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Bone morphogenetic protein and retinoic acid signaling cooperate to induce osteoblast differentiation of preadipocytes

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Mesenchymal cells can differentiate into osteoblasts, adipocytes, myoblasts, or chondroblasts. Whether mesenchymal cells that have initiated differentiation along one lineage can transdifferentiate into another is largely unknown. Using 3T3-F442A preadipocytes, we explored whether extracellular signals could redirect their differentiation from adipocyte into osteoblast. 3T3-F442A cells expressed receptors and Smads required for bone morphogenetic protein (BMP) signaling. BMP-2 increased proliferation and induced the early osteoblast differentiation marker alkaline phosphatase, yet only mildly affected adipogenic differentiation. Retinoic acid inhibited adipose conversion and cooperated with BMP-2 to enhance proliferation, inhibit adipogenesis, and promote early osteoblastic

differentiation. Expression of BMP-RII together with BMP-RIA or BMP-RIB suppressed adipogenesis of 3T3-F442A cells and promoted full osteoblastic differentiation in response to retinoic acid. Osteoblastic differentiation was characterized by induction of cbfa1, osteocalcin, and collagen I expression, and extracellular matrix calcification. These results indicate that 3T3-F442A preadipocytes can be converted into fully differentiated osteoblasts in response to extracellular signaling cues. Furthermore, BMP and retinoic acid signaling cooperate to stimulate cell proliferation, repress adipogenesis, and promote osteoblast differentiation. Finally, BMP-RIA and BMP-RIB induced osteoblast differentiation and repressed adipocytic differentiation to a similar extent.

Introduction

Mesenchymal cells can differentiate into adipocytes, myocytes, chondrocytes, or osteoblasts (Prockop, 1997; Pittenger et al., 1999). How far mesenchymal cells that have initiated differentiation along one defined lineage can be persuaded to transdifferentiate into another lineage is largely unknown. This basic question is of interest to understand not only the regulation of cell fate decisions, but also the etiology of clinical syndromes resulting from misregulation of mesenchymal differentiation. Of particular interest is the regulation of osteoblast versus adipocyte differentiation in the bone marrow. Marrow stromal cell cultures demonstrate the ability of single cell clones to differentiate into osteoblasts, chondrocytes, or adipocytes (Muraglia et al., 2000; Satomura et al., 2000). Additionally, an inverse relationship between osteoblast and adipocyte differentiation has been observed (Beresford et al., 1992). Under conditions leading to bone loss, e.g.,

osteoporosis or osteopenia, the decreased osteoblast number correlates with increased adipocyte differentiation (Meunier et al., 1971; Benayahu et al., 2000), suggesting that adipocytes are generated at the expense of osteoblasts from a common precursor. This hypothesis is supported by the isolation of single cell clones from bone marrow that can differentiate in vitro into either adipocytes or osteoblasts (Park et al., 1999).

Essential to a cell's commitment to a differentiation lineage are the activation of defined transcription factors and repression of others, thus setting the stage for a gene expression pattern characteristic for that particular cell type (Black and Olson, 1998; de Crombrugghe et al., 2000; Rosen et al., 2000; Wagner and Karsenty, 2001). Osteoblastic differentiation is characterized by expression of alkaline phosphatase as an early preosteoblastic marker, later expression of the transcription factor CBFA1 that drives osteoblastic differentiation and the CBFA1 target gene osteocalcin, enhanced collagen I expression, and eventually, calcification of the ECM (Wagner and Karsenty, 2001). Differentiation of adipocytes is initiated through the transcription factors C/EBPβ and C/EBPδ that activate expression of the nuclear receptor PPARγ (Wu et al., 1996; Clarke et al., 1997). PPARγ, as

a heterodimer with RXR, another nuclear receptor, then drives differentiation into lipid-filled, fully differentiated adipocytes (Tontonoz et al., 1994a).

Ectopic expression of the specific transcription factors associated with bone or fat differentiation can activate their respective differentiation programs in mesenchymal cells. Ectopic CBFA1 expression in fibroblasts induces early osteoblastic differentiation (Xiao et al., 1999), but it is not known whether CBFA1 expression in mesenchymal, nonosteoblast cell types (e.g., myoblasts and preadipocytes) can induce osteoblastic differentiation. Similarly, ectopic expression of PPARγ or C/EBP in fibroblasts and some other mesenchymal cells leads to adipocyte differentiation (Tontonoz et al., 1994b; Hu et al., 1995; Yeh et al., 1995), although it is not known whether osteoblasts can be converted into adipocytes by expressing PPARγ.

Signaling by cell–cell contacts, ECM proteins, and secreted differentiation factors, plays a key role in the selection of a differentiation pathway and in progression of differentiation. Among these, the TGF-β family of growth factors plays determinant roles in mesenchymal tissue differentiation. For example, myostatin regulates muscle development (McPherron et al., 1997), whereas bone morphogenetic proteins (BMPs)* and TGF-β regulate skeletal development and homeostasis (Alliston and Derynck, 2000; Hoffmann and Gross, 2001). Some of these functions can be studied in cell culture. Thus, TGF-β inhibits differentiation of myoblasts (Massagué et al., 1986; Olson et al., 1986; Liu et al., 2001), preadipocytes (Ignotz and Massagué, 1985; Choy et al., 2000), and osteoblasts (Centrella et al., 1994; Alliston et al., 2001) in culture. Additionally, BMP-2, -4, and -7 induce expression of alkaline phosphatase and CBFA1 in fibroblasts and myoblasts, and full osteoblastic differentiation of preosteoblasts (Li et al., 1996; Lecanda et al., 1997), whereas BMP-7–transduced fibroblasts can form bone in vivo (Krebsbach et al., 2000).

TGF-β ligands signal through two types of transmembrane serine–threonine kinase receptors. BMP-2 and -4 signal through the type II receptor, BMP-RII, in combination with the type I receptors, BMP-RIA or BMP-RIB, encoded by different genes (ten Dijke et al., 1994; Rosenzweig et al., 1995). BMP-RIA and -RIB have been shown to stimulate osteoblastic differentiation in preosteoblastic cell lines (Ishidou et al., 1995; Fujii et al., 1999). However, studies in bipotential 2T3 calvarial cells proposed that BMP-RIA promotes adipocyte differentiation and BMP-RIB promotes osteoblast differentiation (Chen et al., 1998). Ligand binding results in type II receptor–mediated activation of the type I receptors, which in turn phosphorylate and thereby activate "receptor-activated" Smads (Massagué and Wotton, 2000). BMPs activate Smad1, Smad5, and Smad8, and TGF-β signals primarily through Smad2 and Smad3. The receptor-activated Smads trimerize with Smad4, a component of both the TGF-β and BMP signaling pathways, and translocate into the nucleus, where they cooperate with sequence-specific transcription factors to regulate gene expression in response to ligand. Smad3 acts as a key component of TGF-β–induced repression of adipocyte differentiation

(Choy et al., 2000), and represses CBFA1 activity to inhibit osteoblastic differentiation by TGF-β (Alliston et al., 2001).

Another signaling system that regulates mesenchymal cell differentiation is triggered by retinoic acid. Activation of the nuclear retinoic acid receptor and its combination with RXR initiates transcriptional activation of target genes. Retinoic acid is a potent inhibitor of adipocyte differentiation, in part through inhibition of C/EBPβ transcription (Schwarz et al., 1997), an upstream regulator of PPARγ transcription. Additionally, retinoic acid can also enhance osteoblast differentiation through induction of alkaline phosphatase, osteocalcin, and osteopontin expression (Choong et al., 1993; Sodek et al., 1995).

Little is known about the ability of extracellular signals to induce transdifferentiation from one mesenchymal lineage into another. BMP-2/4 can induce early osteoblastic characteristics in C2C12 myoblasts (Katagiri et al., 1994; Aoki et al., 2001). Also, ectopic BMP receptor and Smad1 or Smad5 expression in C2C12 myoblasts leads to alkaline phosphatase expression, but not full osteoblastic differentiation with ECM calcification (Akiyama et al., 1997; Yamamoto et al., 1997). It is not known whether preadipocytes can be converted into osteoblasts in response to BMPs or any other extracellular stimuli.

