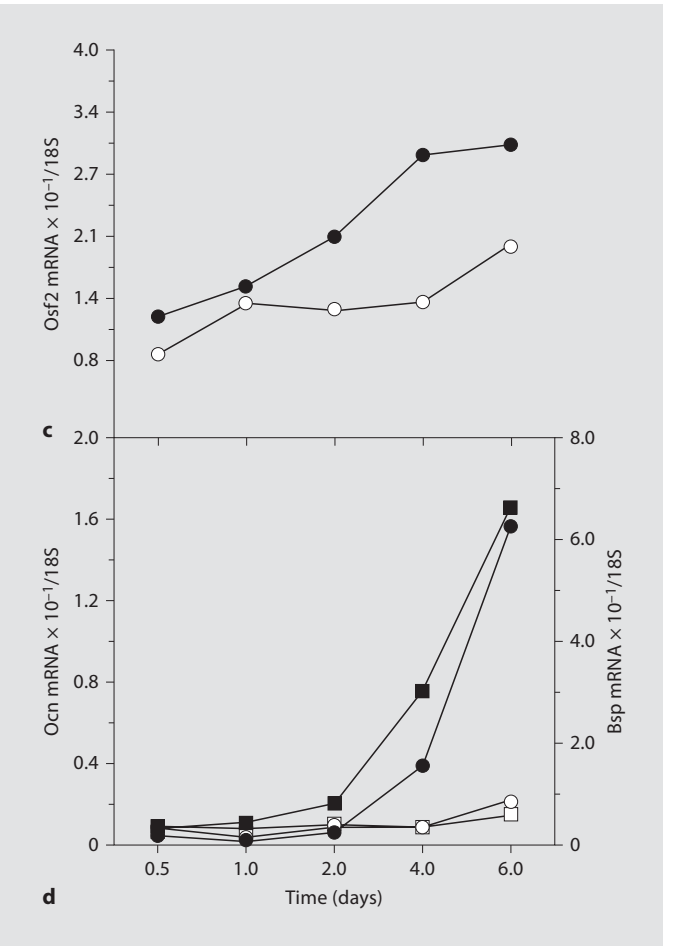


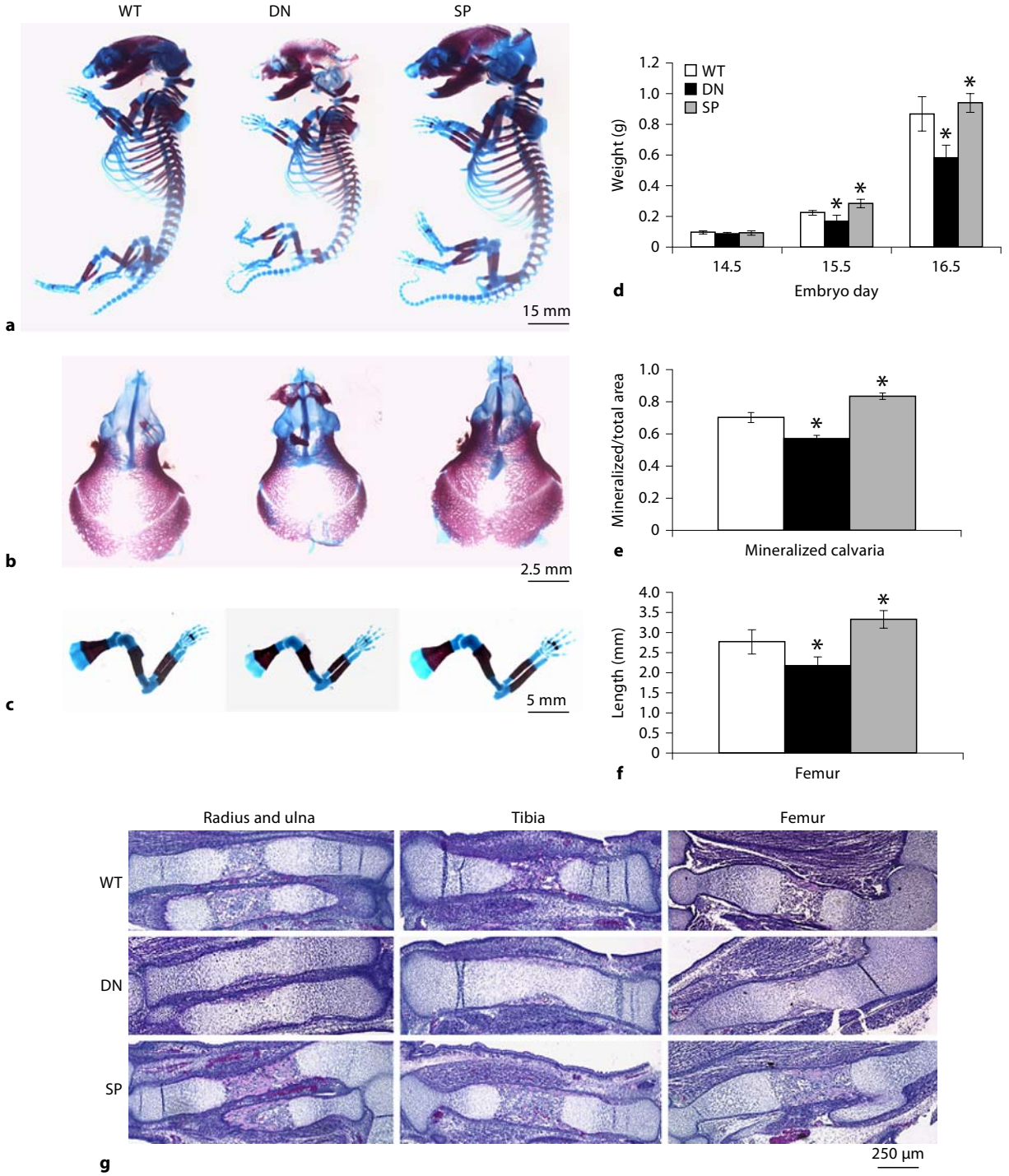
Fig. 2. RUNX2 protein levels are not well correlated with transcriptional activity. MC3T3-E1 clone 4 preosteoblast cells were grown in control (–) or ascorbate-containing medium (+). At the times indicated, RUNX2 protein levels were determined by Western blotting (**a**), while *Runx2* (Osf2), *Ocn* and *Bsp* mRNA levels (**b–d**) were measured on Northern blots (**b**) and quantified by densitometry. **c** Runx2. **d** Ocn and Bsp. Open symbols = Control; closed symbols = ascorbate. ○, ● = Ocn mRNA; □, ■ = Bsp mRNA [Xiao et al. 1998].



(MEK-SP) could induce Ocn expression, while dominant-negative MEK1 (MEK-DN) was inhibitory [Xiao et al., 2000]. Mutational analysis of the Ocn promoter for MAPK-responsive sequence elements established the involvement of 2 RUNX2-binding sites called OSE2a and b. Mutation of either site reduced MAPK responsiveness [Frendo et al., 1998].

As might be expected, activation of MAPK, either by ECM synthesis or transfection with MEK-SP, results in increased RUNX2 phosphorylation [Xiao et al., 2000]. Examination of different truncations of the RUNX2 protein showed that the C-terminal proline/serine/threonine region of RUNX2 was required for both MAPK responsiveness and phosphorylation [Xiao et al., 2000]. More detailed deletional analysis further localized a minimal region for MAPK responsiveness between amino acids 254 and 320 (data not shown). The specific identification and functional significance of ERK/MAPK phosphorylation sites in RUNX2 will be reported elsewhere. A number of other stimuli that act through the ERK/MAPK pathway, including FGFR2 activation by FGF2 and mechanical loading of osteoblasts, also stimulate RUNX2 phosphorylation and transcriptional activity [Wang et al., 2002; Xiao et al., 2002; Ziros et al., 2002; Kim et al., 2003; Kanno et al., 2007].

Fig. 3. Altered skeletal development in *TgMek-dn* and *TgMek-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**, **e** Cranial bones showing differences in mineralization (**b**) and quantification of mineralized area (expressed as percent of total calvarial area) (**e**). **c**, **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 *TgMek-sp* mice. Note the delay in bony collar and trabecular bone in *TgMek-dn* embryos. Statistical analysis: values are expressed as means ± SD, n = 8/group. * Significantly different from wild type at $p < 0.01$. Reproduced from Ge et al. [2007].



