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Repetitive exposure to TGF-β suppresses TGF-β type I receptor expression by differentiated osteoblasts ☆

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Abstract

Transforming growth factor-β (TGF-β) has potent, cell phenotype restricted effects. In bone, it controls multiple activities by osteoblasts through three predominant receptors. Of these, the relative amounts of TGF-β receptor I (TβRI) vary directly with TGF-β sensitivity. The rat TβRI gene promoter includes *cis*-acting elements for transcription factor Runx2. Here we show conservation and selective partitioning of TβRI and retention of TGF-β activity with osteoblast differentiation, Runx2 binding to the TβRI gene promoter on osteoblast chromatin, and decreased promoter activity by Runx2 binding site mutation. Furthermore, in contrast to the stimulatory effects induced by single or limited exposure to TGF-β, we found that osteoblasts became resistant to TGF-β after high dose and repetitive treatment. TβRI protein, mRNA, and gene promoter activity all decreased after three daily TGF-β treatments, in parallel with a reduction in Runx2 protein and Runx dependent gene expression. In this way, sustained TGF-β exposure can limit its own effectiveness by suppressing the expression of its primary signaling receptor. This tightly controlled system may constitute a feedback loop to protect against TGF-β excess, and impose important limitations that are required for the progression of events during skeletal growth, remodeling and repair.

Keywords: Bone; Runx; Gene promoter; Transcription

1. Introduction

Virtually every tissue in the body responds throughout life to systemic and local growth factors that regulate development, healing, or disease. Among these, transforming growth factor β (TGF-β) is an abundant and potent regulator of skeletal cell activity *in vivo* and *in vitro*. In combination with other growth factors, TGF-β positively and negatively affects osteoblast proliferation and differentiation based on its concentration, duration of treatment, and current state of cell differentiation (Centrella et al., 1987, 1994b, 1995).

Many TGF-β sensitive cells express three conventional cell surface TGF-β receptors, designated here as TβRI, TβRII, and TβRIII. Often, TβRI and TβRII together are necessary for TGF-β activity. However, cells that express constitutively active TβRI are ligand independent (Wrana, 1998), consistent with its predominant

Abbreviations: BMP-2, bone morphogenetic protein 2; ChIP, chromatin immunoprecipitation; DNA, deoxyribonucleic acid; EMSA, electrophoretic mobility shift analysis; Ig, immunoglobulin; kDa, kilodalton; nctd, nucleotide; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; RNase, ribonuclease; rRNA, ribosomal RNA; SDS, sodium dodecyl sulfate; TβR, TGF-β receptor; TGF-β, transforming growth factor β.

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role in intracellular signal transduction. Variations in the amount of TβRI relative to TβRII and TβRIII correlate with osteoblast differentiation and occur in response to other growth regulators (Centrella et al., 1988, 1991, 1995; Chang et al., 1998). Moreover, in some cells including osteoblasts, high levels of TβRIII reduce their sensitivity to TGF-β treatment (Ji et al., 1999; Eickelberg et al., 2002). Thus, the relative expression of individual TβRs may restrict the distribution of TGF-β among distinct receptors or receptor complexes, and perhaps determine its overall or specific effects.

Previous studies showed that, in osteoblasts, TβRI mRNA and protein have relatively short half-lives compared to TβRII and TβRIII (Centrella et al., 1995, 1996b). To understand the molecular aspects of TβRI gene expression, its promoter was cloned from a rat genomic library (Ji et al., 1996, 1997), and initial results identified specific *cis*- and *trans*-acting elements that correlate with osteoblast differentiation and with increases and decreases in the essential osteoblast transcription factor Runx2 (Ji et al., 1996, 1998, 2001; Chang et al., 1998). Other studies revealed increases in Runx2 in response to bone morphogenetic protein 2 (BMP-2), and either increases, decreases, or no change in Runx2 in response to TGF-β (Ducy et al., 1997; Alliston et al., 2001; Spinella-Jaegle et al., 2001; Viereck et al., 2002; Miura et al., 2004; Takagi et al., 2004).

Even though TGF-β potently enhances osteoblast proliferation and matrix synthesis *in vitro* and *in vivo* (Centrella et al., 1994b), transgenic overexpression of TGF-β2 or dominant negative TβRII, under control of the osteocalcin gene promoter, disrupt mineral deposition and other aspects of bone remodeling in mice and suggest negative effects by TGF-β on bone integrity (Erlebacher and Derynck, 1996; Filvaroff et al., 1999). Although these results may partly reflect inappropriate temporal or spatial expression of these gene products by highly differentiated osteocalcin-expressing osteoblasts (Lian and Stein, 1995), they predict that persistent TGF-β signaling deregulates osteogenesis. In addition, TGF-β typically increases new TGF-β gene expression in many cells including osteoblasts (Bascom et al., 1989; Kim et al., 1990; Dallas et al., 1994). Therefore, high levels of TGF-β, left unchecked, could have cumulative, possibly detrimental, effects on skeletal tissue.

Based on these observations, we examined if the events they define might functionally intersect in osteoblasts. We asked if discrete retention of TβRI found on clonal highly differentiated osteoblasts also occurs during endogenous osteoblast differentiation *in vitro*, and if Runx2 directly controls endogenous TβRI gene expression. Finally, we assessed persistent exposure to TGF-β on TβRI, Runx2, and TGF-β activity to determine if they converge, either to reactivate or to limit osteoblast activity, and thereby control skeletal tissue homeostasis when TGF-β levels are present in excess.

2. Materials and methods

2.1. Cells

Primary osteoblast-enriched cultures were prepared from parietal bones of 22 day old Sprague–Dawley rat fetuses (Charles River Breeding Laboratories) by methods approved by the Yale Institutional Animal Care and Use Committee. Bone sutures were dissected and cells were released by five sequential collagenase digestions. Cells pooled from the last three digestions express biochemical features that typify differentiating osteoblasts, including a high level of nuclear factor Runx2, parathyroid hormone receptor, type I collagen synthesis, and alkaline phosphatase activity. They also exhibit an increase in osteocalcin expression in response to vitamin D_3, differential sensitivity to TGF-β, BMP-2, and various prostaglandins, and form mineralized nodules under conditions that promote long term differentiation *in vitro*. Cells were plated at 4000/cm^2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 μg/ml ascorbic acid. After reaching confluence, cells were re-fed twice weekly with medium in which serum and ascorbic acid were reduced by 50%. Growth factor treatments were performed in serum-free medium (Centrella et al., 1987, 1994a, 1995, 1996a; McCarthy et al., 1988; Ji et al., 1997, 1998; Carpenter et al., 1998).