In this paper, we evaluated the ability of preadipocytes to differentiate into osteoblasts in response to BMP signaling. As a model system, we used 3T3-F442A cells (Green and Kehinde, 1976), a well-characterized preadipocyte cell line. These cells robustly differentiate into adipocytes in vitro, yield a fully differentiated fat pad after subcutaneous injection into nude mice (Green and Kehinde, 1979; Mandrup et al., 1997), and are not known to differentiate into another cell type. We demonstrate that these cells respond to BMP-2 and that increased BMP signaling represses insulin-stimulated adipocyte differentiation and induces expression of osteoblast differentiation markers. BMP signaling and retinoic acid cooperate to strongly repress adipocyte differentiation, stimulate proliferation, and induce osteoblast differentiation markers at high levels. The cooperation of retinoic acid and BMP signaling results in transdifferentiation of the preadipocytes into fully differentiated osteoblasts with ECM calcification in culture. BMP-RIA and BMP-RIB signaling had similar abilities to inhibit adipocyte differentiation and induce osteoblast differentiation.

Results

3T3-F442A preadipocytes have a functional BMP signaling pathway

3T3-F442A preadipocytes, a widely used model for adipocyte differentiation, fully differentiate into adipocytes after 8 d of insulin treatment. Because BMP signaling has been shown to promote osteoblastic differentiation of several mesenchymal cell lines, we evaluated the presence of the BMP signaling effectors in 3T3-F442A cells. As shown in Fig. 1 A, 3T3-F442A cells expressed mRNAs for BMP-RII, BMP-RIA, and BMP-RIB, both before and after adipocyte differentiation at 8 d after reaching confluence. All three BMP receptor genes were expressed as two mRNA species, which is consistent with previous reports (Nohno et al., 1995; Namiki et al., 1997). Al-

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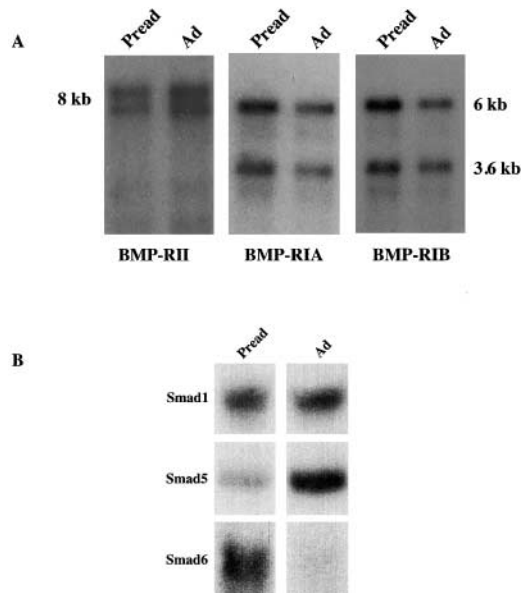*Abbreviation used in this paper: BMP, bone morphogenetic protein.

Figure 1. **Expression of BMP signaling effectors in 3T3-F442A cell, as assessed by Northern blotting.** (A) Detection of mRNAs for BMP-RII, BMP-RIA, and BMP-RIB in mRNA isolated from 3T3-F442A preadipocytes before (pread) and after (ad) adipocyte differentiation. The mRNA sizes are marked. (B) Detection of mRNA for Smad1, 5, and 6 in confluent 3T3-F442A preadipocyte and adipocyte cells.

Figure 2. **Effect of BMP-2 on 3T3-F442A cell proliferation.** Cells were treated with indicated concentrations of BMP-2 and the levels of [³H]thymidine incorporation were determined by liquid scintillation counting.

though BMP-RII mRNAs remained unchanged, the BMP-RIA and -RIB mRNA levels slightly decreased during adipocyte differentiation (Fig. 1 A). The BMP receptors could not be detected using available antibodies (unpublished data).

BMPs transduce signals through activation of Smad1, 5, and 8 (Hoodless et al., 1996; Nakayama et al., 1998; Nishimura et al., 1998). In addition, BMPs induce the expression of Smad6 (Takase et al., 1998), which acts as an inhibitory Smad for BMP signals (Hata et al., 1998). Although we did not detect Smad8 mRNA expression, 3T3-F442A cells expressed mRNAs for Smad1 and Smad5, as well as Smad6 (Fig. 1 B). Smad1 mRNA levels were similar in undifferentiated and differentiated cells, but Smad5 mRNA expression increased and Smad6 mRNA levels decreased with adipogenic differentiation. Together, these data suggest the presence of a fully operative BMP signaling pathway with BMP receptors and BMP Smads in 3T3-F442A cells.

In addition to promoting osteoblastic differentiation, BMP signaling can also enhance proliferation of mesenchymal cells (Chen et al., 1991; Yamaguchi et al., 1991; Lou et al., 1999). Therefore, we treated 3T3-F442A cells with BMP-2 and measured the proliferation response using a [³H]thymidine incorporation assay. As shown in Fig. 2, BMP-2 increased the rate of DNA synthesis in a dose-dependent manner. These results establish the existence of a functional BMP-2 signaling pathway in 3T3-F442A cells.

Effects of BMP-2 and retinoic acid on adipogenic differentiation

Because 3T3-F442A cells expressed BMP signaling effectors and responded to BMP-2, we assessed the effect of BMP-2 on the differentiation of these preadipocytes (Fig.
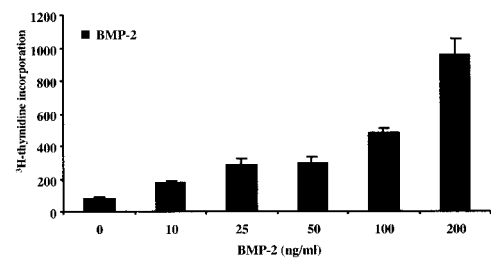
3). BMP-2 altered the appearance of the cells, resulting in an elongated, fibroblastic or osteoblastic appearance (Fig. 3 A, panel c), different from the rounder appearance of untreated 3T3-F442A cells (Fig. 3 A, panel a). BMP-2 also slightly enhanced the low level of adipogenic conversion in the absence of insulin (Fig. 3 A, panel c vs. panel a). Treatment of 3T3-F442A cells with insulin induced adipogenic differentiation with accumulation of lipid and full differentiation after 8 d (Fig. 3 A, panel b). BMP-2 mildly inhibited adipocyte differentiation in the presence of insulin, as assessed by the number of lipid-containing cells using light microscopy (Fig. 3 A, panel d). The adipogenic differentiation in the presence of insulin was also evaluated using Oil Red O staining of lipid accumulation, and the mild inhibition of adipogenic differentiation by BMP-2 was also apparent in this assay (Fig. 3 B). BMP-2 treatment also conferred a decrease in mRNA expression of PPARγ and adipsin, a late marker of adipocyte differentiation (Fig. 3 C). Thus, BMP-2 signaling mildly inhibited insulin-induced adipocyte differentiation.

Because retinoic acid inhibits adipocyte differentiation in culture, including differentiation of 3T3-F442A cells (Kuri-Harcuch, 1982; Schwarz et al., 1997), we evaluated the effect of retinoic acid on adipogenic differentiation in the absence or presence of BMP-2. Retinoic acid induced a fibroblastic morphology (Fig. 3 A, panel e) similar to BMP-2, and strongly inhibited the ability of 3T3-F442A cells to undergo adipocyte differentiation in the presence of insulin (Fig. 3 A, panel f). This inhibition was apparent by a reduction of lipid-filled adipocytes seen microscopically (Fig. 3 A, panel f) and after Oil Red O staining of lipids (Fig. 3 B). Consistent with these results, retinoic acid inhibited the expression of PPARγ and adipsin mRNA (Fig. 3 C).

