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TRIP-1: A Regulator of Osteoblast Function

Diana Metz-Estrella[1,2], **Jennifer H. Jonason**[1], **Tzong-Jen Sheu**[1], **Rachel M. Mroczek-Johnston**[1,2], and **J. Edward Puzas**[1,2]

[1]Center for Musculoskeletal Research, University of Rochester Medical Center, Rochester NY, USA

[2]Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester NY, USA

Abstract

TGFβ receptor interacting protein-1 (TRIP-1) is an intracellular protein expressed in osteoblasts with high affinity for type 5b tartrate resistant acid phosphatase (TRAP). It is suggested that through this interaction, TRIP-1 serves as a positive regulator of TGFβ signaling and osteoblast differentiation during bone remodeling. We show here that TRIP-1 is abundant in osteoblasts *in vivo* and *in vitro*. TRIP-1 mRNA and protein expression were increased at early stages and decreased at later stages during osteoblast differentiation, suggesting a predominant role during early maturation. To investigate a role during bone remodeling, primary osteoblasts were treated with different hormones and factors that are known to affect remodeling. TRIP-1 levels were decreased with dexamethasone and increased with vitamin D_3, DHT, TGFβ1 and BMP-2. Treatment with PTH and β-estradiol did not affect TRIP-1 levels. Transfected siRNA against TRIP-1 inhibited osteoblast differentiation as characterized by a decrease in alkaline phosphatase staining and enzyme activity, and decrease in the expression of collagen I, alkaline phosphatase, Runx2, osteopontin and osteocalcin. The proliferation of osteoblasts was also affected by TRIP-1 siRNA. This particular effect was defined by decreased cell number, marked reduction of cyclin D1, a 38% decrease of cells in S phase ($p<0.001$) and a 97% increase of cells in the G2/M phase ($p<0.01$) of the cell cycle. However, TRIP-1 siRNA did not induce an effect in apoptosis. Using a TGFβ luciferase reporter we found that knocking down TRIP-1 decreased by 40% percent the activation of TGFβ signaling ($p<0.001$). In conclusion, our characterization of TRIP-1 in osteoblasts provides the first evidence of its key role as a positive regulator of osteoblast function.

Keywords

osteoblast; TRIP-1; TGFβ; TRAP; remodeling

Introduction

The TGFβ signaling pathway controls many processes necessary for normal bone development, growth and healing. Activation of TGFβ signaling promotes the proliferation of osteoprogenitor cells and induces their initial differentiation into a more committed osteoblast,[(1)] yet it can also inhibit the final stages of osteoblast maturation.[(2, 3)] The TGFβ receptor interacting protein-1 (TRIP-1) is a WD40 repeat protein that interacts with and is

Corresponding author: J. Edward Puzas, Ph.D., Department of Orthopaedics, Box 665, University of Rochester, School of Medicine and Dentistry, 601 Elmwood Ave., Rochester, NY 14642, phone: 585-275-3664, edward_puzas@urmc.rochester.edu.

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phosphorylated by the type II TGFβ receptor.[4] However, the complete function for TRIP-1 upon its phosphorylation is still unknown. Nevertheless, experiments using TGFβ-responsive reporters have shown that TRIP-1 regulates the transcriptional effects of TGFβ.[5, 6] Moreover, work in our laboratory suggests that in osteoblasts TRIP-1 is a positive regulator of bone formation.[6]

In addition to being recognized as a protein interacting in the TGFβ signaling, TRIP-1 is also known to be subunit of the eukaryotic initiation factor 3 (eIF3) complex, a set of proteins involved in the initiation of protein translation.[7] Thus, in the literature, TRIP-1 has also been identified as eIF3i, eIF3S2, eIF3β, and eIF3p36, among other names. The fact that TRIP-1 can have roles as a translation initiation factor and as a regulator of gene expression suggests it has pleiotropic functions in the cell.

Despite the reported role of TRIP-1 in translation, Masutani et al., found that, TRIP-1 was not necessary for the formation of an active eIF3 complex during the initiation of protein translation of the β-globin mRNA.[8] Suggesting that TRIP-1 could have a different primary role in the cell or that TRIP-1 could be involved in the regulation of translation of specific proteins.

The TGFβ signaling pathway and the process of protein translation have been independently associated with control over cell proliferation. Hence members of their signaling pathways have been scrutinized for their regulation of carcinogenesis. Since TRIP-1 is linked to both pathways, several studies have been conducted relating TRIP-1 expression with a cancer phenotype. In this regard, over expression of TRIP-1 is associated with, increases in cell proliferation and in cell size, anchorage-independent growth, resistance of apoptosis induction, increased global protein synthesis and tumorigenic potential in mice.[9–11] Consistently, knock down of TRIP-1 had opposite effects in the cell phenotype and has been shown to promote epithelial-mesenchymal transition (EMT).[9, 10, 12]

Interest in characterizing TRIP-1 expression and function in osteoblasts began when our lab found TRIP-1 to interact with high affinity with the osteoclast enzyme, tartrate resistant acid phosphatase 5b (TRAP).[6] In this study it was suggested that the interaction of TRIP-1 and TRAP activated the TGFβ pathway and stimulated the expression of markers of osteoblast differentiation. As TRAP can be found on the surface of the resorption lacunae we believe it may serve as a target protein for controlling osteoblast activity during bone remodeling.[6, 13] That is, when osteoblasts bind to resorption surfaces in bone, a series of signaling events develop intracellularly that lead to their activation for bone formation. We believe that TRIP-1 serves as one of these intracellular mediators. In this study we have found evidence that demonstrates TRIP-1's importance in osteoblasts. Moreover, we show that absence of TRIP-1 in osteoblasts can deregulate normal osteoblast differentiation, proliferation, cell cycle progression and TGFβ signaling.

Materials and Methods

Experimental animals

C57BL/6J mice were purchased from Jackson Laboratories. Euthanasia was done in accordance with the University of Rochester Committee on Animal Resources.

Histology and immunohistochemistry

For immunostaining on histological sections, the left hind limb of three mice in each group was harvested. Muscle and soft tissue were removed and specimens were fixed for three days in 10% neutral buffered formalin, decalcified for 21 days in 14% EDTA (pH 7.2), embedded in paraffin, and sectioned at a thickness of 3 μm. Immunohistochemistry on

paraffin sections was performed using standard protocols with an antibody against TRIP-1 (ProteinTech Group, Inc.).

Cell culture

Rat calvarial primary osteoblasts (ROB) were isolated as previously described.[14] ROB cells were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with 5% fetal bovine serum (FBS) (Sigma) and 50 μg/mL ascorbic acid. For osteoblast differentiation assays, a final concentration of 10 mM β-glycerophosphate (BGP) was also added to the media. Mouse calvarial primary osteoblasts (MOB) were isolated using the same procedure used to isolate ROB cells. For this, neonate pups of C57BL/6J mice obtained from Jackson Laboratories were used. MOB cells were cultured in α Minimum Essential Medium (aMEM) with 10% FBS. The MC3T3-E1 subclone 4 cell line (mouse derived calvarial preosteoblast cell line) was purchased from ATCC and cultured in αMEM with 10% FBS. 2T3 cells (mouse derived calvarial osteoblast cell line) were cultured also in αMEM with 10% FBS. ROS 17/2.8 (rat osteosarcoma cell line) were cultured in DMEM/F-12 with 7% FBS. hFOB cells (ATCC) (human fetal osteoblast cell line) were cultured in DMEM/F-12 with 10% FBS supplemented with ascorbic acid and BGP. Saos-2 cells (human osteosarcoma cell line) were cultured in DMEM high glucose with 10% FBS. All media were supplemented with 1% penicillin and streptomycin (Pen/Strep) (Invitrogen). Treatment with dexamethasone (Sigma), vitamin D_3 (1α,25-dihydroxyvitamin D_3) (Sigma), β-estradiol (Sigma) and dihydrotestosterone (DHT) was done in media with charcoal stripped serum. PTH was purchased from BACHEM. Prior to treating with TGFβ1 (R&D Systems) or BMP-2 (R&D Systems), cells were serum starved overnight in Opti-MEM (Invitrogen) that contained 1% Pen/Strep. For siRNA transfections, Lipofectamine RNAiMAX (Invitrogen) was used following the manufacturer's instructions. The ON-TARGETplus SMARTpool siRNA targeting TRIP-1 was purchased from Thermo Scientific as was the ON-TARGETplus Non-targeting siRNA #1 control. The siRNA was used at a concentration of 4 nM for all experiments. For the differentiation assay 20,000 ROB cells were plated in each well of a 6-well plate. Cells were transfected overnight with siRNAs and the next day media was changed to differentiation media with ascorbic acid and BGP.