2.2. Western immunoblots

Cell or nuclear extracts were fractionated on polyacrylamide-SDS gel and electroblotted onto PVDF membranes (NEN Life Sciences) along with pre-stained molecular weight markers. Blots were blocked in 5% fat-free powdered milk, probed with specific primary antibodies (Clontech/BD Bioscience), and reactive bands were visualized with secondary antibody linked to horseradish peroxidase and chemiluminescence (Western Lightning, PerkinElmer Life Sciences). Primary anti-Runx2 (OSI, Inc.), anti-Sp1 and anti-TβRI (Santa Cruz Biotechnology, Inc.) antibodies were used at 1:300 dilution, and goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Inc.) was used at a 1:2500 dilution (Chang et al., 1998).

2.3. mRNA analysis

Total RNA was extracted with acid-guanidine-monothiocyanate, precipitated with isopropyl alcohol, and dissolved in sterile water. Runx2 mRNA was assessed by fractionation on a 1.5% agarose/2.2 M formaldehyde gel, blotting on charged nylon, and hybridization with ^{32}P-labeled Runx2 cDNA encompassing 2.25-kb restriction fragment of the murine Runx2 (previously termed PEBP2αA1) gene. rRNA was assessed by ethidium staining of a parallel sample. TβRI mRNA was assessed by ribonuclease protection assay with ^{32}P-labeled antisense probes for TβRI (275 nucleotides) by comparison to 18S rRNA (80 nucleotides), collection with isopropyl alcohol, and fractionation on a 5% denaturing polyacrylamide gel. Radiolabeled products were visualized by autoradiography (Centrella et al., 1995; Chang et al., 1998).

2.4. Alkaline phosphatase

Cell layers were lysed by freeze thawing and extracted with 0.5% Triton X-100. Enzyme activity was assessed in cell extracts by hydrolysis of *p*-nitrophenol phosphate, measured at 410 nm after 30 min of incubation at 37 °C. Data are expressed as pmol of *p*-nitrophenol released per minute per μg protein, as determined by dye binding (Centrella et al., 1995).

2.5. Osteocalcin assay

Aliquots of 100 μl of culture medium were combined with radioiodinated rat osteocalcin and measured in triplicate by radioimmunoassay, using species specific goat anti-rat osteocalcin and rat osteocalcin standards (Carpenter et al., 1998).

2.6. Protein synthesis

Cells were incubated with 5 μCi/ml [^{3}H-2,3]proline (2.5 Ci/mmol) for the last 2 h of culture. Cell layers were lysed by freeze thawing and extracted with 0.5% Triton X-100. Samples were precipitated with cold 10% trichloroacetic acid, and acid insoluble material was collected by centrifugation. Precipitates were extracted with acetone, dried, and rehydrated. Total protein synthesis was measured in the precipitates, and [^{3}H-2,3] proline incorporated into collagenase digestible protein (collagen) or noncollagen protein was determined with bacterial collagenase free of nonspecific protease activity (Centrella et al., 1987, 1995).

2.7. TGF-β binding

Cells were incubated with 150 pM of ^{125}I-TGF-β1 (4000 Ci/mmol) for 3 h at 4 °C in serum-free medium containing 4 mg/ml bovine serum albumin. Unbound radioligand was removed by multiple washings, and bound TGF-β was covalently crosslinked with 0.2 mM disuccinimidyl suberate (Pierce). Cells were extracted, equal amounts of cell extracts were collected with specific TβR antiserum, immunoprecipitates were fractionated on a denaturing 4–10% gradient polyacrylamide gel, and TβR patterns were visualized by autoradiography (Centrella et al., 1995).

2.8. Transfections

TGF-β dependent gene expression was assessed with SBE4 reporter plasmid driven by four Smad response elements (Zawel et al., 1998; McCarthy et al., 2003), and TβRI gene promoter activity was assessed with reporter plasmid driven by a 0.54 kbase fragment of the rat TβRI gene promoter, each cloned upstream of firefly luciferase cDNA (Ji et al., 1998, 2001). Specific Runx response elements in the TβRI gene promoter activity were assessed by PCR generated mutations (in bold) in Runx binding site PS2 at −540 to −546 bp (5′-*AACCGCG*-3′ to 5′-*AACC***T***CG*-3′), and Runx binding site PS3 at −307 to −313 bp (5′-*AGCCACA*-3′ to 5′-*AGCC***T***CA*-3′), or by restriction site truncation. Runx dependent gene expression was examined on native TβRI promoter, and on reporter plasmid pRCP/L driven by Runx and C/EBP response elements inserted upstream of a minimal TATA box and firefly luciferase cDNA. The upstream insert only binds Runx2 in the absence of C/EBP activation and nuclear translocation (McCarthy et al., 2000). Promoter–reporter plasmid constructs and empty parental vectors were pre-titrated for optimal expression efficiency and transfected with reagent TransIT LT1 (Mirus). Cells at 50–70% culture confluence (25,000–30,000 per cm^2) were exposed to 50 ng per cm^2 of plasmid DNA for 16 h in medium containing 0.8% fetal bovine serum, and then supplemented to achieve 5% serum. Cells were cultured and then treated as indicated in each figure in serum-free medium, rinsed, lysed, and nuclear-free supernatants were analyzed for reporter gene activity and corrected for protein content. Transfection efficiency was assessed in parallel with positive and negative reporter plasmids (Ji et al., 1996, 1998).

2.9. Electrophoretic mobility shift analysis (EMSA)

Double strand oligonucleotide probe [5′-AAGGAAGTT-G*AACCGCG*GGATTGGACCG-3′] containing the Runx binding sequence (italics) from region PS2 (Ji et al., 1998) in the rat TβRI gene promoter was labeled with [^{32}P]dCTP and Klenow fragment of *E. coli* DNA polymerase I, and gel purified. Nuclear protein extract (5 μg) was preincubated with no addition, nonimmune IgG, or rabbit anti-Runx2 antibody (Oncogene Research Products, Cambridge, MA), and then supplemented with [^{32}P]-labeled probe. Specificity was determined with unlabeled oligonucleotide PS2, with PS2 with a mutated Runx binding site (PS2μ [5′-AAGGAAGTT-G*AACC***T***CG*GGATTGGACCG-3′]), with oligonucleotide containing an alternate consensus Runx binding sequence [5′-CGTATT*AACCACA*ATACTCG-3′] (Ji et al., 1998), or an Sp1 binding sequence [5′-GTACATTCGATC*GGGCGGGG*CGA-GCGATC-3′] (Ji et al., 1997), and then combined with osteoblast nuclear extract. Protein bound DNA complexes were resolved on a 5% nondenaturing polyacrylamide gel and examined by autoradiography (Chang et al., 1998; Ji et al., 1998).