Combination treatment of BMP-2 and retinoic acid on 3T3-F442A cells was also investigated. In the absence of insulin, BMP-2 and retinoic acid induced a more pronounced elongated, fibroblastic appearance than either component alone, suggesting cooperation between BMP-2 and retinoic acid signaling (Fig. 3 A, panel g). In the presence of insulin, the combination of both ligands was sufficient to prevent adipogenesis, as assessed morphologically (Fig. 3 A, panel h) and using Oil Red O staining (Fig. 3 B). The combination of BMP-2 and retinoic acid did not decrease the expression of PPARγ mRNA beyond what was seen with BMP-2 alone (Fig. 3 C). However, their combined inhibitory effect on adipsin mRNA expression
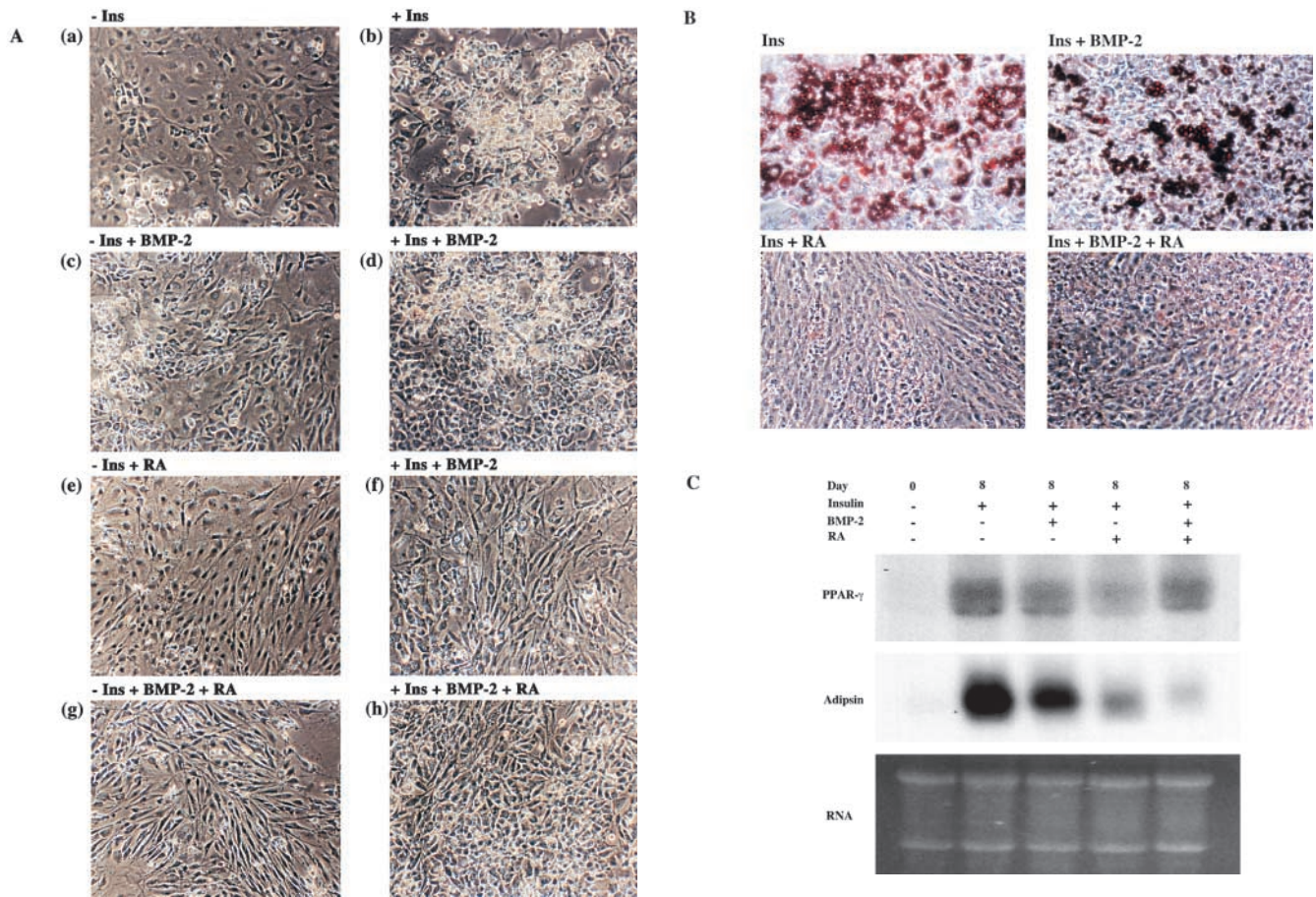
Figure 3. Effects of insulin, BMP-2, and retinoic acid treatments on 3T3-F442A cell morphology, lipid accumulation, and adipocyte gene expression. (A) Morphology of 3T3-F442A cells, cultured in the presence of 10% FBS, in the absence (a, c, e, and g) or presence (b, d, f, and h) of 5 μg/ml insulin (a and b, no addition; c and d, 100 ng/ml BMP-2; e and f, 10^{-6} M retinoic acid; g and h, 100 ng/ml BMP-2 and 10^{-6} M retinoic acid. (B) Oil Red O staining of lipids in cells cultured in the presence of 5 μg/ml insulin, 100 ng/ml BMP-2, 10^{-6} M retinoic acid, or 100 ng/ml BMP-2 with 10^{-6} M retinoic acid. (C) Northern blot detection of mRNAs for PPARγ and adipsin, after 8 d of insulin treatment in the presence or absence of BMP-2 and retinoic acid. Insulin, BMP-2, and retinoic acid were used at the same concentrations as in A and B. Ethidium bromide staining of RNA (bottom) demonstrates equal loading. RA, retinoic acid; Ins, insulin.

was striking (Fig. 3 C), and consistent with a synergy of BMP-2 and retinoic acid signaling.

BMP-2 and retinoic acid induce alkaline phosphatase expression and cooperate

The reported ability of BMP-2 to promote osteoblastic differentiation led us to evaluate the effect of BMP-2 on the expression of alkaline phosphatase, an early marker of osteoblast differentiation. As can be seen in Fig. 4 A, BMP-2 induced the expression of alkaline phosphatase in confluent 3T3-F442A cells in a dose-dependent manner, suggesting that these cells have the potential to transdifferentiate to osteoblasts. Cotreatment with insulin, which promotes adipogenesis, did not repress the expression of alkaline phosphatase. Instead, insulin minimally but consistently enhanced the induction of alkaline phosphatase expression by BMP-2 (Fig. 4 B), even though the cells were undergoing nearly quantitative adipose conversion (Fig. 3, A and B). BMP-2 did not induce an advanced osteoblastic phenotype, which was apparent from the absence of cbfa1 or osteocalcin mRNA expression (unpublished data). Preosteoblasts can undergo full osteoblast differentiation with ECM

mineralization, when cultured as confluent monolayers in the presence of β-glycerophosphate and ascorbic acid for extended periods. However, also under these conditions did we not detect cbfa1 or osteocalcin mRNA expression, and we did not observe mineralization of the ECM (unpublished data).

Because retinoic acid inhibited adipocyte differentiation and can enhance osteoblast differentiation (Choong et al., 1993; Sodek et al., 1995), we determined its effect on osteoblast differentiation of 3T3-F442A cells. Enzymatic assays revealed that retinoic acid induced a dose-dependent increase in alkaline phosphatase activity, which was already apparent at a level of 1 nM (Fig. 4 C). Treatment with 1 μM retinoic acid induced a sixfold increase over basal activity. Thus, retinoic acid both suppressed adipocyte differentiation and promoted expression of an early osteoblast marker. However, continuous treatment with 1 μM retinoic acid, even in the presence of β-glycerophosphate and ascorbic acid, failed to yield cbfa1 or osteocalcin mRNA expression or detectable mineralization (unpublished data).