Western blotting

Cells were lysed with NP-40 lysis buffer (0.5% NP-40, 50 mM Tris HCl, 150 mM NaCl, pH 7.4) containing Halt proteinase and phosphatase inhibitor cocktail (Thermo Scientific). Protein concentration was quantified using the Bio-Rad Protein Assay (Bio-Rad) in a DU 640 Beckman Coulter Spectrophotometer. Laemmli sample buffer was added and samples were boiled for 8 minutes at 95°C. Proteins were separated by SDS-PAGE using the NuPAGE Novex 10% Bis-Tris Gel Systems (Invitrogen) and MOPS SDS running buffer (Invitrogen). The iBlot gel transfer device (Invitrogen) was used for all transfers. After transfer to a nitrocellulose membrane, proteins were incubated with either 5% BSA diluted in TBS 0.1% Tween 20 (TBST) (for P-Smad3 blotting) or 5% non-fat dry milk diluted in PBS 0.1% Tween 20 (PBST) (for all other antibodies) for one hour. Antibodies were diluted in 5% milk, except for P-Smad3 which was diluted in 5% BSA. The antibodies used were: TRIP-1 antibody (ProteinTech Group, Inc.), β-Actin (Sigma), Smad3 (Cell Signaling), P-Smad3 (Cell Signaling), cyclin D1 (Cell Signaling). Membranes were incubated with antibodies overnight. Species-specific goat antibodies conjugated to HRP (BioRad) were used as secondary antibodies. Antibody detection was done using the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). Quantification of band intensities were performed using Image J software and results were normalized to β-Actin expression levels and to control conditions.

Immunocytochemistry and confocal microscopy

ROB cells were grown in chamber slides. Before staining, cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences), diluted in PBS for 10 minutes. Cells were permeabilized with 0.1% Triton X-100/PBS for 20 minutes at room temperature and then blocked for one hour in 1% BSA, 10% normal donkey serum (Jackson ImmunoResearch), 0.3M glycine. The primary antibody against TRIP-1 was purchased from Abcam, diluted in PBST to a concentration of 5 μg/mL, and incubated on cells overnight at 4°C. Donkey anti-rabbit antibody conjugated to FITC (Jackson Immunoresearch) was used as the secondary antibody. ProLong Gold Antifade Reagent with DAPI (Invitrogen) was used to mount the slides and stain the nuclei. Imaging was performed in a FV1000 Olympus Laser Scanning Confocal Microscope.

Luciferase assays

MC3T3-E1 cells were co-transfected with a TGFβ-responsive (4XSBE) luciferase reporter, an SV40-Renilla expression plasmid (as transfection control) and with either control or TRIP-1 siRNA. Forty-eight hours after transfection, cells were treated with 5 ng/mL of TGFβ1 for 24 hours. Cells were lysed using the 1X passive lysis buffer provided in the Dual-Luciferase Reporter Assay System (Promega). Luciferase assays were carried out according to the manufacturer's instructions. An Optocomp 1 luminometer was used for the readings (MGM Instruments, Inc.).

Real-time PCR

Messenger RNA was collected using the RNeasy Plus Mini kit (QIAGEN). RNA concentration was quantified using a Nanodrop ND-1000 Spectrophotometer. The iScript kit (Bio-Rad) was used to prepare cDNA. PerfeCta SYBR Green FastMix (Quanta Biosciences) was used for all real-time PCR in a thermo cycler (Corbett Research). All results were normalized to β-Actin expression levels. The sequence of the specific primers used for collagen I (*Col1a1*), osteocalcin (*Bglap*), osteopontin (*Spp1*), *Runx2*, alkaline phosphatase (*Alpl)*, mouse TRIP-1 *(Eif3i)*, rat TRIP-1 (*Eif3i*) and β-Actin *(Actb)* can be found in Table 1.

Alkaline phosphatase staining and activity

Cells were fixed with 4% paraformaldehyde. To stain for alkaline phosphatase, 1-Step NBT/BCIP reagent (Thermo Scientific) was used according to the manufacture's instructions. To measure alkaline phosphatase activity, 20 μL of total lysate was mixed in 1.0 mL of substrate solution (50% ddH_20, 50% 2-amino-2-methyl-1-propanol (AMP) buffer pH 10.2 and one p-nitrophenylphosphate tablet (5mg) (Sigma) per 2.5 mL of total substrate solution). This was incubated at 37°C, 5% CO_2 until sufficient color was seen. The reaction was stopped with 500 μL of 0.3 M Na_3PO_4. Absorbance was measured at 410 nm using a spectrophotometer. Reactions were done in triplicates with a blank reaction was used as a control.

Cell number count

The nuclei of ROB cells (plated in 6 cm dishes) transfected with control or TRIP-1 siRNA were stained with Hoechst dye, 24, 48 or 72 hours after transfection. After a 10-minute incubation with the nuclear dye the cells were imaged in fluorescent and bright fields. The number of nuclei per image field was counted and the brightfield image was used to confirm the presence of a cell. Four different image fields were obtained from one sample per time point and the number of cells counted per image were averaged and graphed.

BrdU labeling and cell cycle analysis

ROB cells (150,000 cells per plate) were grown in 10 cm dishes. Cells were transfected with siRNA as described above and on the third day after transfection, cells were labeled with BrdU (BD Biosciences) for 4 hours. Cells were then trypsinized, fixed in ethanol and permeabilized with 2M HCl with 0.5% Triton X-100 (Bio-Rad). For staining, an anti-BrdU FITC-conjugated antibody (BD Biosciences) and propidium iodide were used. RNase A (Sigma) was used at a 100 μg/mL concentration. Reactions were carried out in triplicates. Flow cytometry of the cells was done with a FACSCanto II. FlowJo 8.8.4 Software was used to analyze the data.

Apoptosis assays

For all apoptosis assays, ROB cells were transfected with control or TRIP-1 siRNA as described above. Twenty-four hours after transfection, activation of caspase 3/7 was measured using the Caspase-Glo 3/7 luminescencent assay (Promega) on 12 samples (plated in a 96 well plate) for each siRNA reaction. Alternatively, 48 hours after transfection, assessment of apoptosis by TUNEL was carried out using the In Situ Cell Death Detection Kit (Roche) on 5 samples (plated in a 96 well plate) for each siRNA reaction. Flow cytometry analysis of annexin V and propidium iodide analysis was done using the Dead Cell Apoptosis Kit (Invitrogen) on 3 samples (plated in 10 cm dishes) for each siRNA reaction 72 hours after transfection. The graph presented represents the percentage of cells gated as annexin V positive and propidium iodide negative. Using the same cells used in the procedure for cell number count, condensed nuclei were counted after administering Hoechst dye to live ROB cells. Fluorescent condensed nuclei were counted only if the bright field image showed features of an apoptotic cell.

Statistics

Results are expressed as the mean ± standard deviation. Statistical significance was identified by Student's *t*-test or one-way ANOVA where appropriate. Unless specified differently, the results presented are from a single experiment that was reproduced at least three times.