2.10. Chromatin immunoprecipitation

Cells were formaldehyde crosslinked, collected by scraping, and detergent lysed, and chromatin was fragmented by sonication to approximately 700 bp. Samples were diluted and pre-cleared with Protein A Sepharose, incubated with nonimmune IgG or anti-Runx2 antibody, and collected with Protein A Sepharose. They were digested sequentially with RNase A and proteinase K, incubated overnight at 68 °C to reverse crosslinking, extracted with phenol and chloroform, and precipitated with ethanol. Equal aliquots of extracted DNA were amplified by PCR with primers corresponding to the sequences at nucleotides −640 to −617, and −405 to −378 that encompass Runx response element PS2 at nucleotides −546 to −540 in the rat TβRI gene promoter (GenBank accession # U48401) (Ji et al., 1997; Im et al., 2004). Specific PCR product of 262 bp was visualized by ethidium staining.

2.11. Statistical analysis

Statistical differences in biochemical assays were assessed by one-way analysis of variance and Student–Newman–Keuls *post hoc* analysis, using SigmaStat software (Jandel Corporation) from six or more replicate samples and two or more different cell preparations. A significant difference was assumed by a P value

of <0.05. Protein and mRNA levels assessed by visual methods represent analogous findings from two or more studies, and when expressed graphically, represent results from three or more studies.

3. Results

3.1. TβRs and TGF-β activity during osteoblast differentiation

Several lines of evidence show that TβRs are differentially regulated on osteoblasts. For example, TβRI preferentially persists relative to TβRII and TβRIII on clonal highly differentiated ROS 17/2.8 rat osteosarcoma derived osteoblasts, or when primary osteoblast cultures are induced with BMP-2 (Centrella et al., 1995, 1997). Through endogenous regulatory events that occur during long term culture, primary osteoblasts express several markers associated with differentiation (Centrella et al., 1996a; Ji et al., 1997; Lian et al., 2003). In parallel with the high levels of nuclear transcription factor Runx2 protein and mRNA (Fig. 1A), alkaline phosphatase enzyme activity, immunoreactive osteocalcin, and new collagen synthesis (Fig. 1B) that mark this progression *in vitro*, more differentiated osteoblasts also exhibited a transition in cell surface TβRs (Fig. 1C). In the younger, confluent osteoblast cultures, all three TβRs were evident by radioligand binding. At this point, TβRI and TβRIII each associated with TβRII by co-collection with antibody specific for the various TβRs (lanes 1–3).