Because BMP-2 and retinoic acid signaling cooperated to suppress adipocyte differentiation, we assessed their com-
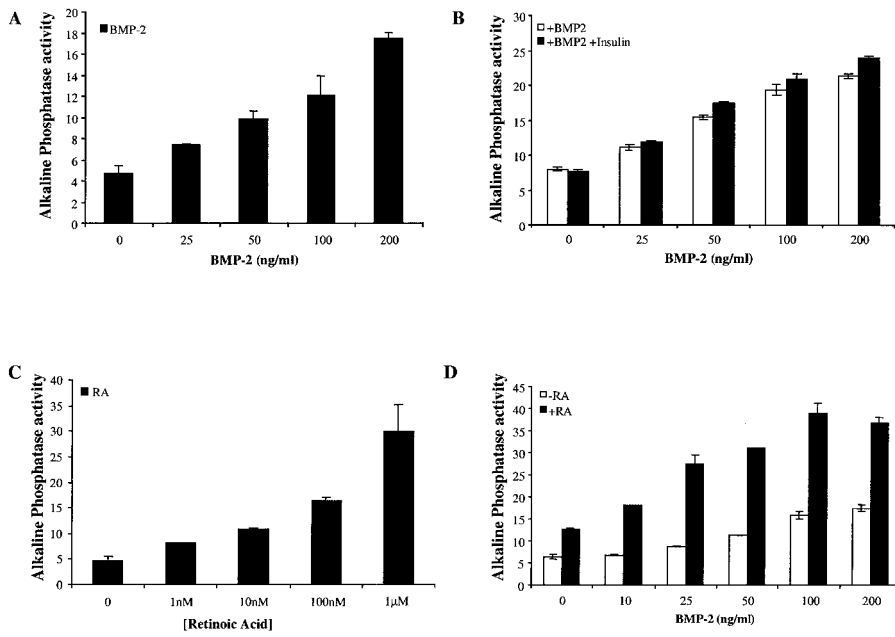
Figure 4. **Effects of BMP-2, insulin, and retinoic acid on alkaline phosphatase expression in 3T3-F442A cells.** Dose-dependent induction by (A) BMP-2, (B) BMP-2 with or without 5 μg/ml insulin, (C) retinoic acid, and (D) BMP-2 with or without 10 nM retinoic acid. Confluent cells were treated with the ligands for 48 h followed by assessment of alkaline phosphatase activity.

bined effect on osteoblastic differentiation and proliferation of 3T3-F442A cells. 10 nM retinoic acid alone only moderately induced alkaline phosphatase expression, but strongly synergized with BMP-2 in inducing the expression of alkaline phosphatase (Fig. 4 D). A similar cooperativeness was also seen when we measured the cell proliferation in [^{3}H]thymidine incorporation assays (Fig. 5). Although retinoic acid alone had only a mild effect on cell proliferation, it strongly enhanced BMP-2–induced DNA synthesis to a level that greatly exceeded the maximum proliferation attained by BMP-2 alone.

Together, these results suggest that BMPs and retinoic acid cooperate in the regulation of mesenchymal differentiation along the adipocyte and osteoblast lineages. Therefore, we treated 3T3-F442A cells for long periods with both ligands in the presence of β-glycerophosphate and ascorbic acid. However, these cells did not show detectable mRNA

Figure 6. **Western blot detection of BMP-RII, RIA, and RIB in stably infected 3T3-F442A cells, and effect of BMP-2 and retinoic acid on [^{3}H]thymidine incorporation.** Il-RIA and Il-RIB cells have increased levels of BMP-RII and BMP-RIA and BMP-RIB, respectively, whereas LL cells are control-infected 3T3-F442A cells. (A) Expression of Flag-tagged BMP-RII and HA-tagged BMP-RIA or BMP-RIB in the retrovirally infected, stable 3T3-F442A cell lines. Receptors were immunoprecipitated with anti-Flag–conjugated beads (for BMP-RII) or anti-HA antibody with protein A–Sepharose beads (for BMP-RIA and BMP-RIB) followed by Western blotting and chemiluminescence detection. (B) LL and Il-RIB cells were treated with BMP-2 at the indicated concentrations in the presence or absence of 10 nM retinoic acid, and then the levels of cellular [^{3}H]thymidine incorporation were determined.
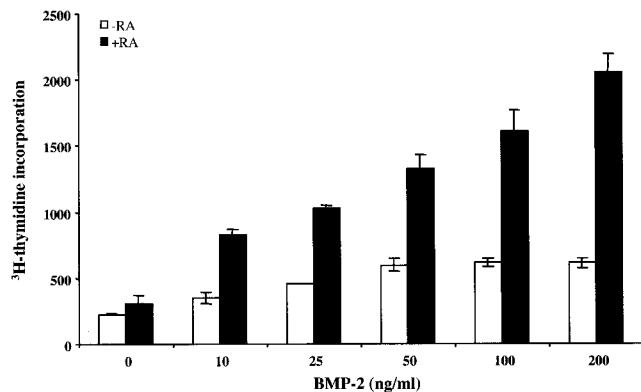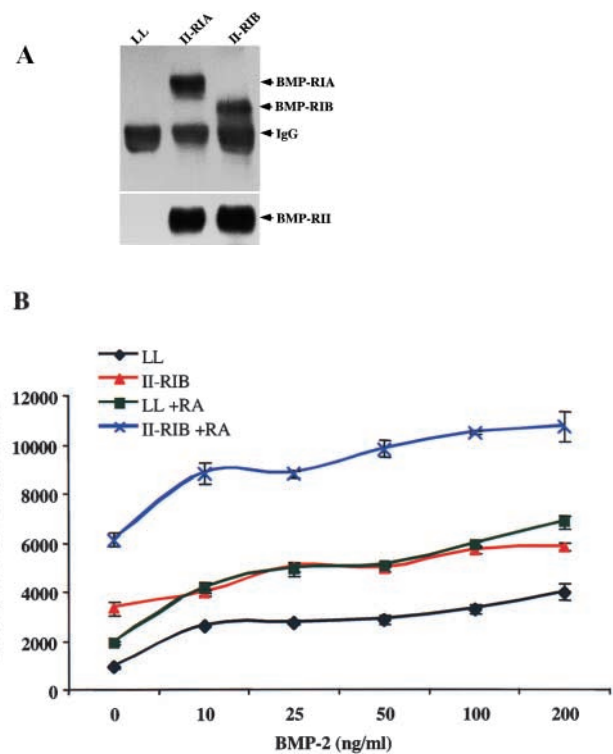

Figure 5. **Synergy of BMP-2 and retinoic acid in the stimulation of cell proliferation.** Cells were treated with BMP-2 at the indicated concentrations in the presence or absence of 10 nM retinoic acid, and then the levels of cellular [^{3}H]thymidine incorporation were determined. RA, retinoic acid.

Figure 7. **Adipocyte differentiation potential of stable BMP receptor–expressing cells.** II-RIA and II-RIB cells have increased levels of BMP-RII and BMP-RIA and BMP-RIB, respectively, whereas LL cells are control-infected 3T3-F442A cells. (A) Oil Red O staining of lipids after 8 d of insulin treatment. LL cells show a high level of adipocyte differentiation, whereas adipocyte differentiation of II-RIA and II-RIB cells is impaired. (B) mRNA expression of PPARγ and adipsin in LL and II-RIB cells, after 8 d under conditions of adipogenic differentiation (+ insulin) or in the presence of fetal bovine serum. Retinoic acid and/or BMP-2 were added during this time, as indicated. Ethidium bromide staining of RNA demonstrates equal loading. RA, retinoic acid.
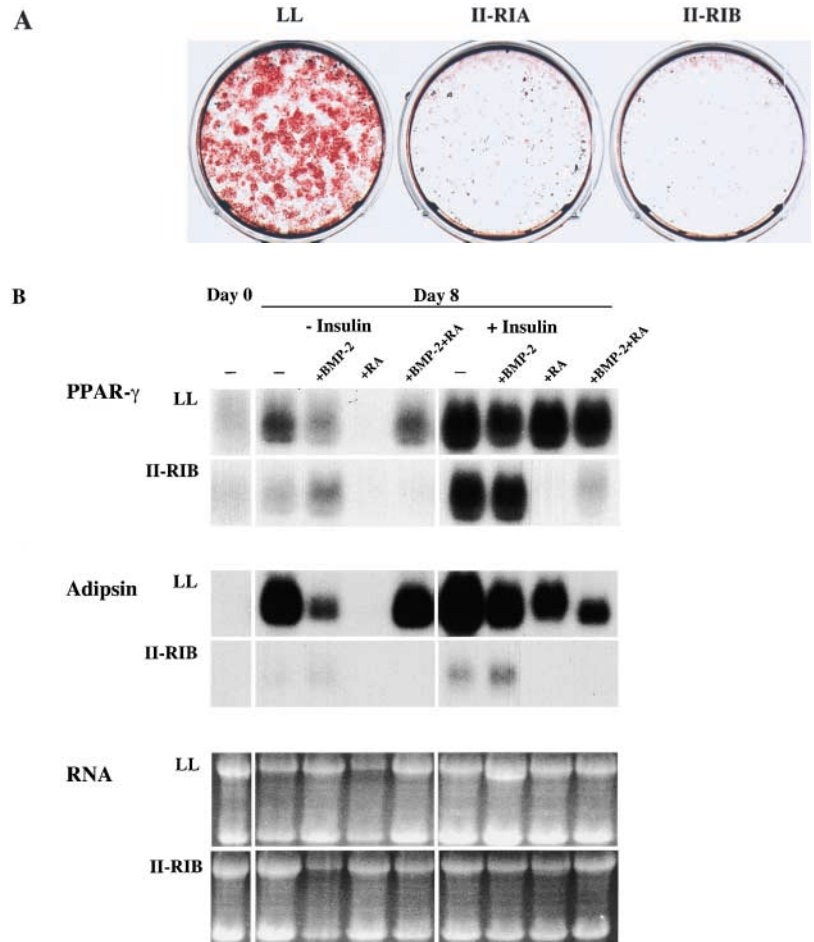
expression of cbfa1 and osteocalcin, and we did not observe mineralization of the ECM (unpublished data).