In vivo Manipulation of ERK/MAPK Signaling Alters Bone Development

We took a transgenic approach to examine the importance of the ERK/MAPK pathway and RUNX2 phosphorylation in osteoblast function in vivo [Ge et al., 2007]. Mice were generated using a 0.6-kb mOG2 promoter to specifically drive MEK-SP and MEK-DN expression in osteoblasts. Transgene expression was detected only in bones and had a developmental time course of expression that parallels expression of the endogenous Ocn gene. ERK1/2 phosphorylation in calvarial osteoblasts derived from these animals was shown to increase by 50% in cells from *Mek-sp* mice and to decrease by 50% in *Mek-dn* cells [Ge et al., 2007]. Thus, transgene expression leads to subtle changes in MAPK activity that resemble fluctuations normally induced by physiological stimuli. Skeletal whole mounts revealed that *Mek-dn* decreased skeletal size and calvarial mineralization, while these parameters were increased in *Mek-sp* mice (fig. 3a–f). Histology of long bones revealed an additional interesting difference between wild-type and transgenic mice (fig. 3g). At E15.5, long bones are normally undergoing endochondral ossification in diaphyseal regions. However, in *Mek-dn* mice, this process is drastically delayed with only early bony collar formation being visible. In contrast, in *Mek-sp* mice, endochondral bone formation is accelerated.

Transgenic modification of osteoblast MAPK activity was also found to alter RUNX2 phosphorylation and transcriptional activity. RUNX2 phosphorylation was increased in *Mek-sp* cells as was luciferase activity of an *Ocn* reporter gene. Also, as expected, *Mek-sp* stimulated in vitro osteoblast differentiation as measured by induction of osteoblast marker mRNAs or mineralization, while differentiation was inhibited in Mek-dn cells [Ge et al., 2007].