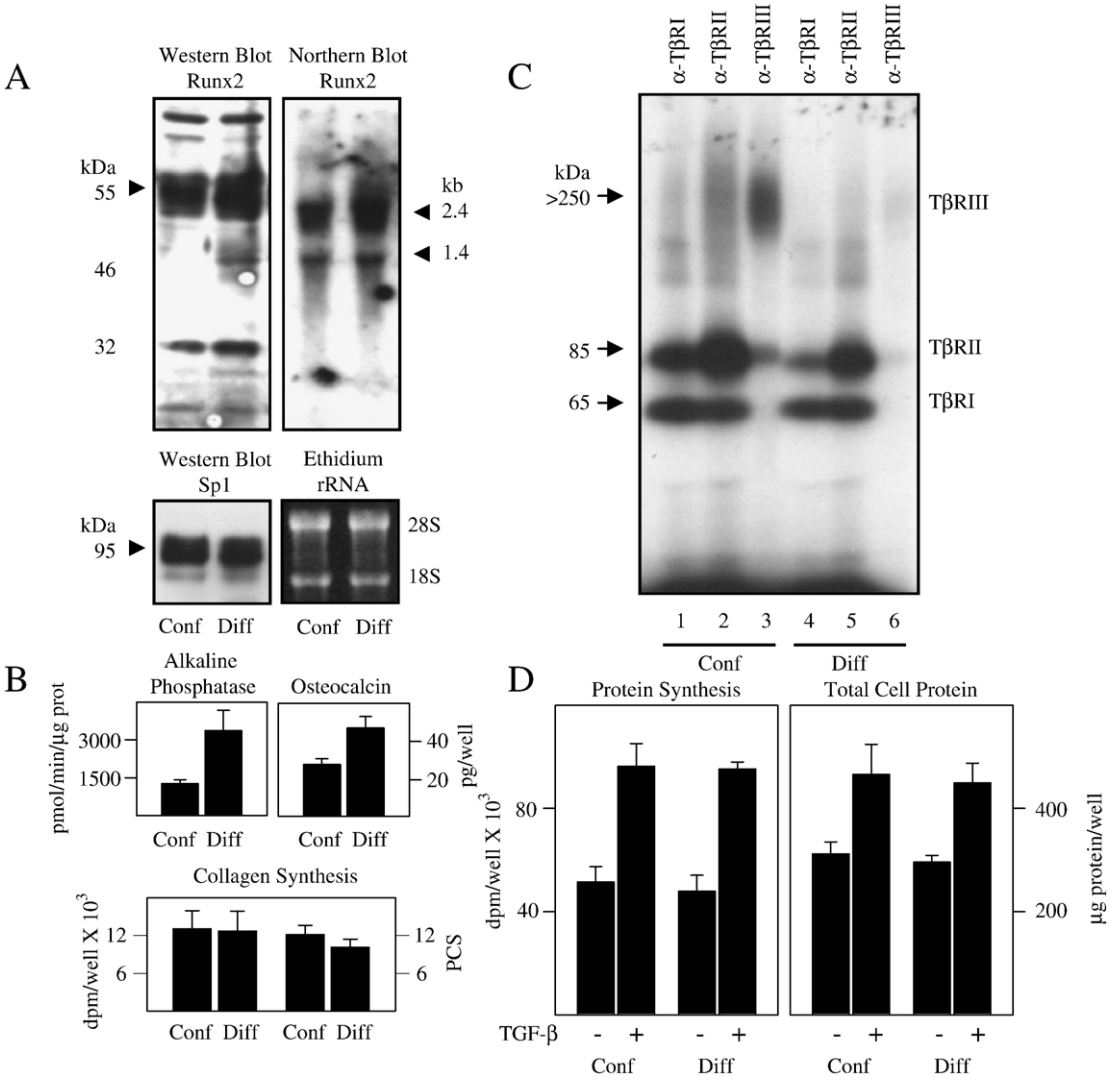

Fig. 1. Osteoblast differentiation, TβR levels, and TGF-β activity. Primary fetal rat osteoblast cultures were maintained for 8–10 days to achieve confluence (Conf) or incubated an additional three weeks to promote further differentiation (Diff). In (A), nuclei were extracted and Runx2 and Sp1 were assessed by Western blots with specific antibodies (left panels), or total RNA was extracted to assess Runx2 mRNA by Northern blot, or rRNA by ethidium staining (right panels). In (B), alkaline phosphatase activity was measured in cell lysates by substrate conversion and spectrophotometry, and osteocalcin was measured in the culture medium by radioimmunoassay (upper panels). The rates of collagen synthesis and percent collagen synthesis (PCS) were measured by pulse-labeling with [^{3}H]proline during the last 2 h of culture, and differential sensitivity to purified bacterial collagenase (lower panel). Alkaline phosphatase activity and osteocalcin expression were significantly greater in differentiated cell cultures ($P<0.05$). In (C), cell surface TβRs were examined by binding and crosslinking to ^{125}I-TGF-β1, extraction, and collection with antibody specific to the individual TβRs. In (D), cells were treated with vehicle (−) or 120 pM TGF-β1 (+) for 24 h, and the rate of protein synthesis was measured by pulse-labeling with [^{3}H]proline during the last 24 h of culture and acid precipitation (left panel), or total protein was measured by dye binding (right panel). TGF-β treatment significantly increased protein synthesis rates and total protein ($P<0.05$).

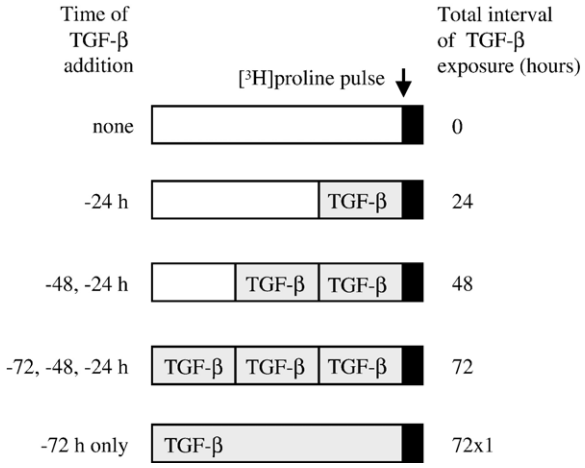

Fig. 2. TGF-β treatment protocol. Confluent fetal rat osteoblasts were exposed to serum-free medium supplemented with vehicle (none) or TGF-β1 at 24 h intervals for a total of 24, 48 or 72 h, as indicated, or for a total of 72 h after one treatment at the beginning of the experiment (72 × 1). Clear boxes indicate interval without TGF-β1 exposure, and filled boxes indicate interval of TGF-β1 exposure. All cells that were not treated with TGF-β1 were supplemented with vehicle at the same time points. Black boxes indicate the last 2 h of incubation when cells were labeled with [^{3}H]proline to compare the cumulative effects of the various TGF-β treatment intervals on the rate of protein synthesis at the same time.

After three weeks of differentiation, osteoblasts still exhibited cell surface TβRI and TβRII (lanes 4, 5), but little TβRIII (lane 6). A fraction of TβRI remained associated with TβRII when the proteins were collected with anti-TβRII antibody (lane 5). However, by comparison to complexes collected with anti-TβRI antibody from confluent cultures (lane 1), 50–60% less TβRII was recovered in complex with TβRI from more differentiated osteoblasts (lane 4), suggesting that some portion of TβRI partitioned independently of TβRII after TGF-β binding. This could result from a decrease in total cell surface TβRII or TβRIII, which both appear to decline during osteoblast differentiation (Centrella et al., 1995; Ji et al., 1999; Chang et al., 2002), or from other, presently unknown, features of TβRI or TβRII. Therefore, TβRI persisted on more differentiated osteoblasts independent of TβRIII expression and in part independent of complex formation with TβRII. Treatment with TGF-β at 120 pM, which maximally stimulates protein synthesis in these cells (Centrella et al., 1995, 1997), was similarly active in both confluent and more differentiated osteoblast cultures (Fig. 1D). Consequently, the relative independence of TβRI found in more differentiated osteoblasts does not prevent TGF-β dependent activation of these cells. These findings recall the selective effects of BMP-2 on the differential expression or retention of TβRI, TβRII, and TβRIII on osteoblasts, and the persistent stimulatory effect by TGF-β on protein synthesis (Centrella et al., 1995, 1997).

3.2. Repeated exposure to TGF-β suppresses TGF-β activity by osteoblasts

Our earlier studies showed that a single exposure to TGF-β dose- and time-dependently enhances new protein synthesis by confluent osteoblasts, which accrues for at least 72 h (Centrella