Enhanced BMP receptor signaling and retinoic acid cooperate to stimulate cell proliferation

The cooperation of BMP-2 and retinoic acid resulted in alkaline phosphatase expression, but not in cbfa1, osteocalcin expression, or deposition of a mineralized matrix. Therefore, we explored the effects of enhanced BMP sensitivity and BMP receptor signaling by increasing the levels of BMP receptors on adipogenic and osteoblastic differentiation. BMP-RIA and BMP-RIB were mutated in their GS domain to confer a low level of constitutive signaling (Hoodless et al., 1996), which could be enhanced by addition of exogenous ligand. Ectopic expression of these basally activated BMP-RIA or -RIB, in the absence of overexpressed BMP-RII, had little effect on differentiation (unpublished data). Because efficient ligand binding requires both ectodomains of BMP-RII and BMP-RIA or BMP-RIB (Liu et al., 1995; Rosenzweig et al., 1995), we generated retrovirally infected cells that expressed both BMP-RII and BMP-RIA or BMP-RIB receptors. These cells, named II-RIA and II-RIB, expressed both Flag-tagged BMP-RII and HA-tagged BMP-RIA or -RIB, with the expected sizes of 80 kD and 60 or 55 kD, respectively (Fig. 6 A). The II-RIA and II-RIB cells, as well as the control cells, infected with empty control vectors (LL cells), were then evaluated for their adipocytic and os-

teoblastic differentiation potential. Because II-RIA cells behaved similarly to II-RIB cells in their differentiation characteristics, we will focus our description on the characterization of the II-RIB cells.

In the absence of added BMP-2, the rate of [³H]thymidine incorporation of II-RIB cells was considerably above that of control LL cells in the presence of saturating levels of BMP-2 (Fig. 6 B). Such enhanced proliferation is consistent with the mitogenic effect of BMP-2 on 3T3-F442A cells (Fig. 2) and the increased sensitivity and receptor signaling by the overexpressed BMP-RII and BMP-RIB receptors (Hoodless et al., 1996). Addition of BMP-2 further enhanced the [³H]thymidine incorporation (Fig. 6 B). Retinoic acid at 10 nM enhanced the proliferation of LL cells and more strongly that of the II-RIB cells (Fig. 6 B), thereby reflecting a cooperativeness between retinoic acid and BMP receptor signaling, as observed in the parental cells (Fig. 5). The resulting proliferation rate of II-RIB cells was much higher than what could be achieved in the parental cells.

BMP signaling and retinoic acid cooperate to inhibit adipocyte differentiation

Increased BMP signaling had a dramatic effect on the adipocyte differentiation of these stable cells. Under adipogenic conditions (i.e., in the presence of insulin), both II-RIA and II-RIB cells displayed less lipid accumulation, as visualized using Oil Red O staining, whereas LL cells had normal dif-
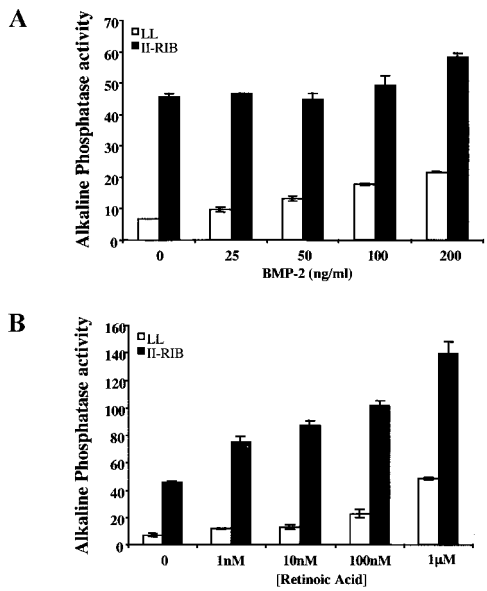
Figure 8. **Effects of BMP-2 and retinoic acid on osteoblastic differentiation of II-RIB and LL cells.** (A) BMP-2 induction of alkaline phosphatase activity in LL and II-RIB cells. (B) Retinoic acid induction of alkaline phosphatase activity in LL and II-RIB cells. In A and B, confluent cells were treated with ligand for 48 h, and alkaline phosphatase activity in the cell lysates was measured. (C) Morphology of LL and II-RIB cells cultured for 8 d in the presence of 10^{-6} M retinoic acid. (D) Effects of BMP-2 and retinoic acid on mRNA expression of the osteoblast differentiation genes, CBFA1, osteocalcin, and collagen type I. Ethidium bromide staining of RNA (bottom) demonstrates equal loading. RA, retinoic acid.
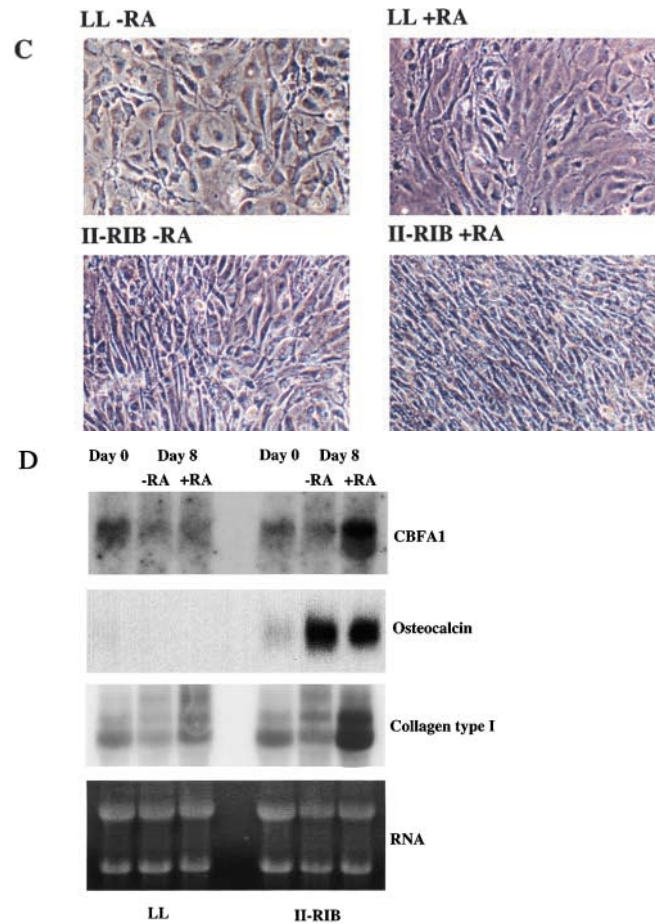
ferentiation characteristics (Fig. 7 A) with increased PPARγ and adipsin mRNA levels (Fig. 7 B, right lanes). The II-RIB cells had only slightly lower PPARγ mRNA expression than LL cells at confluence after 8 d in the presence of insulin, but adipsin mRNA expression was strongly inhibited (Fig. 7 B), demonstrating that BMP signaling primarily blocks the later stages of adipogenesis.