Results

TRIP-1 is present in osteoblasts

The interaction between TRIP-1 and TRAP implied that TRIP-1 has an osteoblast-specific function. Therefore, we deemed it important to provide a characterization of its expression in osteoblasts. Immunohistochemistry for TRIP-1 was used to evaluate possible differences in TRIP-1 expression between genders and age in leg bones of mice. Results show TRIP-1 was highly expressed in trabecular osteoblasts (Fig. 1A) and in chondrocytes (Fig. 1A and B). However, no significant differences were found in the femur or tibia of young, old, female or male mice (data not shown). Confocal imaging was used to characterize TRIP-1 endogenous distribution in rat primary calvarial osteoblasts (ROB) cells. TRIP-1 was found ubiquitously distributed in osteoblasts and had a punctuated pattern of expression (Fig. 1C). Images of different sections through the Z plane of the cells showed TRIP-1 to be present in the nuclei of osteoblasts (Fig. 1C). In addition, the presence of TRIP-1 in osteoblasts was further confirmed using protein lysates from primary mouse calvarial osteoblasts (MOB) and ROB as well as other well-known osteoblastic cell lines (i.e., MC3T3-E1, 2T3, hFOB, ROS 17/2.8 and Saos-2) (Fig. 1D). To further characterize TRIP-1 expression in osteoblasts, its protein and mRNA levels were evaluated throughout the differentiation process. These data show that TRIP-1 mRNA increased and then decreased with time in culture (Fig. 2A). TRIP-1's protein followed a trend where its expression peaked at days 3 and 9 in culture and

progressively decreased thereafter (Fig. 2B). Thus, these results show that TRIP-1 expression changes with osteoblast differentiation.

Hormones and growth factors regulate TRIP-1 expression in osteoblasts

Hormones are active regulators of bone remodeling and can directly affect osteoblast function. Therefore, hormones that affect osteoblasts during bone remodeling were investigated for their potential to induce changes in TRIP-1 expression. For this, the glucocorticoid analog dexamethasone, the active form of vitamin D (1,25-dihydroxyvitamin D_3), parathyroid hormone (PTH), the androgen, dihydroxytestosterone (DHT) and the estrogen, β-estradiol were used. In humans excess glucocorticoids are known to cause osteoporosis, whereas Vitamin D, PTH, testosterone and estrogen are used in clinical practice to increase bone mass. Western blotting results show that dexamethasone decreased TRIP-1 levels, but vitamin D_3 and DHT increased TRIP-1 levels. PTH and β-estradiol had not effect (Fig. 3). Thus, these data revealed that dexamethasone, vitamin D_3, and DHT are regulators of TRIP-1 expression in osteoblasts.

In osteoblasts, TGFβ1 and BMP-2 are both growth factors of the TGFβ superfamily. TGFβ1 is a stimulator of proliferation and of the early stages of differentiation, and an inhibitor of late stages of differentiation. BMP-2 is a well-defined osteoinducer. Treating ROB for 24 hours with increasing concentrations of TGFβ1 or BMP-2, increased TRIP-1 levels (Fig. 3). Thus revealing that TGFβ1 and BMP-2 are positive regulators of TRIP-1 expression in osteoblasts.

However, activating the Wnt signaling pathway with the synthetic GSK-3 inhibitor BIO did not induce changes in TRIP-1 expression (data not shown).

Osteoblast function is compromised when TRIP-1 expression levels are knocked down

To investigate the importance of TRIP-1 for osteoblast function, siRNA was used to knock its protein levels down in ROB. ROB cells were then cultured in differentiation media for 5 days. Western blotting and real-time PCR confirmed that TRIP-1 levels were efficiently knocked down throughout the assay (Supplemental Fig. 1A and C). Quantification of band density revealed that maximum reduction in TRIP-1 levels occurred at day 3 after transfection (i.e., 84% lower than control) (Supplemental Fig. 1B).

To investigate the phenotype of osteoblasts with reduced TRIP-1 levels, alkaline phosphatase staining and activity assays and real-time PCR for osteoblast markers were performed. Alkaline phosphatase staining increased with time in control conditions but much less staining was found with TRIP-1 siRNA at all time points (Fig. 4A). This trend was also observed when alkaline phosphatase activity was measured (Fig. 4B). Furthermore, real-time PCR for Runx2, collagen I, alkaline phosphatase, osteopontin and osteocalcin confirmed that osteoblasts with reduced TRIP-1 levels were not able to express differentiation markers at a level similar to control osteoblasts (Fig. 4C).

When ROB transfected with TRIP-1 siRNA were evaluated microscopically an obvious decrease in cell number was observed (Fig. 5A). This was also quantified and the decrease was apparent at the 3rd day after transfection, which is when most TRIP-1 is knocked down (Fig. 5A). To initially investigate a possible proliferation defect, cyclin D1 levels were evaluated (Fig. 5B). Cyclin D1 is a protein abundant during the proliferative stages of osteoblasts and important for cell cycle progression. Western blotting data show that TRIP-1 siRNA abolished cyclin D1 expression (Fig. 5B) thus, suggesting a proliferation defect due to TRIP-1 siRNA. To investigate this further, flow cytometry for BrdU and propidium iodide was performed in ROB transfected with control or TRIP-1 siRNA (Fig. 5C and D). The results show that TRIP-1 siRNA produced a 38% decrease in BrdU incorporation and

doubled the percentage of cells in the G2/M phase (Fig. 5C and D). Thus, decreased TRIP-1 levels in osteoblasts inhibit cell proliferation and induce a cell cycle arrest at the G2/M phase.

Since apoptosis can also be contributing to the decrease in cell number, assays to measure markers of apoptosis were evaluated. After testing different conditions and different assays, no evidence of an increase in apoptosis was found when TRIP-1 was knocked down (Fig. 6).

It is known that TRIP-1 can serve as a regulator of gene expression.[5] Previous published work suggests that TRIP-1 can be a positive regulator of TGFβ signaling in osteoblasts.[6] Consistently, knocking down TRIP-1 in MC3T3-E1 preosteoblasts decreased basal (43%) and activated (42%) TGFβ signaling (Fig. 7). In addition, phosphorylated Smad3 levels were also decreased when TRIP-1 was knocked down. But no changes were found in the phosphorylation levels of Smad2 or p38 with TRIP-1 siRNA (data not shown). As observed with ROB in Figure 3, treating MC3T3-E1 with TGFβ1 also increased TRIP-1 levels (Fig. 7B).

Discussion

The concept of coupling between osteoclastic bone resorption and osteoblastic bone formation has long been a principle that defines the process of bone remodeling. Coupling encompasses not only the dependence in the activities of osteoclasts with that of osteoblasts, and vice versa; but also their spatial synchrony. Our lab discovered that TRAP bound to the resorbed surface could serve as a protein regulating this coupling, mediating this by inducing recruitment and activation of osteoblasts for bone formation.[6, 13] One of TRAP's interacting partner, TRIP-1,[6] has been defined in this study as a positive regulator of osteoblast function. Thus suggesting a mechanism occurring during coupling for activation of osteoblasts for bone formation and redefining TRIP-1 as an important protein for osteoblast activity.

Since is it implied that TRIP-1 has an osteoblast-specific function, a characterization of its expression in osteoblasts was initially carried out. Histological analysis of mouse embryo sections had demonstrated that TRIP-1 is abundantly expressed in cells of mesenchymal origin, including the periosteum and hypertrophic chondrocytes.[4] In our study, TRIP-1 was found to be an abundant protein in osteoblasts, both *in vivo* and *in vitro* (Fig. 1). However, its expression is progressively reduced during osteoblast differentiation (Fig. 2), suggesting a more active role during the early stages of this process. This is consistent with TRIP-1 being a positive regulator of the TGFβ signaling pathway and TGFβ being an activator of osteoblast function during the initial differentiation process.[15]

Confocal microscopy using an antibody against endogenous TRIP-1 in osteoblasts (Fig. 1C) confirms results obtained by Anhelmann et al. in NIH3T3 cells.[9] In both cell types, TRIP-1 presence was found to be ubiquitous in the cell and its expression pattern had a speckled appearance. Interestingly, when evaluating the nuclear area in the Z plane of the cell TRIP-1 was found to be present at high levels in the nucleus. This finding is particularly important considering the hypothesis that TRIP-1 has a role as a regulator of gene transcription.