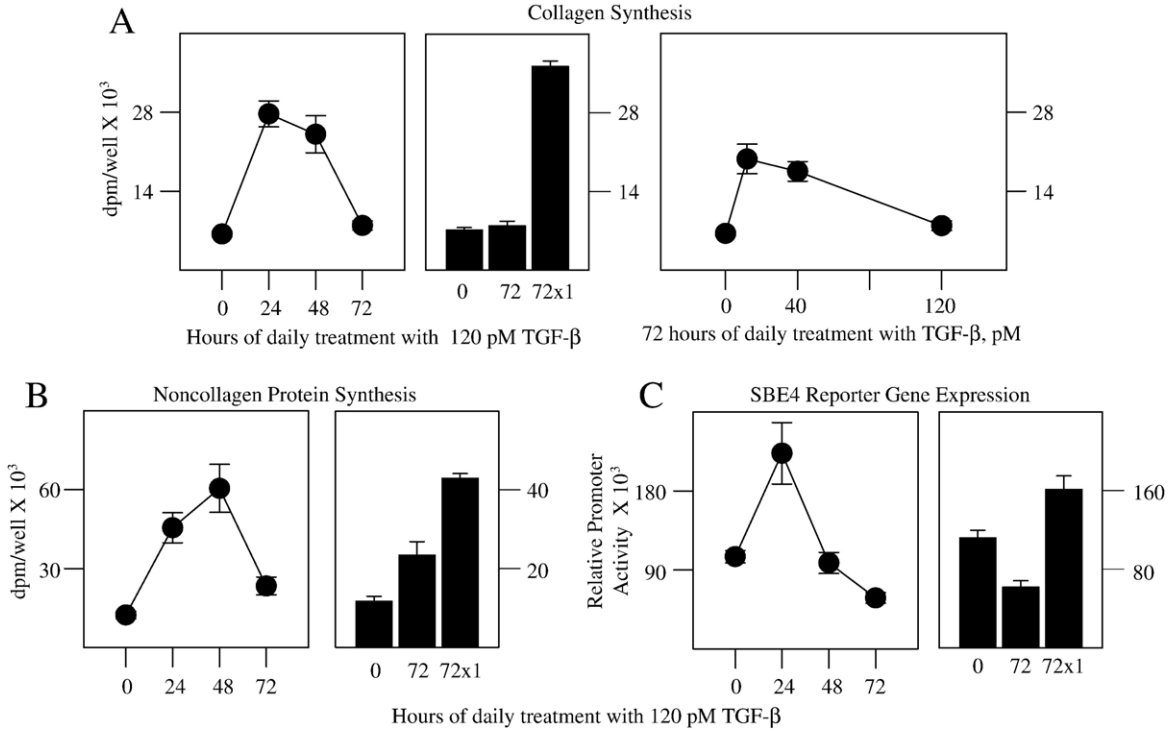

Fig. 3. Desensitization by repetitive TGF-β treatment. Confluent fetal rat osteoblasts were treated with vehicle (0) or TGF-β1 as indicated in Fig. 2. In (A) and (B), collagen and noncollagen protein synthesis were measured by pulse-labeling with [^{3}H]proline during the last 2 h of culture, and differential sensitivity to purified bacterial collagenase. In (C), Smad dependent gene activation was measured by relative expression of the reporter gene luciferase in cells transfected with reporter plasmid SBE4, driven by four Smad binding elements. TGF-β significantly increased collagen and noncollagen protein synthesis after one treatment for a total of 24 or 72 h, or after two daily treatments for a total of 48 h, and significantly increased Smad dependent gene activation after one treatment for a total of 24 or 72 h ($P<0.05$).

et al., 1992; Jay et al., 1996). Consequently, biochemical events initiated by a single TGF-β treatment can have a protracted stimulatory effect on this important aspect of bone growth and repair. Although cell surface TβR levels down regulate soon after TGF-β treatment, they recur within 24 h (Centrella et al., 1996b) and could thereby re-sensitize osteoblasts to additional ligand engagement. Therefore, the benefit of one exposure to TGF-β might be augmented by recurrent treatment. To address this, cells were re-fed serum-free medium to minimize serum derived growth factor effects and supplemented with TGF-β at 24 hour intervals for a total of 24, 48 or 72 h, as indicated in Fig. 2. Surprisingly, in contrast to the progressive time dependent increase in collagen and noncollagen protein synthesis that occurs over 72 h in response to a single TGF-β exposure at the beginning of culture as previously reported (Centrella et al., 1992; Jay et al., 1996), the stimulatory effect of two repeated treatments over 48 h was limited to that by one 24 hour exposure. After three repeated treatments over 72 h, the effect of TGF-β was restrained even further, to nearly the level in untreated cells (left panels, Fig. 3A,B). This attenuated effect by three repeated TGF-β treatments was dose-related and more pronounced at higher TGF-β concentrations (right panel, Fig. 3A). Also, the stimulatory effect of TGF-β on gene expression through the Smad dependent reporter plasmid SBE4 was severely limited after repeated exposure. In this case, Smad dependent gene expression was refractory after two TGF-β treatments and suppressed after three treatments, perhaps from differences between this and other TGF-β sensitive signaling and effector systems (left panel, Fig. 3C). In all cases, however, when TGF-β was added only once at the beginning of the total 72 hour interval (designated as 72×1), its stimulatory effect was retained (middle panel Fig. 3A, and right panels Fig. 3B,C). Since protein synthesis rates never fell significantly below basal levels, which were equivalent at 0, 24, 48, or 72 h in untreated cells ((Centrella et al., 1992; Jay et al., 1996) and our current results), repetitive exposure to TGF-β had no general deleterious effect on osteoblast activity. These results also could not be explained by a total lack of TβR recycling (Centrella et al., 1996b; Di Guglielmo et al., 2003), inasmuch as repetitive TGF-β exposure, and therefore TβR reactivation, was required for their expression.

3.3. Repeated exposure to TGF-β reduces new TβRI expression

Differential retention of TβRI in combination with its short protein and mRNA half-lives ((Centrella et al., 1995, 1996b) and Fig. 1C), its rapid loss in response to glucocorticoid (Centrella et al., 1991, 1997; Chang et al., 1998), and its essential role in signal transduction (Wrana et al., 1992; Wrana, 1998), predicts that a variation in its expression might account in part for changes in TGF-β sensitivity after repeated ligand exposure. Indeed, repeated treatment with TGF-β caused a significant reduction in total 53 kDa TβRI protein and an increase in lower molecular mass protein bands reactive with anti-TβRI antibody. Similarly, the amount of cell surface TβRI (which migrates at 65 kDa in complex with TGF-β monomers on reducing polyacrylamide gels) was also strongly decreased (Fig. 4A). After two and three daily TGF-β treatments, TβRI levels were decreased by 50% (right panel, Fig. 4A). The level of TβRI mRNA transiently increased after 24 h of TGF-β treatment, but also decreased by about 50% after three repeated treatments (Fig. 4B). Accordingly, three repeated TGF-β treatments significantly suppressed TβRI gene promoter activity, whereas a single initial treatment had no effect (Fig. 4C). Therefore, repeated exposure to TGF-β engendered a lower level of TβRI, which appears to result from continued TβRI turnover and less new TβRI gene expression.