Under these adipogenic conditions, retinoic acid cooperated with the inhibitory effect of increased BMP receptor signaling. In LL cells, retinoic acid did not affect the induction of PPARγ mRNA, but inhibited adipsin mRNA expression (Fig. 7 B). In II-RIB cells, however, both genes were strongly inhibited by retinoic acid (Fig. 7 B), much more so than by combined retinoic acid/BMP-2 treatment of parental cells (Fig. 3 C). Clearly, BMP and retinoic signaling cooperated to inhibit the adipogenic differentiation of II-RIB cells, and the combination of both signaling pathways was required to block induction of PPARγ and adipsin expression.

Under nonadipogenic conditions (i.e., in the absence of insulin), LL cells showed a different interaction between BMP and retinoic acid signaling. Treatment of LL cells with BMP-2 for 8 d at confluence in the presence of serum slightly decreased PPARγ mRNA, and strongly decreased adipsin mRNA expression, whereas retinoic acid blocked the expression of both genes (Fig. 7, left lanes). However, in the presence of BMP-2, retinoic acid slightly enhanced the ex-

pression of PPARγ mRNA, but not adipsin mRNA (Fig. 7, lane 5 vs. lane 3). Under nonadipogenic conditions, retinoic acid, in the absence or presence of BMP-2, blocked PPARγ and adipsin mRNA expression in II-RIB cells. Thus, increased BMP receptor levels profoundly affected the response to BMP-2, with or without retinoic acid, under nonadipogenic conditions.

BMP signaling and retinoic acid cooperate to confer full osteoblastic differentiation

BMP-2 and retinoic acid cooperatively induced the expression of alkaline phosphatase in parental 3T3-F442A preadipocytes (Fig. 4 D). Therefore, we explored this cooperation in osteoblastic differentiation of II-RIA and II-RIB cells. II-RIB cells had a basal level of alkaline phosphatase expression that was much higher than that of parental cells treated with 200 ng/ml BMP-2, i.e., 45.5 ± 1.3 (Fig. 8 A) versus 17.4 ± 0.6 U/mg protein/h (Fig. 4 A). This activity was slightly enhanced by adding exogenous BMP-2, and more strongly enhanced by retinoic acid, with levels reaching 139.52 ± 8.58 U/mg protein/h at 1 μM retinoic acid (Fig. 8 B). These observations reflect the cooperation of BMP-2 and retinoic acid signaling in osteoblastic differentiation.

This cooperation was also apparent in the cell morphology of II-RIB cells (Fig. 8 C). II-RIB cells had an elongated spindle-like morphology when compared with the more extended and flatter morphology of the LL cells. Addition of retinoic

Figure 9. **von Kossa staining of confluent LL, II-RIA, and II-RIB cells and microscopic visualization of the differentiated osteoblast phenotype.** (A) von Kossa staining of LL, II-RIA, and II-RIB cells, cultured in the presence of β-glycerophosphate and ascorbic acid for 25 d, in the absence or presence of 10^{-6} M retinoic acid. (B) Microscopic visualization of LL or II-RIB cells cultured for 25 d in the presence of β-glycerophosphate and ascorbic acid, and 10^{-6} M retinoic acid. RA, retinoic acid.
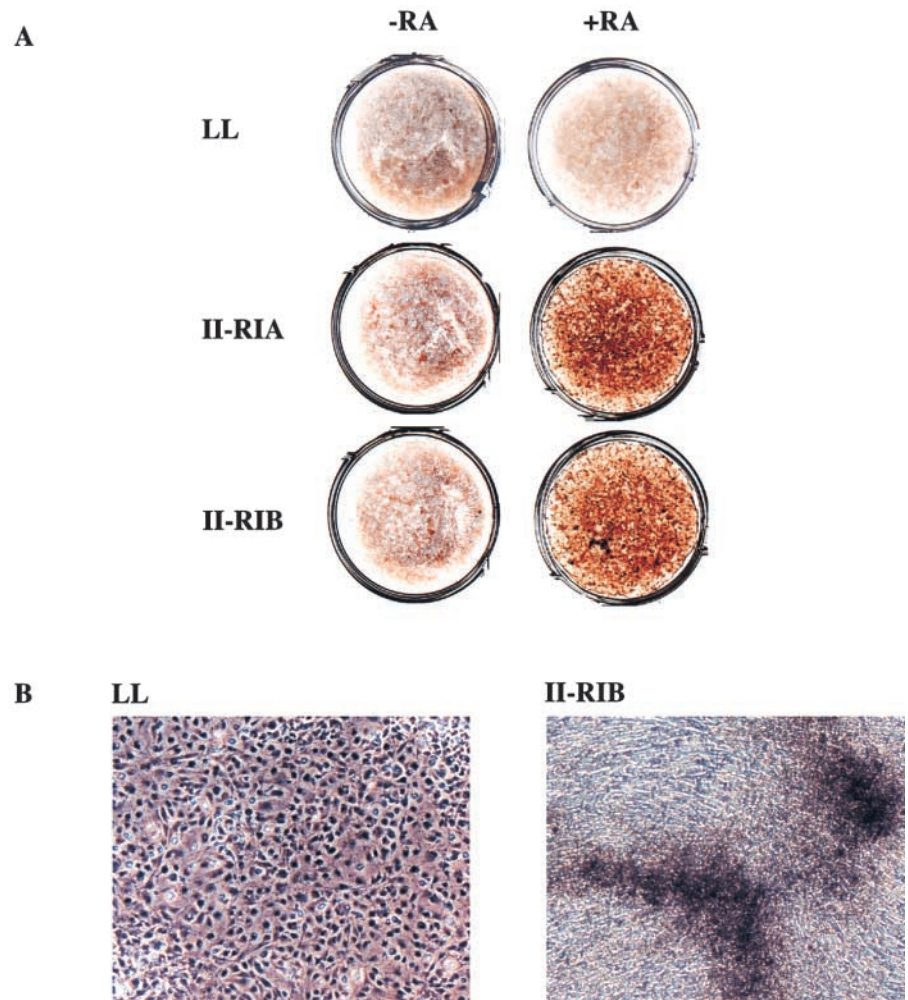
acid induced a mildly elongated phenotype in LL cells, and a dense, elongated phenotype in II-RIB cells, reminiscent of confluent osteoblast cultures, before mineralization.

Osteoblastic maturation is accompanied by induction of expression of CBFA1 and several late marker genes such as osteocalcin and collagen type I. Although osteocalcin is involved in the formation of hydroxyapatite crystals in the mineralization process, it is the extracellular collagen type I matrix that is ultimately mineralized (Wagner and Karsenty, 2001). Thus, we evaluated the expression of these genes in II-RIB cells (Fig. 8 D). Retinoic acid induced the expression of cbfa1 mRNA in II-RIB cells, but not in LL cells. In contrast to LL cells, confluent II-RIB cells expressed osteocalcin mRNA, but appeared to do so without a concomitant increase in cbfa1 mRNA expression. The levels of collagen I mRNA expression were similar in LL and II-RIB cells, but retinoic acid strongly increased its expression in II-RIB cells, and not in control LL cells. Together, these findings demonstrate the expression of osteoblastic marker genes in II-RIB cells, and the cooperation of retinoic acid with BMP receptor signaling in inducing the cbfa1 and collagen I gene expression.