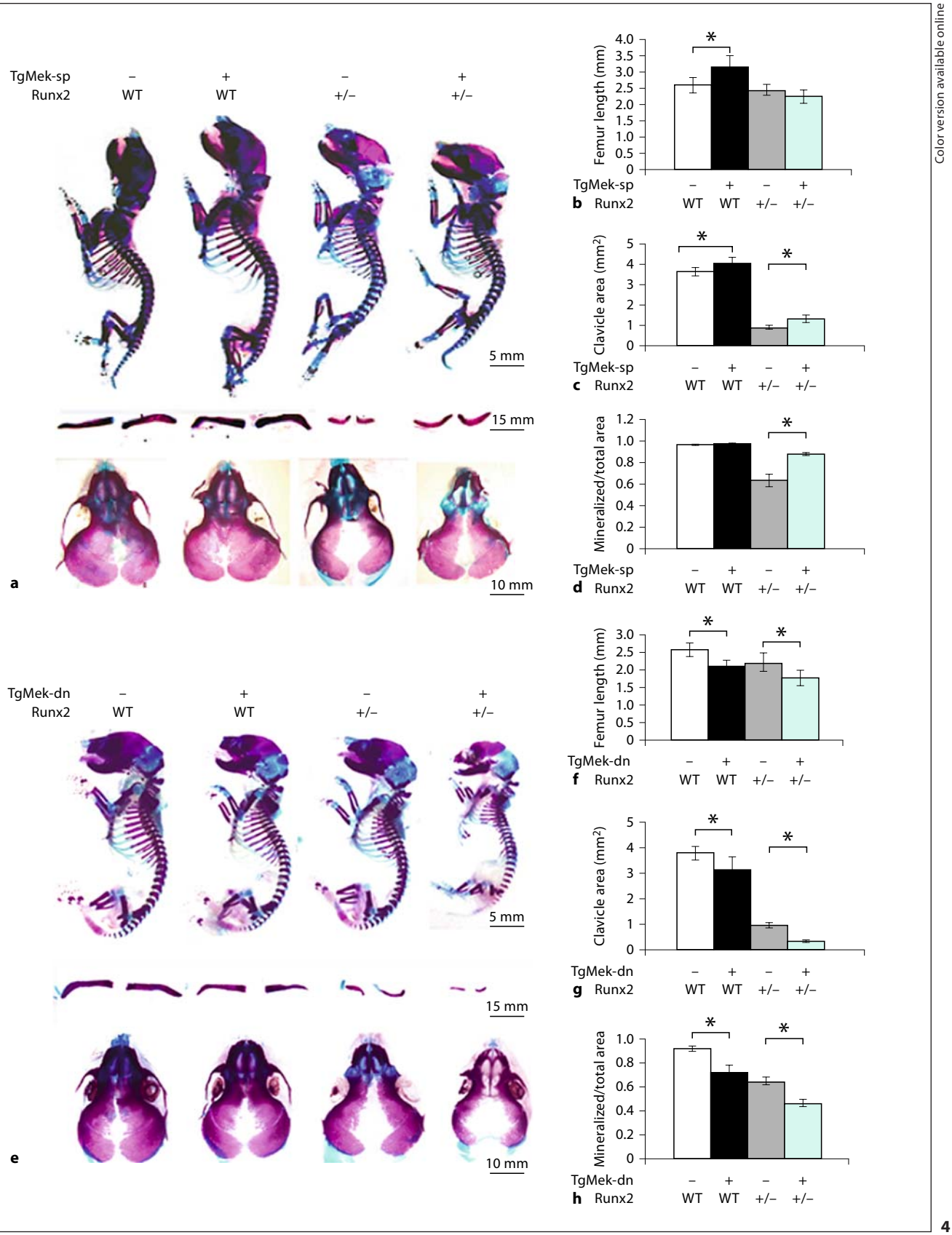
To provide evidence that MAPK effects on skeletal development are mediated by RUNX2, we used a genetic approach. *Runx2*$^{+/-}$ mice are known to have a characteristic phenotype (hypoplastic clavicles, patent fontanelles) that resembles the human genetic disease cleidocranial dysplasia [Otto et al., 1997]. We reasoned that if MAPK acts by altering RUNX2 activity, calvaria and clavicles should be selectively sensitive to the *Mek* transgene when RUNX2 is limiting (that is, in *Runx2*$^{+/-}$ mice). To test this, *Runx2*$^{+/-}$ mice were crossed with *Mek-sp* or *Mek-dn* transgenic lines and the resulting skeletal phenotypes were examined at E19 (fig. 4). Note the reduced calvarial mineralization and tiny clavicles in the *Runx2*$^{+/-}$ embryos. The presence of the *Mek-sp* transgene led to a clear rescue of the cleidocranial dysplasia phenotype with increased clavicle size and calvarial mineralization. In the presence of *Mek-dn*, effects on *Runx2*$^{+/-}$ mice were even more dramatic. In this case, the *Mek-dn* transgene exacerbated effects of *Runx2* haploinsufficiency with a further reduction in calvarial mineralization and near disappearance of clavicles. Notably, *Runx2*$^{+/-}$ *Mek-sp* embryos did not survive the birth process due to severe skeletal defects. These experiments provide strong evidence that the ERK/MAPK pathway, via actions on RUNX2 transcriptional activity, is important for normal osteoblast differentiation and bone formation in vivo.

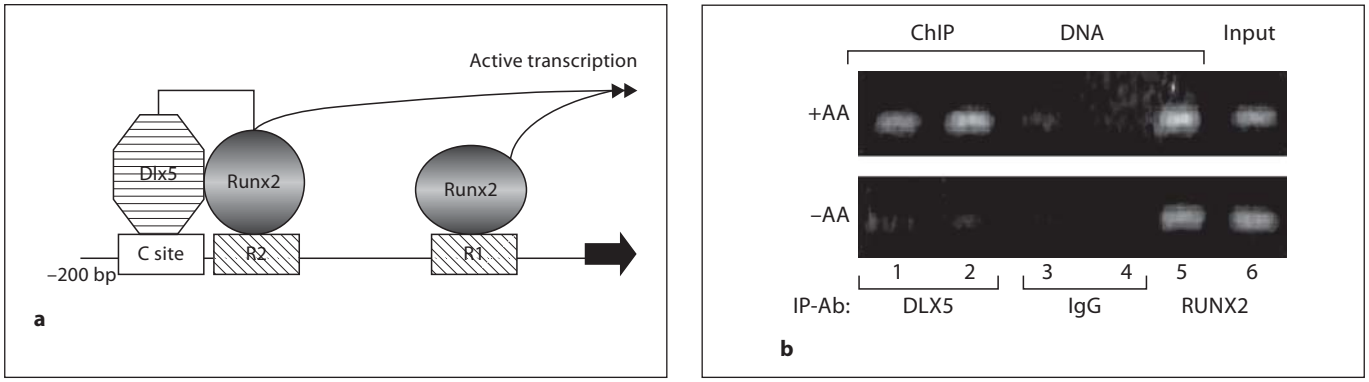
RUNX2 Interaction with DLX5 Is Necessary for Bone-Specific Activation of *Bsp*