3.4. Runx2 regulates the TβRI gene promoter in osteoblasts

The 3′ 0.7 kb region of the rat TβRI gene promoter contains multiple *cis*-acting regulatory elements (Ji et al., 1997, 1998), including a large CpG island and multiple Sp1 binding sites consistent with basal constitutive TβRI expression by many cells (Ji et al., 1997), and four nuclear factor Runx2 binding sites consistent with persistent Runx2 and TβRI expression during osteoblast differentiation ((Ji et al., 1998) and Fig. 1A). Osteoblast

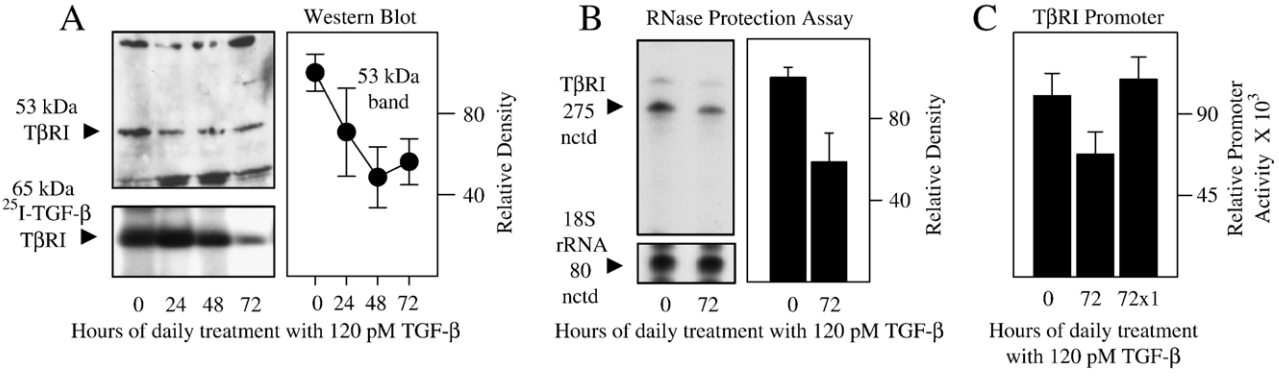

Fig. 4. Decreased TβRI expression after repeated TGF-β treatment. Confluent fetal rat osteoblasts were treated with vehicle (0) or TGF-β1 as indicated in Fig. 2. In (A), cells were extracted and intact TβRI at 53 kDa was assessed by polyacrylamide gel analysis and Western blot with specific antibody. Cell surface TβRI, which migrates at 65 kDa in complex with ^{125}I-TGF-β on reducing SDS PAGE, was assessed by radioligand binding, crosslinking, and autoradiography. Immunoreactive TβRI was measured by densitometry relative to untreated cells. In (B), total RNA was assessed by RNase protection with a ^{32}P-labeled probe of 275 nucleotides (nctd) specific for rat TβRI and a ^{32}P-labeled probe of 80 nctd specific for 18S rRNA. The amounts of TβRI mRNA were measured by densitometry relative to 18S rRNA. In (C), TβRI gene promoter activity was measured by relative expression of the reporter gene luciferase in cells transfected with reporter plasmid driven by a 0.54 kbase 3′ fragment of the rat TβRI gene promoter. Repetitive treatments with TGF-β significantly suppressed TβRI protein, mRNA, and gene promoter activity ($P<0.05$).

nuclear protein specifically associated *in vitro* with radiolabeled oligonucleotide encoding a high affinity Runx2 binding site termed PS2 from the TβRI promoter. Radiolabeled complex formation was specifically disrupted by anti-Runx2 antibody, by unlabeled PS2 or a separate consensus Runx2 binding sequence, and less so by unlabeled probe containing a point mutation in the Runx binding site or by an Sp1 specific probe ((Chang et al., 1998; Ji et al., 1998) and right panel Fig. 5A). Accordingly, nuclear Runx2 formed an endogenous physical complex with osteoblast chromatin specified by this region of the TβRI promoter in osteoblasts, as detected by protein to DNA crosslinking, collection with anti-Runx2 antibody, and sequence specific PCR analysis. No complex formation occurred with nonimmune IgG or nonspecific primers (Fig. 5B). We earlier showed that the Runx binding sites PS1, PS3, and PS4 (depicted in Fig. 5D) also bind osteoblast derived Runx2 *in vitro* (Ji et al., 1998). Of these, we also found Runx2 binding to PS3 by chromatin binding analysis, but have not yet resolved PS4 or PS1 in this way. When PS2 and PS3 were mutated within this highly active TβRI promoter fragment, or when the TβRI promoter sequence was truncated to eliminate PS2, promoter dependent reporter gene expression was decreased by 50% (Fig. 5C). The remaining level of gene promoter activity may derive from essential Sp1 binding sequences within the basal TβRI gene promoter, or perhaps from the remaining Runx binding sequences PS4 and PS1. These finding are consistent with previous studies showing that increases in Runx2 expression in Runx deficient cells, and decreases in Runx2 in osteoblasts, correlate directly with TβRI gene expression (Ji et al., 1996, 1998, 2001). These new results from chromatin binding and site-directed mutations authenticate a definitive rather than a correlative role for Runx2 in TβRI expression, by which it can control the effectiveness of TGF-β on osteoblast activity.

3.5. Repeated exposure to TGF-β suppresses nuclear Runx2 levels

TGF-β exposure decreased full length 55 kDa Runx2 in osteoblasts, and increased lower molecular mass immunoreactive protein bands (Fig. 6A, left panel). After three repeated treatments, 55 kDa Runx2 was reduced by 50–60% (Fig. 6A, right panel). Repeated TGF-β treatment also decreased the amount of functional Runx2 binding to DNA (Fig. 6B). Lower amounts of Runx2 occur in less osteoblast-like periosteal cells