Finally, II-RIA, II-RIB, and control LL cells were examined for their ability to undergo ECM mineralization, when cultured at confluence in the presence of β-glycerophosphate and ascorbic acid. Matrix mineralization was visualized using von Kossa staining. In the absence of retinoic acid, II-RIA and II-RIB cell cultures did not undergo mineralization. In contrast, II-RIA and II-RIB cells both elaborated a high level of ECM mineralization when cultured in the presence of retinoic acid (Fig. 9 A). Microscopic analysis of these retinoic acid–treated cells confirmed the presence of mineralized regions (Fig. 9 B). Control LL cells did not have any matrix mineralization, either in the absence or presence of retinoic acid, which is similar to parental 3T3-F442A cells (unpublished data). These results indicate that increased BMP receptor signaling and retinoic acid cooperate to induce full osteoblastic differentiation of 3T3-F442A preadipocytes.

Discussion

In this paper, we explored whether extracellular signals could redirect cells that were destined to differentiate along the adipocyte lineage, to differentiate into osteoblasts. BMP signaling is relevant for osteoblast differentiation, but in how far BMP signaling can drive transdifferentiation from other mesenchymal cell types to osteoblasts is unclear. C2C12 myoblasts can be induced to express some early osteoblast markers in response to elevated BMP signaling (Katagiri et al., 1994; Aoki et al., 2001); however, this stimulation was inadequate for osteoblast differentiation and mineralization. To explore whether BMP signaling can redirect committed

preadipocytes to fully differentiate into osteoblasts, we studied 3T3-F442A preadipocytes. In contrast to primary cultures from adipose tissue, this cell line has defined characteristics, is homogeneous, and is not known to differentiate into any other cell type. The basal expression of PPARγ (Choy et al., 2000) and the differentiation into fat pads in vivo (Green and Kehinde, 1976; Mandrup et al., 1997) reflect the commitment of 3T3-F442A cells to adipogenic differentiation.

Our results allowed for several major conclusions: (1) 3T3-F442A preadipocytes express the effectors of the BMP receptor–signaling system and respond to BMP-2; (2) BMP receptor signaling cooperates with retinoic acid signaling to repress adipogenesis and promote osteoblastic differentiation; (3) 3T3-F442A preadipocytes can be converted into fully differentiated osteoblasts that are able to undergo calcification in cell culture; and (4) BMP-RIA and BMP-RIB are comparable in their abilities to induce osteoblastic differentiation and repress adipose differentiation.

3T3-F442A cells express BMP signaling mediators and respond to BMP-2

Our data demonstrate that 3T3-F442A cells express the mediators for a functional BMP signaling pathway. We show that BMP-RII, -RIA, and -RIB mRNAs are present in 3T3-F442A cells, as well as Smad1 and Smad5, and the inhibitory Smad6, in addition to Smad4 as reported previously (Choy et al., 2000). Smad5 mRNA levels were enhanced during adipocyte differentiation, whereas Smad1 mRNA levels were unchanged. Smad6 mRNA was down-regulated during differentiation, similarly to the down-regulation of Smad7 (Choy et al., 2000).

The presence of a functional BMP signaling mechanism was also apparent from the responses of 3T3-F442A cells to BMP-2. BMP-2 increased proliferation and induced a change in cell shape and expression of alkaline phosphatase. The mitogenic response and induction of alkaline phosphatase are consistent with the responses of some other mesenchymal cell lines, including preosteoblasts, to BMP-2 or BMP-4 (Chen et al., 1991; Yamaguchi et al., 1991; Lou et al., 1999).

Cooperation of BMP receptor and retinoic acid signaling

To our knowledge, our results provide the first evidence for cooperation of retinoic acid and BMP signaling in cell differentiation, proliferation, and gene expression. BMP-2 and retinoic acid were shown to cooperate to induce apoptosis (Glozak and Rogers, 1996; Rodriguez-Leon et al., 1999). The cooperation of retinoic acid with BMP signaling resulted in inhibition of adipocyte differentiation and induction of osteoblast differentiation, as well as proliferation. This cooperation occurred at a low level of retinoic acid (10 nM), which by itself had only a minimal effect.

The cooperation in cell differentiation may, in part, result directly from the ability of retinoic acid to inhibit adipocyte differentiation because the strong adipogenic tendency of 3T3-F442A cells could prevent their osteoblastic potential from being realized. Indeed, unidentified factors released by adipocytes inhibit osteoblast activity (Maurin et al., 2000), and blocking adipocyte differentiation by retinoic acid treatment may eliminate these inhibitors. However, our results indicate that the activity of retinoic acid to promote BMP-

induced osteoblastic differentiation goes beyond simply inhibiting adipocyte differentiation. For example, BMP signaling and retinoic acid cooperate to induce expression of the cbfa1 and collagen type I genes that are critical for bone mineralization. Because retinoic acid can increase BMP expression (Gazit et al., 1993; Helvering et al., 2000; Paralkar et al., 2002), such cooperation could result from the induction of BMP ligands by retinoic acid. However, in 3T3-F442A cells, we did not see an increase of the low levels of endogenous mRNAs for BMP-2, -4, and -6 in response to retinoic acid (unpublished data), and the strong effects of retinoic acid on gene expression and differentiation reflect a more direct cooperation of these two signaling pathways. In addition to interactions with indirect, BMP-induced transcriptional responses, the activated retinoic acid receptor could also directly interact with BMP-activated Smads to repress or activate transcription of genes involved in cell cycle regulation and osteoblastic or adipocytic differentiation. Although Smads are not known to interact with the retinoic acid receptor, they can associate and cooperate with several nuclear receptors, including the glucocorticoid, androgen, and vitamin D3 receptors (Song et al., 1999; Yanagisawa et al., 1999; Hayes et al., 2001). Perhaps the cooperation of retinoic acid with BMP signaling may provide a mechanism for retinoic acid–induced osteoblast differentiation observed in some preosteoblast cell lines (Choong et al., 1993; Kodama et al., 1997).

Preadipocytes can be converted to fully differentiated osteoblasts

Several mesenchymal cell systems respond to BMPs by inducing genes that are expressed early during osteoblast differentiation, but only preosteoblastic cells can go as far as matrix mineralization (Li et al., 1996; Lecanda et al., 1997). For a cell to convert from one cell type to another, there must be suppression of differentiation of the original cell type with promotion of differentiation to the new type. We now show that enhancement of BMP signaling combined with retinoic acid signaling fully converts 3T3-F442A preadipocytes into osteoblasts, with induction of cbfa1 and osteocalcin expression and ECM mineralization, while blocking adipose differentiation. These results suggests that, under physiological conditions, retinoids could cooperate with increased BMP ligand, BMP receptor, or Smad expression to redirect preadipocytes into the osteoblastic lineage.

In parental 3T3-F442A cells, BMP-2 only weakly inhibited insulin-induced adipogenesis and, in the absence of insulin, slightly promoted adipocyte differentiation. This weak stimulation may be due to endogenous expression of BMP-RIA, which can promote adipogenesis in 2T3 calvarial cells (Chen et al., 1998), and is consistent with reports that BMP-2 sometimes promotes or inhibits adipogenesis (Wang et al., 1993; Gimble et al., 1995). Similar to results in C2C12 myoblasts (Katagiri et al., 1994), BMP-2 induced alkaline phosphatase expression in 3T3-F442A cells, but did not confer a more differentiated osteoblastic phenotype. Furthermore, alkaline phosphatase induction by BMP-2 was not accompanied by a reduction in adipogenesis; instead, cells expressing alkaline phosphate were capable of quantitative adipose conversion. Thus, induction of alka-

line phosphatase is compatible with nonosteoblastic differentiation, in agreement with a previous demonstration of alkaline phosphatase expression in preadipocytes (Dorheim et al., 1993). In fact, insulin even slightly enhanced BMP-2–induced alkaline phosphatase expression as it promoted adipogenesis.

Only at higher BMP receptor levels, in the retrovirally infected cells, did BMP signaling strongly decrease adipocyte differentiation, while promoting osteoblastic differentiation. Full osteoblastic differentiation resulted from combining retinoic acid treatment with high level BMP signaling. Under these conditions, adipocyte differentiation was fully repressed. Interestingly, osteocalcin was expressed in II-RIB cells in the absence of retinoic acid, suggesting that osteocalcin expression is not sufficient for matrix mineralization. Indeed, mineralization was observed only when collagen type I expression was induced.