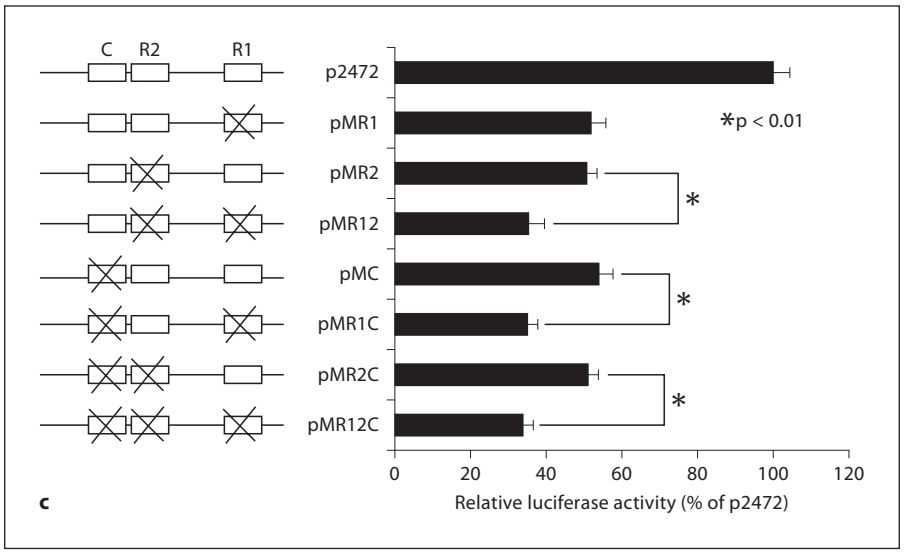
RUNX2 directly or indirectly interacts with a large number of nuclear factors. For example, CBF-β forms heterodimers with all members of the Runx family [Ito, 2004], while ATF4-RUNX2 interactions may mediate some of the responses of osteoblasts to parathyroid hormone [Jiang et al., 2004; Xiao et al., 2005]. SMAD proteins, mediators of BMP/TGF-β actions, can also stimulate RUNX2 activity [Afzal et al., 2005]. In addition, there are a number of inhibitory factors. SOX9, mentioned above, suppresses RUNX2-dependent chondrocyte hypertrophy [Zhou et al., 2006]. TWIST may prevent RUNX2 stimulation of mineralization in developing cranial bones to prevent craniosynostosis [Bialek et al., 2004]. Lastly, histone deacetylases are known to bind RUNX2 and keep chromatin in a deacetylated, inactive state [Imai et al., 2004].

Studies on *Bsp* revealed an important regulatory function of interactions between RUNX2 and DLX5 [Roca et al., 2005]. We previously characterized a 2.4-kb promoter

Fig. 4. Genetic interactions between *Mek-dn* and *Mek-sp* transgenes and *Runx2*. *TgMek-dn* or *TgMek-sp* mice were crossed with RUNX2$^{+/-}$ mice to generate the genotypes indicated. **a–d** Partial rescue of cleidocranial dysplasia 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) (**d**). **e–h** Increased severity of cleidocranial dysplasia phenotype with *Mek-dn*. Groups are as in panels **a–d**. Statistical analysis: values are expressed as means ± SD, n = 8/group. Comparisons are indicated by bars. * $p < 0.01$. Reproduced from Ge et al. [2007].