Since TRIP-1 has been implicated as a regulator of osteoblast function during bone remodeling,[6] hormones and growth factors known to be regulators of bone formation *in vivo* were evaluated for their potential on regulating TRIP-1 levels *in vitro* (Fig. 3). Among the molecules evaluated, dexamethasone, vitamin D_3, DHT, TGFβ1 and BMP-2 were found to be regulators of TRIP-1 expression in osteoblasts (Fig. 3).

In the literature there are differences when establishing the effects of glucocorticoids in osteoblasts. In the clinical practice glucocorticoids are used as immunosuppressant for the treatment of conditions such as asthma, multiple sclerosis, or rheumatoid arthritis.[16–18] In all of these, osteoporosis has resulted as an outcome of excess steroid hormones in the body. *In vitro*, glucocorticoids have been shown to both induce and repress osteoblast differentiation.[19, 20] In the TGFβ pathway, glucocorticoids diminished the TGFβ-induced response in osteoblasts.[21] Consistent with the proposed role for TRIP-1 as a positive regulator of osteoblasts and the TGFβ signaling, treatment with dexamethasone decreased TRIP-1 levels in osteoblasts (Fig. 3).

Vitamin D_3 is known to act on rat bone marrow stromal cells, increasing their proliferation and alkaline phosphatase activity.[22] Additionally, in human bone marrow stromal cells it has been found to have synergistic actions with TGFβ to further increase alkaline phosphatase activity.[23] Since we found that vitamin D_3 increased TRIP-1 levels we can hypothesize that TRIP-1 could be, in part, a mediator of vitamin D_3 effects in osteoblasts (Fig. 3).

No difference was found in TRIP-1 expression when histological sections of female vs. male and young vs. aged wild type mice were compared (data not shown). However, treating osteoblasts with DHT increased TRIP-1 levels, while β-estradiol had no effect (Fig. 3). This suggests an androgen-specific regulation of TRIP-1 expression. On the other hand, the absence of changes in TRIP-1 expression with β-estradiol treatment (Fig. 3) correlate with the lack of significant changes observed *in vivo* when comparing TRIP-1 protein levels of young and old female mice (data not shown).

PTH was also evaluated for its potential for regulating TRIP-1 expression. Despite using different concentrations and treatment conditions, treating rat or mouse (data not shown) calvarial osteoblasts with PTH did not induce significant changes in TRIP-1 levels (Fig. 3).

In human renal epithelial cells TGFβ1 increased TRIP-1 protein levels.[24] However, regulation of TRIP-1 levels by BMP-2 has not been investigated until now. Treating osteoblasts with TGFβ1 or BMP-2 increased TRIP-1 levels (Fig. 3). Due to the importance of both of these signaling pathways for osteoblast function these data further supports a role for TRIP-1 in the osteo-inductive properties of these growth factors.

In this study it was demonstrated that osteoblasts have impaired differentiation and proliferation when TRIP-1 levels are decreased. It is possible that this phenotype is a result of a decrease in TGFβ signaling and consequently a decrease in the transcriptional activation of TGFβ-regulated genes involved in proliferation and differentiation of osteoblasts. Since TRIP-1 is a subunit involved in the initiation of translation,[7] another possibility is that the observed phenotype is a consequence of reduced translation of specific mRNAs involved in the proliferation and differentiation of osteoblasts. However, the finding that TRIP-1 siRNA did not affect β-Actin protein levels (Fig. 5B, 7B and Supplementary Fig. 1) revealed that general protein translation was not affected by decreased TRIP-1 levels and further supports the osteoblast-specific role for TRIP-1.

The observed decrease in mRNA levels of collagen I, alkaline phosphatase, Runx2, osteopontin and osteocalcin (Fig. 4C), implied that their transcription was affected or that translational defects were preceding their transcription. In this regard, it is possible that the expression of a transcription factor important for induction of the osteoblast phenotype is compromised. This could be a likely possibility because besides finding a decrease in Runx2 mRNA levels (Fig. 4C), its protein levels were also decreased with TRIP-1 siRNA (data not shown). However, with the data so far obtained it is difficult to assess if the decrease in osteoblast markers was directly related to decreases in Runx2 expression.

After observing a decrease in cell number, the ablated cyclin D1 levels suggested reduced proliferation in osteoblasts transfected with TRIP-1 siRNA (Fig. 5B). In the cell cycle, cyclin D1 regulates the G1/S transition and its levels are usually elevated during proliferation.[25, 26] Flow cytometry data confirmed decreased incorporation of BrdU and also revealed a G2/M phase arrest in cells lacking TRIP-1 (Fig. 5C and D). Consistent with our results, NIH3T3 cells over expressing TRIP-1 show an opposite effect, an increase in cell proliferation and a reduction in the percentage of cells in G2/M phase.[9, 11] Thus, TRIP-1 is important for osteoblast progression through the cell cycle.

The data presented in this study revealed that apoptosis was not involved in the decrease in cell number observed with TRIP-1 siRNA (Fig. 6). However, the possibility of apoptosis occurring after the observed cell cycle arrest (i.e., after day 3 after transfection) was not evaluated.

Consistent with our hypothesis that TRIP-1 is a positive regulator of TGFβ signaling in osteoblasts, MC3T3-E1 pre-osteoblasts transfected with TRIP-1 siRNA have reduced activation of a TGFβ-responsive reporter (Fig. 7A). Interestingly, TRIP-1 siRNA also reduced the phosphorylation levels of Smad3 (Fig. 7B). In human lung epithelial cells, reduction of TRIP-1 levels causes an opposite effect, phospho-Smad3 levels are upregulated and the TGFβ-mediated response is increased.[12] Despite the disagreement, this implies a possible new role for TRIP-1 as a regulator of Smad3 phosphorylation, although the end result may be cell type specific. The finding that phosphorylation of Smad2 or p38 were not affected by TRIP-1 siRNA suggests that TRIP-1's effects are specific for Smad3 and that it may act primarily through the canonical TGFβ signaling pathway.

Mice that lack the type II TGFβ receptor in skull mesenchyme present a similar phenotype to our model of decreased TRIP-1 expression in isolated rat calvarial osteoblasts.[27] The skull mesenchyme of $Prx1/Cre^{+}$; $TGFBR2^{f/f}$ mice show a decrease in osteoblast proliferation and differentiation but no effect on apoptosis. These parallel phenotypes further demonstrate that the type II TGFβ receptor is a regulator of TRIP-1 function and that TRIP-1 is important in this signaling pathway in calvarial osteoprogenitors.

One aspect of TRIP-1 characterization not evaluated in this study is the role of phosphorylation in its function. It has been reported that TRIP-1 is phosphorylated on serine and threonine residues by the type II TGFβ receptor and by mTOR.[4, 9] Additionally, mutations in the putative consensus motif recognized by mTOR (i.e. mTOS motif) rescues the oncogenic phenotype seen in NIH3T3 cells when TRIP-1 is over expressed.[9] Implying that TRIP-1 phosphorylation could be an important modification for controlling its activity. A comprehensive study of changes in TRIP-1 phosphorylation upon treatment with growth factors and during osteoblast differentiation is necessary to decipher in detail its mechanism of action.