BMP-RIA and BMP-RIB both inhibit adipocyte and stimulate osteoblast differentiation

Lastly, our results provide evidence that BMP-RIA and BMP-RIB, in combination with BMP-RII, exert similar effects on adipocyte and osteoblast differentiation of 3T3-F442A cells. Our observations seem to contrast with a previous conclusion, obtained using the calvarial 2T3 cell line, that signaling through BMP-RIB promotes osteoblast differentiation, whereas signaling through BMP-RIA promotes adipocyte differentiation (Chen et al., 1998). Because both receptors are expressed in 3T3-F442A cells, it is conceivable that conflicting signals by these two receptors could contribute to the inability of BMP ligand to promote terminal osteoblast differentiation. However, 3T3-F442A cells, stably expressing either activated BMP-RIA or -RIB, along with BMP-RII, showed a similar inhibition of adipocyte differentiation and similarly elevated levels of osteoblast differentiation. They also exhibited similar proliferation rates and alkaline phosphatase expression levels, and similar cooperation with retinoic acid (unpublished data). The comparable activities of BMP-RIA and -RIB are consistent with their abilities to activate Smad1 and 5, and to induce alkaline phosphatase expression in transfected C2C12 cells (Akiyama et al., 1997). The difference in cell line and differentiation conditions, as well as the experimental design, may have led to the observed differences between BMP-RIA and BMP-RIB in 2T3 versus 3T3-F442A cells. Future studies will characterize the molecular mechanisms of the cooperation of retinoic acid with BMP signaling through either BMP receptor system in the inhibition of adipocyte and promotion of osteoblast differentiation.

Materials and methods

Construction of expression plasmids
For retroviral infection, cDNAs encoding human BMP-RII, BMP-RIA, or BMP-RIB were subcloned in retroviral expression plasmids. The coding sequence for NH$_2$ terminally Flag-tagged BMP-RII (a gift from J. Massagué, Memorial Sloan-Kettering Cancer Center, New York, NY) was cloned into LNCX (Miller and Rosman, 1989). The sequences for NH$_2$ terminally HA-tagged BMP-RIA and BMP-RIB with the activating Q233D and Q203D mutations, respectively (Hoodless et al., 1996; gifts from L. Attisano, University of Toronto, Toronto, Canada) were subcloned into LPCX (Choy et al., 2000).

Cell culture and generation of stable cell lines
The murine preadipocyte cell line 3T3-F442A (Green and Kehinde, 1976) was purchased from H. Green (Harvard Medical School, Boston, MA). The cells were maintained at subconfluence in DME with 10% bovine calf serum (HyClone). For adipocyte differentiation, cells were grown to confluence in DME with 10% FBS (Life Technologies) and the culture medium was supplemented with 5 μg/ml insulin (Sigma-Aldrich). For osteoblast differentiation, cells were grown to confluence in DME with 10% FBS. The culture medium was supplemented with 5 mM β-glycerophosphate and 100 μg/ml ascorbic acid (Sigma-Aldrich), to allow osteoblast differentiation. BMP-2 was purchased from R&D Systems, and all trans-retinoic acid was purchased from Sigma-Aldrich.

For generating recombinant expression viruses, we used Phoenix E cells (provided by G. Nolan, Stanford University, Stanford, CA). Cells were maintained, and retroviruses were generated and used to infect 3T3-F442A cells by a spin-infection method, as described previously (Choy et al., 2000). 48 h later, selection was initiated, either with 1 mg/ml G418 (Life Technologies) or 2 μg/ml puromycin (Calbiochem) as appropriate.

von Kossa staining, [³H]thymidine incorporation, and alkaline phosphatase assays
Stably infected, BMP receptor–expressing 3T3-F442A cells were cultured for 25 d in osteoblast differentiation conditions as described above. Mineralization of the ECM was visualized using the histochemical von Kossa staining method (Bhargava et al., 1988).

Rates of DNA synthesis, which correlate with cell proliferation, were determined by [³H]thymidine incorporation assays as described previously (Choy et al., 2000). BMP-2 and/or retinoic acid were added for 24 h before addition of [³H]thymidine (2 Ci/mmol; NEN Life Science Products) which was allowed to incorporate for 3 h, followed by processing of the cell lysates for liquid scintillation counting.

Alkaline phosphatase assays were performed using a Sigma-Aldrich diagnostic kit. Cells were seeded at a density of 50,000 cells per well of a 12-well dish, and allowed to grow to confluence for 2 d. Medium was changed to contain BMP-2 and/or retinoic acid. After 2 d of treatment, cells were lysed with 0.01% SDS in PBS, and the lysates were scraped and collected. 10 μl lysate was added to 100 μl prewarmed PNPP substrate and buffer mixture, and the reaction proceeded at 37°C. The reaction was stopped by adding 1 ml 0.05 N NaOH and the OD was read at 420 nm. Concentration of alkaline phosphatase was determined by comparison to a PNP standard curve. All values were normalized against protein concentration determined using the Bio-Rad Laboratories protein assay.

Northern blot analysis
Total RNA was isolated from 3T3-F442A cells using the SV RNA isolation kit (Promega), whereas poly(A$^+$) mRNA was isolated using the PolyA Tract mRNA isolation kit (Promega). For Northern blot analysis, 1 μg mRNA or 10 μg total RNA was precipitated and separated in a 1% formaldehyde agarose gel and blotted overnight into BioTrans membrane (ICN Biomedicals). ³²P-labeled cDNA probes were prepared using the random priming oligonucleotide method (Feinberg and Vogelstein, 1983) using α-[³²P]dCTP (6,000 Ci/mmol; NEN Life Science Products). Hybridization and washes were performed as described previously (Ausubel et al., 1994). For Northern blots using total RNA, equal loading was verified by ethidium bromide staining of 18 S- and 28 S-RNA bands.

Probes used for Northern blot analysis were as follows: cDNAs for the BMP receptors (BMP-RIA, BMP-RIB, and BMP-RII [GenBank EMBL/DDBJ accession no. NM 004329, NM 007560, and NM 001204, respectively] ten Dijke et al., 1994; Liu et al., 1995) were obtained from J. Massagué and L. Attisano. Cbfa1 (GenBank EMBL/DDBJ accession no. AF010284; Ducy et al., 1997) and osteocalcin (GenBank EMBL/DDBJ accession no. NM 031368; Celeste et al., 1986) cDNAs were gifts from P. Ducy and G. Karsenty (Baylor College of Medicine, Houston, TX). A collagen type I cDNA probe was generated by RT-PCR of 3T3-F442A RNA using the forward primer 5′-ATGTTCAGCTTTGTGGACCTCC-3′ and the reverse primer 5′-CGTATTGTTCCGGGCAGAAAGC-3′ (GenBank EMBL/DDBJ accession no. X54876). cDNAs for PPAR-γ2 (GenBank EMBL/DDBJ accession no. NM 015869; Tontonoz et al., 1994a) and adipsin (GenBank EMBL/DDBJ accession no. NM 013459; Cook et al., 1985) were gifts from B. Spiegelman (Dana Farber Cancer Institute, Harvard Medical School, Boston, MA).

Western blot analysis
To detect Flag-tagged BMP-RII and HA-tagged BMP-RIA or -RIB, stably infected 3T3-F442A cells were grown to confluence in 10-cm-diam dishes. Cells were washed with PBS and lysed in 1 ml lysis buffer containing 25

mM Tris, pH 7.5, 300 mM NaCl, 1% Triton X-100, 20 µg/ml leupeptin, 10 µM PMSF, and 20 µg/ml aprotinin. Flag-tagged BMP-RII was immunoprecipitated using anti-Flag M2 agarose affinity gel (Sigma-Aldrich). HA-tagged BMP-RIA and -RIB were immunoprecipitated using anti-HA.11 antibody (Covance, Inc.). Immunoprecipitates were analyzed by SDS-PAGE and blotted, and the immunoprecipitated receptors were visualized by chemiluminescence detection (Amersham Biosciences).

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