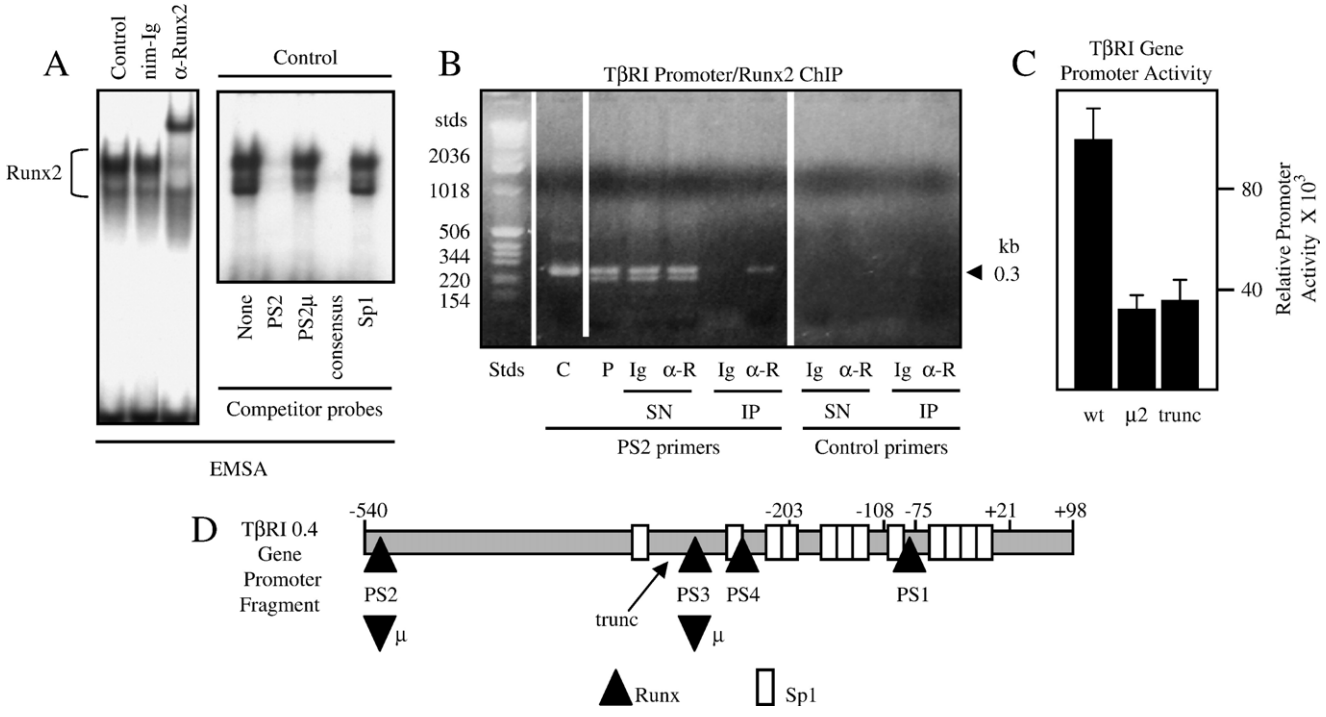

Fig. 5. Endogenous Runx2 and TβRI gene expression in osteoblasts. In (A) nuclei from confluent osteoblasts were extracted and Runx2 was assessed by EMSA. In the left panel, extract was tested with no addition (Control), with nonimmune immunoglobulin (nim-Ig), or anti-Runx2 antibody (α-Runx2) before adding [^{32}P]-labeled oligonucleotide probe with Runx2 binding sequence PS2 specified by rat TβRI gene promoter DNA. In the right panel, nuclear extract was combined with [^{32}P]-labeled-PS2 without (None) or with unlabeled native oligonucleotide (PS2), with a mutated Runx binding site (PS2μ), with an alternate Runx binding sequence (consensus), or with an Sp1 binding sequence (Sp1) (Ji et al., 1998). In (B), endogenous Runx2 binding to TβRI promoter DNA in osteoblast chromatin was determined by chromatin immunoprecipitation (ChIP) and PCR with primers that flank Runx binding element PS2, or control primers. A sizing ladder (stds) is shown on the left. Lane C shows PCR product from plasmid DNA containing the TβRI gene promoter. Lane P shows PCR product of fragmented chromatin before immunoprecipitation. Remaining lanes show PCR products from chromatin in the supernatant (SN) or immunoprecipitate (IP) after collection with nonimmune IgG (Ig) or Runx2 antibody (α-R). EMSA and ChIP also showed Runx2 binding to DNA and chromatin specified by the rat TβRI element PS3 (Ji et al., 1998). In (C), TβRI gene promoter activity was measured in cells transfected with plasmid containing native rat TβRI gene promoter DNA (wt), with mutated Runx2 elements PS2 and PS3 (μ2), or truncated to eliminate upstream DNA (trunc). Reporter gene activity was significantly decreased in osteoblasts transfected with the mutated or truncated TβRI promoter/reporter plasmids ($P<0.05$). Panel D shows the positions of the various previously characterized Runx (triangles) and Sp1 (rectangles) binding sites (Ji et al., 1997, 1998). Mutated Runx sites are denoted by upside down triangles. The truncation site is denoted by the arrow.