In the published literature there seems to be no clear agreement of the role of TRIP-1 in the cell. Results seem diverse and contradictory. However, in osteoblasts, we found that TRIP-1 is a positive regulator of TGFβ signaling and through its interaction with TRAP possesses a cell specific role. TRIP-1 is a target of hormones and growth factors that are known to affect bone formation. We found that it is differentially regulated during osteoblast maturation and is more abundantly expressed during the early stages of this process. Furthermore, we show that TRIP-1's presence in these cells is essential for their proliferation and differentiation. Our results support the importance of further investigations on the mechanisms of action of TRIP-1 in osteoblasts, specifically during bone remodeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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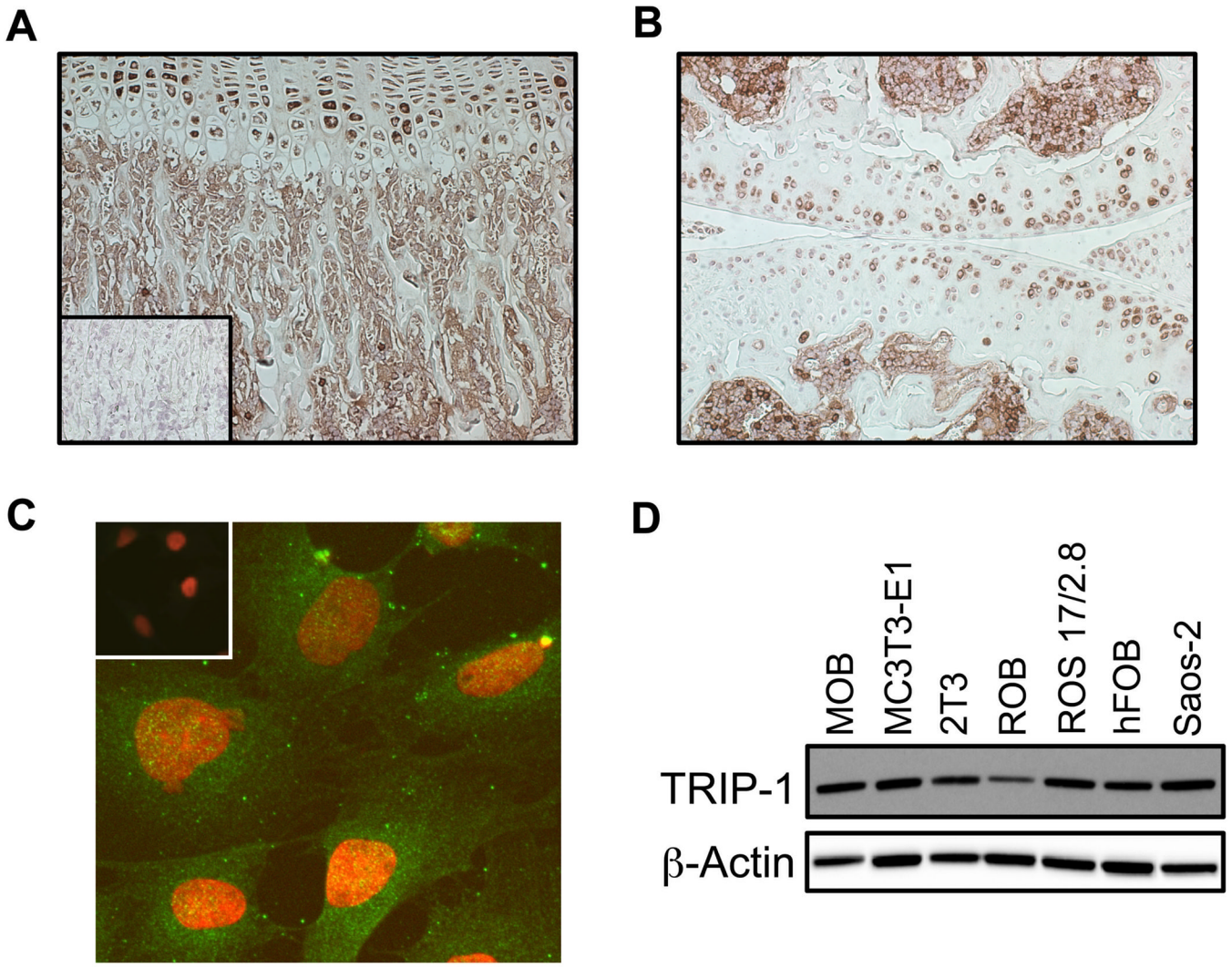

Fig. 1.

TRIP-1 is expressed in osteoblasts. (*A*) Expression of TRIP-1 in the trabecular region and growth plate of mouse tibia. Inset shows a control section with no primary antibody. (*B*) TRIP-1 immunohistochemistry in the mouse knee joint (femur on top and tibia on bottom). (*C*) Confocal microscopy analysis of rat primary calvarial osteoblasts (ROB) stained for TRIP-1 in green and DAPI in red. Inset shows control cells with no primary antibody. (*D*) Western blotting for TRIP-1 expression in different osteoblastic cells.

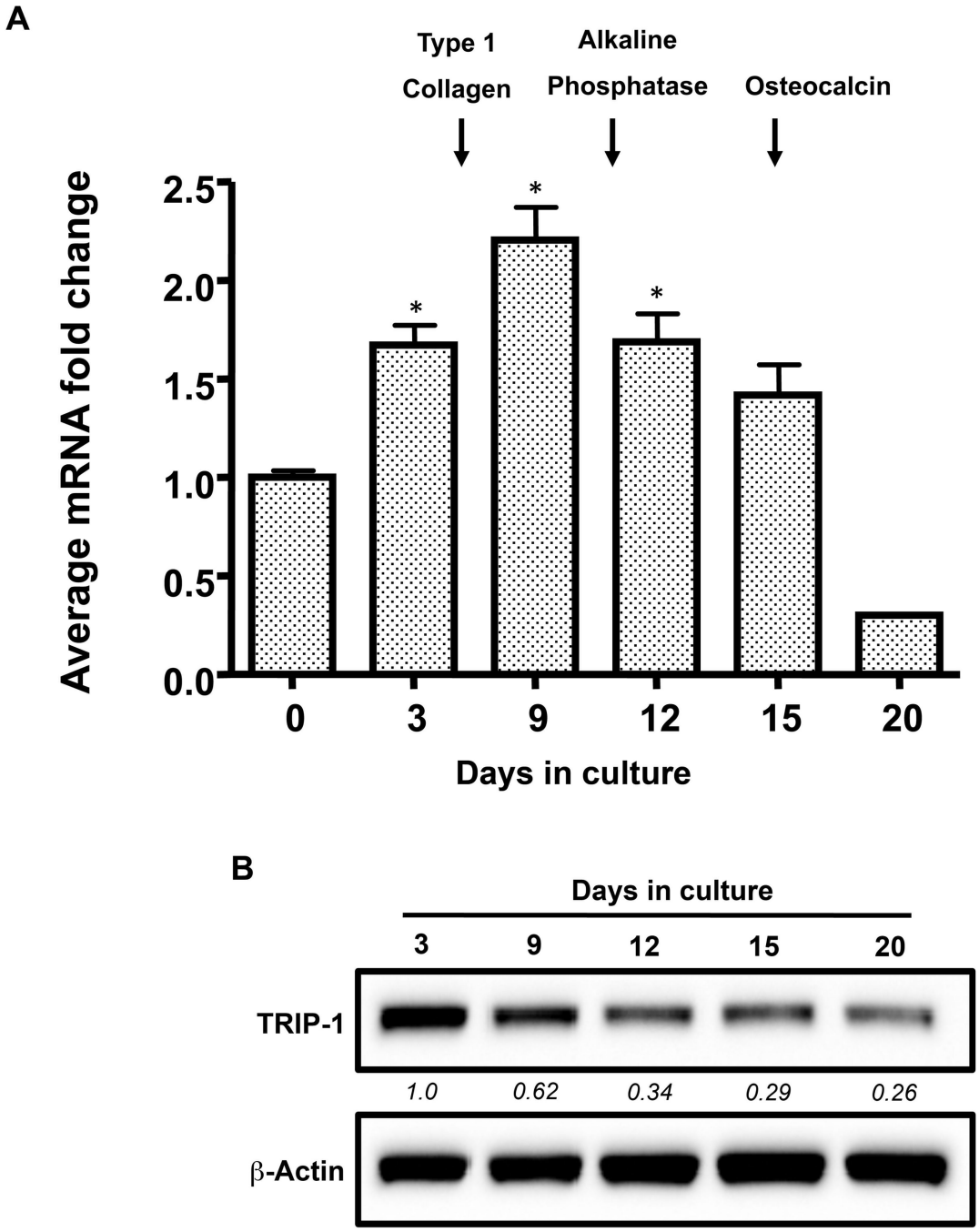

Fig. 2.