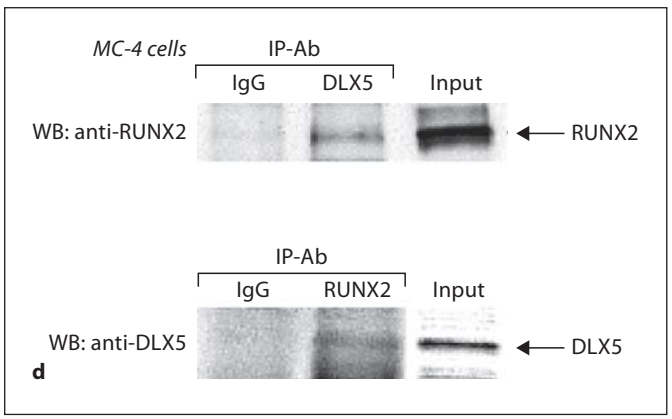


Fig. 5. In vivo binding of RUNX2 and DLX5 to chromatin sites in differentiated and undifferentiated cells. **a** Schematic of the proximal *Bsp* promoter. RUNX2 and homeodomain (DLX5) protein-binding sites are indicated. **b** Comparison of *Bsp* chromatin occupancy by RUNX2 and DLX5. Chromatin immunoprecipitation assays were used to detect RUNX2 and DLX5 bound to the proximal *Bsp* promoter in control (–AA) and differentiated (+AA) MC3T3-E1 clone 4 cells. Antibodies used for chromatin immunoprecipitation are indicated. Note that RUNX2 remains chromatin associated regardless of differentiated state, while Dlx5 is only present in differentiated cells. **c** Functional interaction between R2 and C sites. 2.5-kb Bsp-luc reporter constructs containing all possible combinations of R1, R2 and C site mutations were transfected into MC3T3-E1 cl4 cells and grown under differentiating conditions. Note that mutation of either R2 or C is as inhibitory as mutation of both sites, while mutation of either site together with R1 gives maximal inhibition. **d** Protein-protein interactions between DLX5 and RUNX2. Pull-down assays with nuclear extracts from differentiated MC3T3 cells were used to show physical association between these 2 factors [Roca et al., 2005]. ChIP = Chromatin immunoprecipitation; IP-Ab = chromatin immunoprecipitation antibodies; WB = Western blot.

fragment of this gene and showed that it contains sufficient information to drive osteoblast-selective expression in vivo [Benson et al., 2000; Gopalakrishnan et al., 2003]. Detailed characterization of the proximal Bsp promoter identified 2 RUNX2-binding sites (R1, R2) and an adjacent homeodomain protein-binding site (C site) that binds DLX5 (fig. 5a). Interestingly, levels of RUNX2 associated with chromatin do not appreciably change during osteoblast differentiation. In contrast, DLX5 only becomes chromatin associated in differentiated cells (fig. 5b). However, total DLX5 protein was equivalent in control and differentiated cells indicating that the affin-

ity of Dlx5 for chromatin (and possibly Runx2) increases with differentiation. Subsequent analysis revealed that RUNX2 must be associated with both sites for full activity of the Bsp promoter. Furthermore, the R2 site and the C site functionally cooperate to regulate promoter activity (fig. 5c). Consistent with this observation, RUNX2 and DLX5 physically interact as measured by pull-down assays (fig. 5d).

Summary

As one of the earliest determinants of the bone cell lineage, RUNX2 first appears soon after the formation of mesenchymal condensations. Although present throughout subsequent stages of osteoblast differentiation, RUNX2 activity is highly regulated. As was discussed, this regulation can be the result of RUNX2 phosphorylation or interaction with partner proteins such as DLX5, in both cases leading to activation of RUNX2 transcriptional activity. A number of outstanding questions remain regarding RUNX2 function. First, it is not known if RUNX2 becomes associated with all target genes it will eventually regulate soon after it is first expressed or if it associates with different genes at different stages of differentiation. If the former is true, then RUNX2 may be largely reliant on the posttranslation controls and accessory factor binding described here to become active at the appropriate time. If the latter is the case, RUNX2 synthesis and degradation may be more dynamic with transcriptional activity being dependent on RUNX2 protein levels as well as levels of accessory factors. A second area relates to the degree to which posttranslational modification of RUNX2 can control its activity. As noted above, there are now several examples of RUNX2 being positively or negatively regulated by phosphorylation. However, other types of modification such as acetylation have also been reported [Jeon et al., 2006]. It will be important to understand how these different modifications are coordinated to control RUNX2 activity. Lastly, the full breadth of factors associating with RUNX2 is presently unknown, but current evidence suggests that RUNX2 likely exists in large macromolecular complexes that have a punctate distribution in the nucleus [Zaidi et al., 2002]. A major challenge will be to isolate and characterize these complexes and determine how their composition is regulated during skeletal development.

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