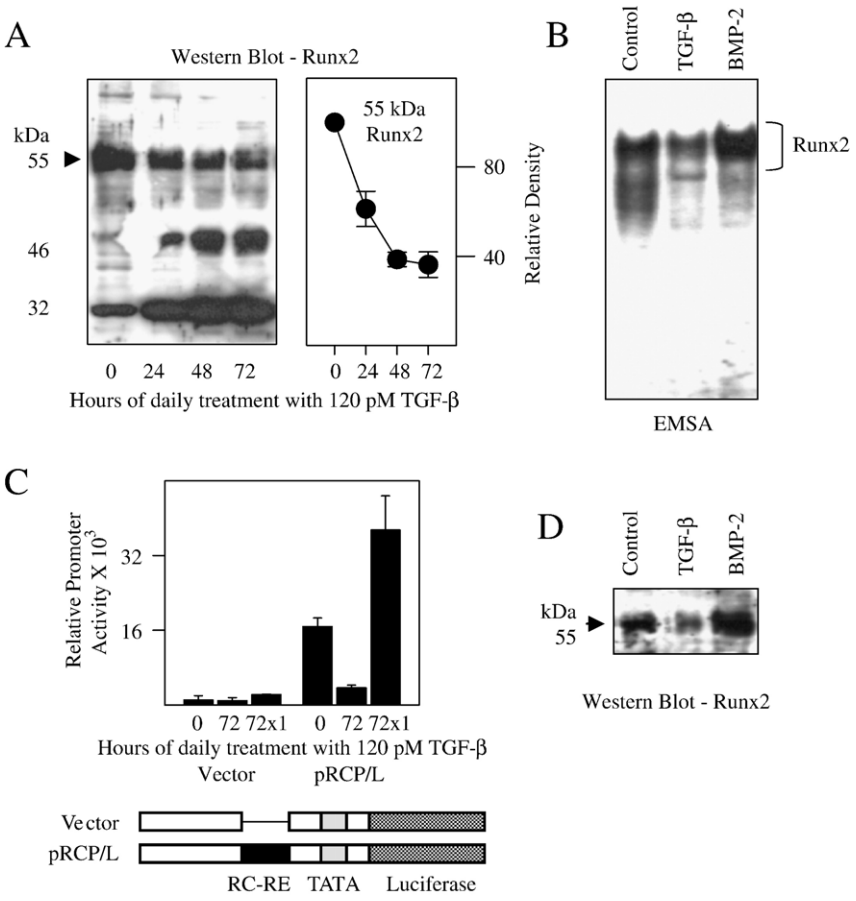

Fig. 6. Decreased Runx2 expression and activity after repeated TGF-β treatment. Confluent fetal rat osteoblasts were treated with vehicle (0) or TGF-β1 as indicated in Fig. 2. In (A), nuclei were extracted and Runx2 was assessed by Western blot with specific antibody. The amount of 55 kDa immunoreactive Runx2 was measured by densitometry relative to untreated cells. In (B), Runx binding to a DNA was assessed by EMSA using extracts from osteoblasts repetitively treated for 72 h with 120 pM TGF-β1 or 1 nM BMP-2. In (C), cells were transfected with vector reporter plasmid containing a minimal TATA box, or pRCP/L, which contains an upstream consensus Runx binding element. Cells were treated with TGF-β1 as in indicated in Fig. 2 and relative reporter gene activity was measured. TGF-β significantly decreased reporter gene activation by plasmid pRCP/L after three repetitive treatments, and significantly increased reporter gene activation after one treatment for a total of 72 h ($P<0.05$). In (D), nuclei were extracted and Runx2 was assessed by Western blot with specific antibody.

from fetal rat parietal bone (Ji et al., 1998), and repeated exposure to TGF-β also decreased the levels of Runx2 and TβRI in these cells (data not shown). To assess Runx2 transcriptional potential independently of the TβRI gene promoter, osteoblasts were transfected with reporter plasmid driven by DNA containing a consensus Runx binding sequence and a minimal TATA box (McCarthy et al., 2000). Three repetitive daily TGF-β treatments over 72 h potently reduced reporter gene activity to levels approaching the activity of parental plasmid vector (Fig. 6C). In contrast, a single exposure to TGF-β at the beginning of the 72 hour treatment interval enhanced reporter gene expression, but we have not determined if this relates to changes in Runx2 levels or to cumulative effects on other transcriptional components. Nonetheless, these results show that a decrease in endogenous Runx2 in osteoblasts repeatedly treated with TGF-β significantly limited Runx dependent gene expression. Finally, unlike the inhibitory effect of TGF-β, parallel repetitive treatments with BMP-2 increased overall Runx2 protein levels and DNA binding, predicting some degree of growth factor specificity (Fig. 6B,D).

4. Discussion

Our current studies demonstrate differential retention of TβRI on osteoblasts that express high levels of nuclear factor Runx2, Runx2 binding directly to TβRI gene promoter DNA within osteoblast chromatin, and less TβRI gene promoter activity by Runx binding site mutation and truncation. Runx2, TβRI, and TGF-β sensitivity all decreased after repeated TGF-β treatment. Inherent differences in synthesis, stability, or secretion prevent direct comparisons among the various decreases in protein synthesis, gene promoter activity, or steady state mRNA or protein levels in TGF-β treated osteoblasts. Nevertheless, the loss of TβRI appears related to a decrease in endogenous Runx2. This may result from changes in Runx2 expression or its degradation, predicted by a rapid increase in lower molecular mass protein bands that react with anti-Runx2 antibody. Increases in Runx2 degradation and decreases in Runx dependent gene expression also occur when rodent osteoblasts are exposed to glucocorticoid (Chang et al., 1998), after long term induction of signals generated by high intracellular cAMP (Selvamurugan et al., 2000), or through

interactions with transcription factor Smad6 (Shen et al., 2006). Many gene products in addition to TβRI are important Runx2 targets, but may be regulated in different ways. For example, three other Runx sensitive gene products, osteocalcin, osteopontin, and collagenase 3, can either increase or decrease in response to TGF-β in osteoblasts (Noda, 1989; Harris et al., 1994; Staal et al., 1996; Fromigue et al., 1998; Hefferan et al., 2000; Alliston et al., 2001; Lilli et al., 2002; Zhang et al., 2003; Lieb et al., 2004; Selvamurugan et al., 2004; Varghese et al., 2005). These differences could relate to variations in TGF-β dose or exposure intervals, which may be resolved by more systematic evaluations.

Notably, homozygous disruption of the murine Runx2 gene severely compromises osteoblast and skeletal tissue development, whereas Runx2 heterozygosity resembles the human condition of cleidocranial dysplasia (Komori et al., 1997; Otto et al., 1997). An important earlier study showed that targeted overexpression of TGF-β2 *in vivo* by highly differentiated osteoblasts also produced a strikingly similar cleidocranial dysplasia-like phenotype in mice (Erlebacher and Derynck, 1996). Our study shows that, like Runx2 heterozygosity, persistent TGF-β treatment *in vitro* reduces Runx2 expression by osteoblasts by approximately 50–60%. Together, these observations suggest that the cleidocranial dysplasia-like phenotype that occurs when osteoblasts overexpress persistently high levels of TGF-β2 might also result from partial loss of Runx2.

We found that osteoblasts were highly resistant to TGF-β after three repetitive treatments. This could result in part from transient cell surface TβR down regulation (Centrella et al., 1996b) in combination with less TβRI re-synthesis as we show here. Still, decreases in Runx2 dependent TβRI expression cannot fully account for TGF-β insensitivity since repetitive treatment, and therefore continued TβR activation, is necessary for this effect. Ligand binding studies show that after 24 and 48 h of TGF-β treatment, TβRIII levels increase relative to TβRI (unpublished results), which may also contribute to lower TGF-β activity (Ji et al., 1999; Eickelberg et al., 2002). Furthermore, other transcription factors potently interact with Runx2 (McCarthy et al., 2000, 2003; Lian et al., 2003; Shen et al., 2006). Therefore, variations in TβRI expression relative to TβRIII, and in other Runx2 sensitive gene products or interacting proteins could contribute to the overall TGF-β insensitivity that we observed.

In summary, our new findings further define a critical role for Runx2 on TβRI expression by osteoblasts, and that Runx2 may act independently of other active elements such as Sp1 that drive basal TβRI synthesis by many cells (Ji et al., 1997). They emphasize the significance of regulatory mechanisms, generated in part by TGF-β itself that can limit its own effects within the skeleton, which may occur to maintain a balanced and controlled progression of events required for proper bone remodeling. Studies like these may further improve our understanding of development and disease in other cell types where Runx2 or other Runx isoforms are expressed, and where TGF-β activity has prominent biological effects.

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