TRIP-1 expression changes during osteoblast differentiation. (*A*) Compilation of 3 independent experiments (except 20 days in culture which is from one experiment) where TRIP-1 mRNA expression values were normalized to β-Actin expression and to the first time point collected. Peak gene expression during this culture period of collagen I, alkaline phosphatase and osteocalcin are shown. One-way ANOVA followed by Dunnet's multiple comparison test was used for statistical analysis; *, $p<0.01$. (*B*) Western blotting of TRIP-1 expression. Numbers under the bands represent quantification of band intensity relative to β-Actin, normalized to day 3 in culture. The TRIP-1 protein expression blot is representative of four independent experiments.

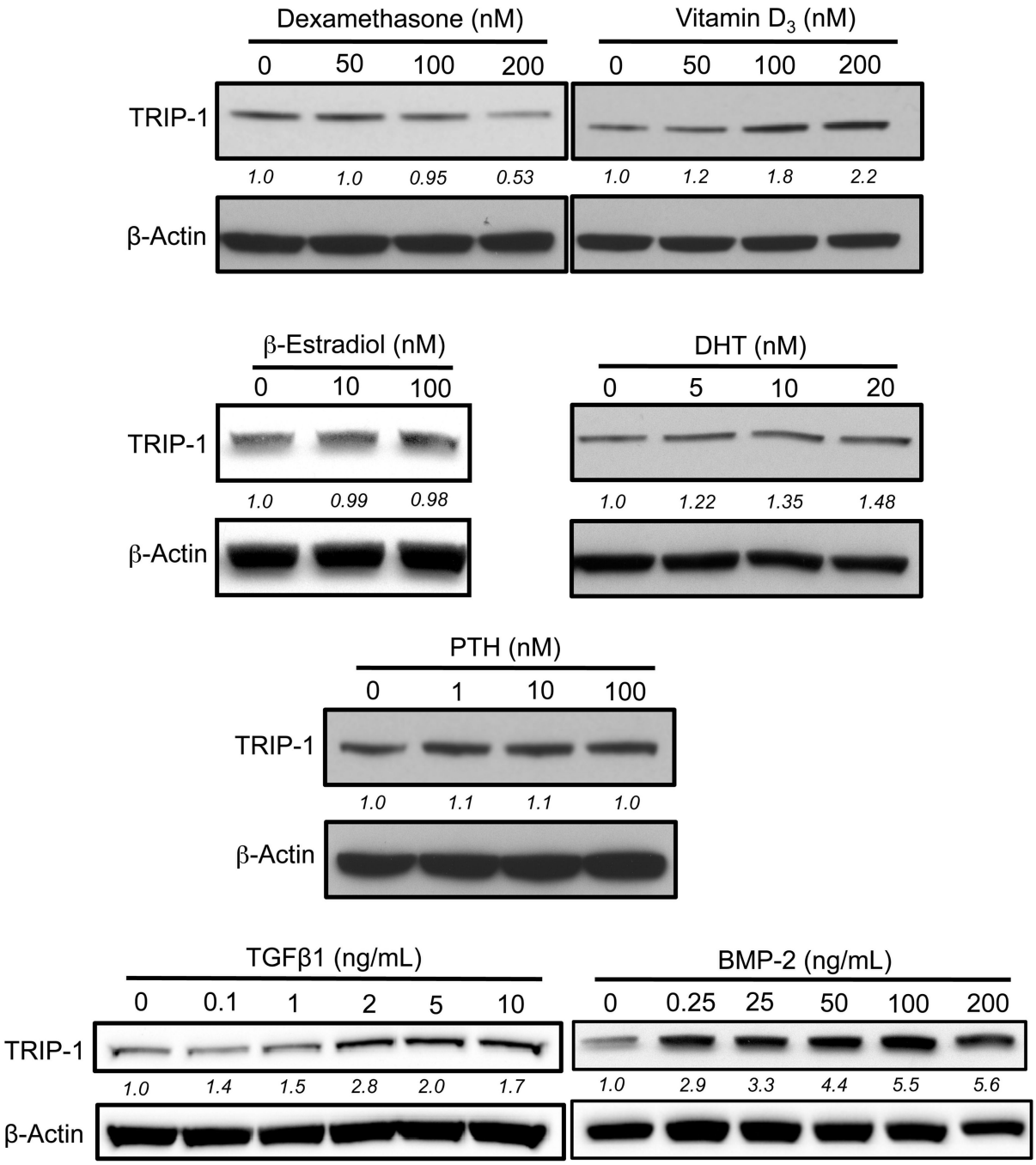

Fig. 3.

Hormones and growth factors alter TRIP-1 expression. Western blotting of TRIP-1 after treating ROB with different concentrations of dexamethasone, vitamin D_3, β-estradiol, DHT or PTH for 48 hours or with TGFβ1 or BMP-2 for 24 hours. These results have been reproduced at least twice more.

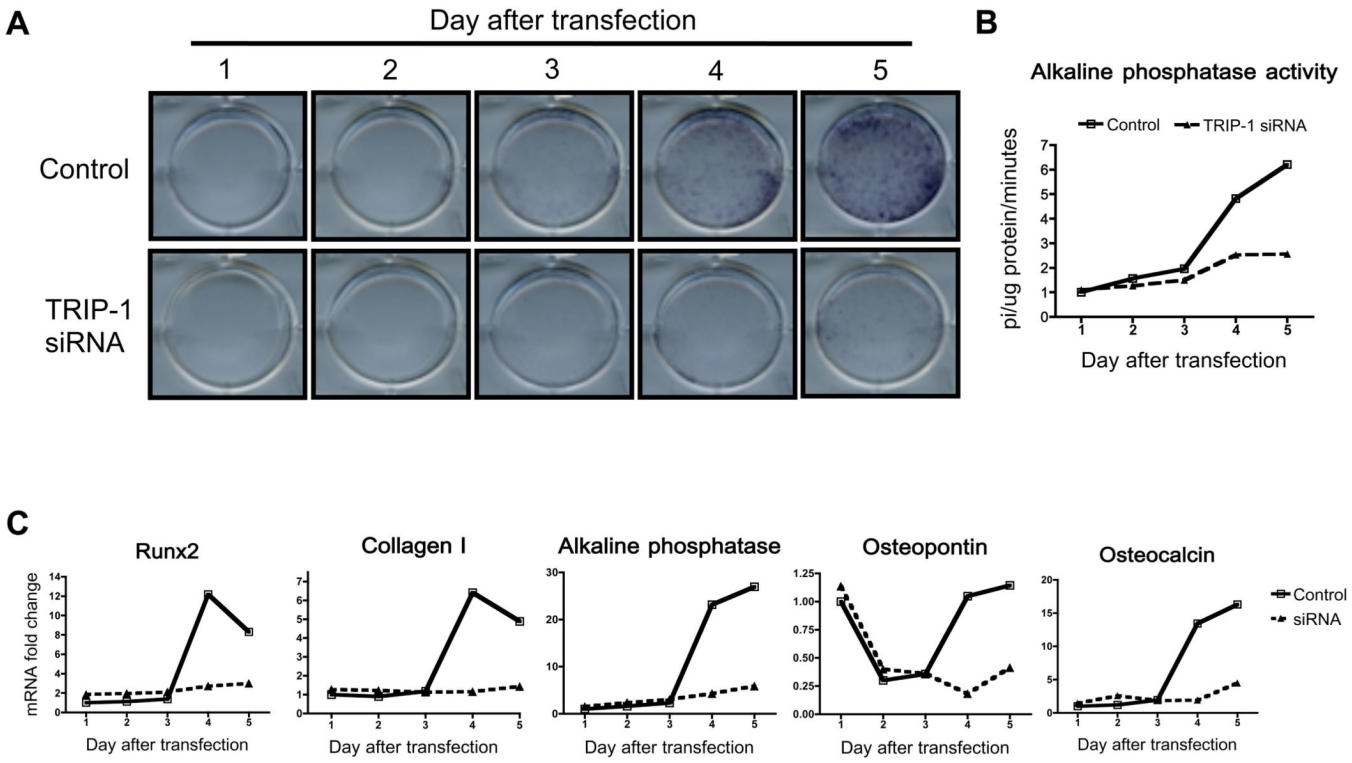

Fig. 4.

TRIP-1 siRNA compromises osteoblast differentiation. ROB transfected with control or TRIP-1 siRNA were evaluated for (*A*) alkaline phosphatase staining, (*B*) alkaline phosphatase activity, and (*C*) mRNA levels of collagen I, Runx2, alkaline phosphatase, osteopontin, and osteocalcin.

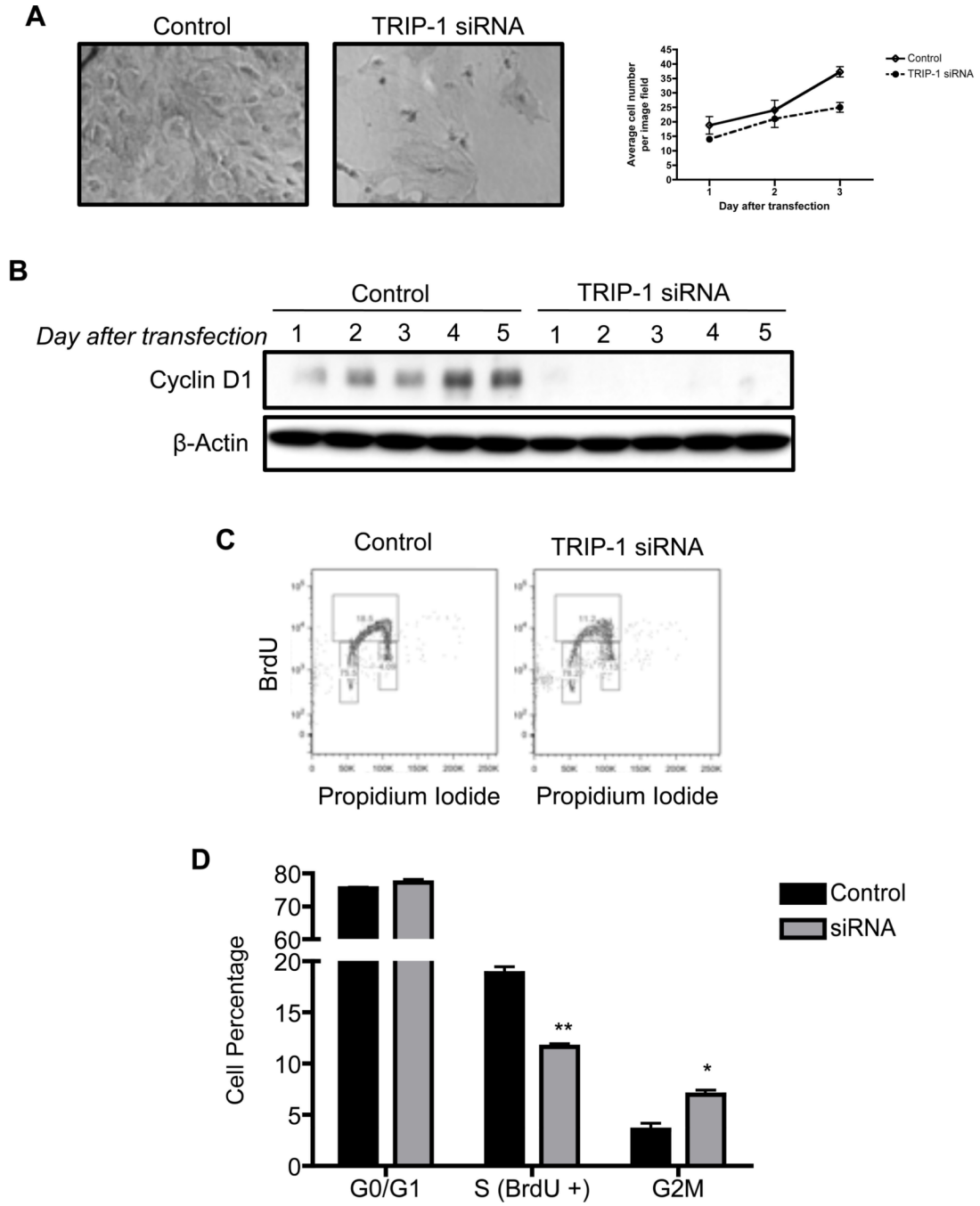

Fig. 5.

TRIP-1 loss-of-function decreases osteoblast proliferation. (*A*) Images of osteoblasts three days after transfection with control or TRIP-1 siRNA. Osteoblasts per image field were quantified during the first three days after siRNA transfection. (*B*) Western blotting of cyclin D1 expression after control or TRIP-1 siRNA transfection. (*C–D*) Flow cytometry of BrdU and propidium iodide incorporation in osteoblasts transfected with control or TRIP-1 siRNA. (*C*) Representative gating of cell distribution plots. (*D*) Average percentage of cells in G0/G1 phase, S phase and G2/M phase. Error bars represent standard deviation of three different samples. Student's *t*-test was used for statistical analysis; *, $p<0.01$; **, $p<0.001$.

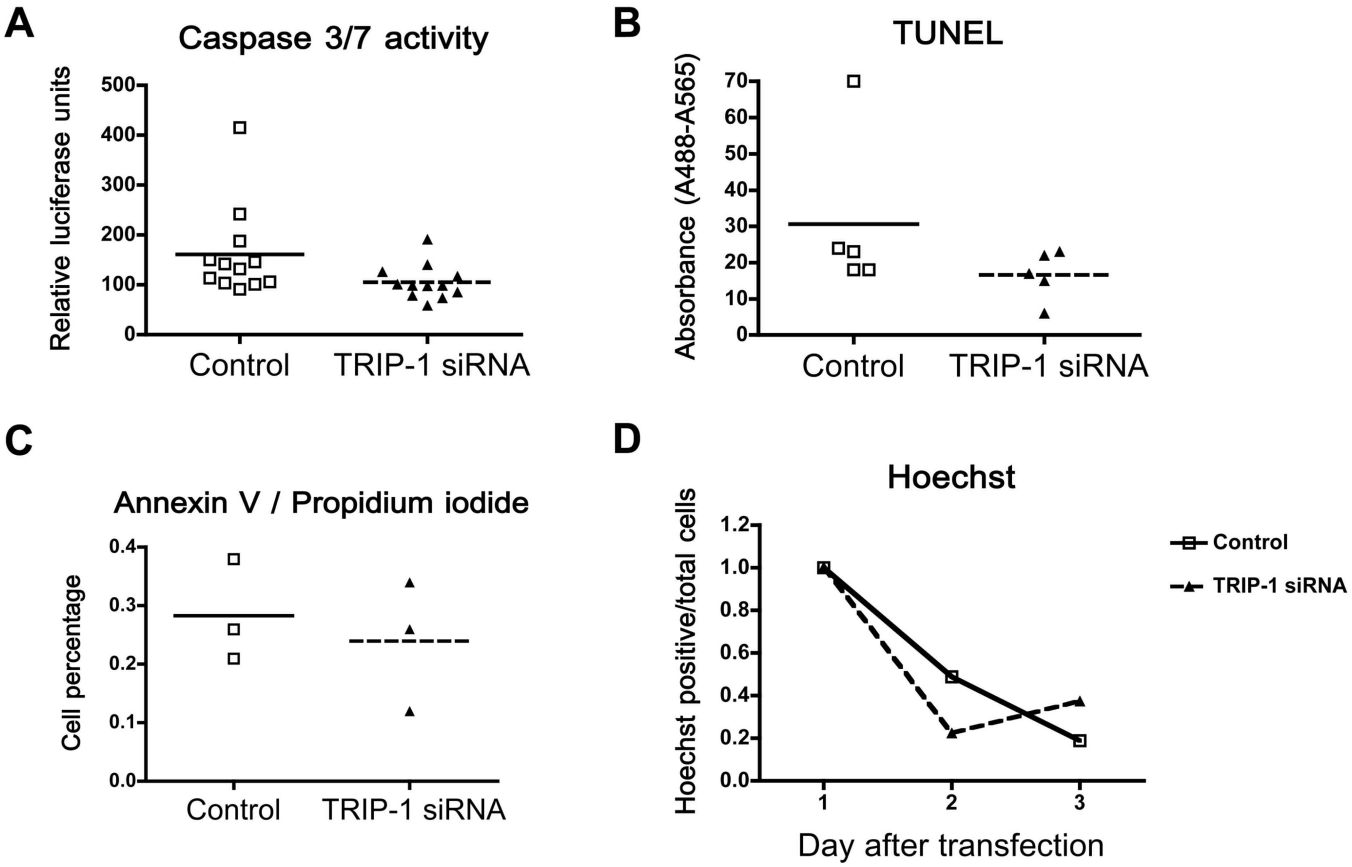

Fig. 6.

Knocking down TRIP-1 does not induce apoptosis of ROB. Apoptosis was evaluated using the following techniques: (*A*) caspase 3/7 activity, (*B*) TUNEL ELISA assay, (*C*) flow cytometry for annexin V positive and PI negative cells; respectively at 24, 48 and 72 hours after transfection and (*D*) Hoechst dye was used to count condensed nuclei during the first three days after transfection.

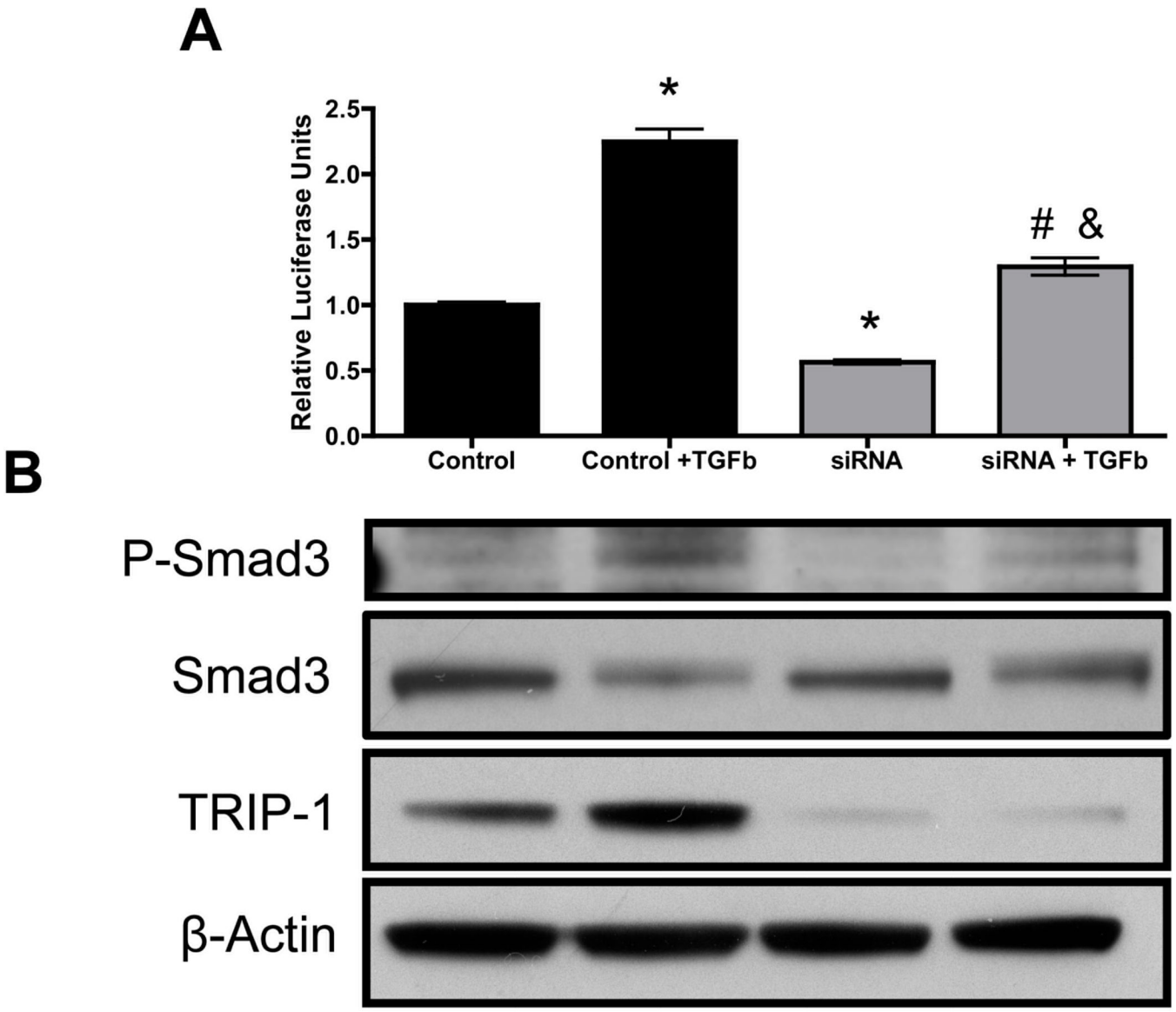

Fig. 7.

Knocking down TRIP-1 reduces TGFβ signaling. MC3T3-E1 preosteoblasts were transfected with a TGFβ-responsive luciferase reporter and with either control or TRIP-1 siRNA. Cells were treated with TGFβ1 (5 ng/mL) 48 hours after transfection and lysed 72 hours after transfection. (*A*) Luciferase assay results are a compilation of three independent experiments done in triplicate. One-way ANOVA followed by Bonferroni's multiple comparison test was used for statistical analysis; *, $p>0.001$, versus Control; #, $p>0.001$, versus Control + TGFβ1; &, $p>0.001$, versus siRNA. (*B*) Western blotting for P-Smad3, Smad3, TRIP-1 and β-Actin.

Table 1

Primer sequences for real-time PCR

Rat collagen I	5’-TGG TTT GGA GAG AGC ATG ACC GA-3’
	5’-TTG GTC CAT GTA GGC TAC GCT GTT-3’
Rat Runx2	5’-GGA GGG CCG TGG GTT CT-3’
	5’-TTT AGG GCG CAT TCC TCA TC-3’
Rat alkaline phosphatase	5’-TGA TCA CTC CCA CGT TTT CA-3’
	5’-CTG GGC CTG GTA GTT GTT GT-3’
Rat osteocalcin	5’-CTG ACA AAG CCT TCA TGT CCA AGC-3’
	5’-TCC AAG TCC ATT GTT GAG GTA GCG-3’
Rat osteopontin	5’-TAT CAA GGT CAT CCC AGT TGC CCA-3’
	5’-ATC CAG CTG ACT TGA CTC ATG GCT-3’
Mouse TRIP-1	5’-GCA GAT GGG GTA TCA GTG CT-3’
	5’-ACG TTC ACC AAC ACC TCT CC-3’
Rat TRIP-1	5’-ACC TGT GGC TTT GAC TTT GG-3’
	5’-AGA CTT GGC GCT GTA CTG GT-3’
Rat/mouse β-Actin	5’-AGA TGT GGA TCA GCA AGC AG-3’
	5’-GCG CAA GTT AGG TTT TGT CA-3’