Biochemical and Biophysical Research Communications 422 (2012) 488–493

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Thrombospondin-1 inhibits osteogenic differentiation of human mesenchymal stem cells through latent TGF-β activation

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ARTICLE INFO

*Article history:*
Received 1 May 2012
Available online 11 May 2012

*Keywords:*
Thrombospondin-1
Transforming growth factor-β
Osteoblast
Mesenchymal stem cell

ABSTRACT

Transforming growth factor-β (TGF-β) is a critical regulator of bone development and remodeling. TGF-β must be activated from its latent form in order to signal. Thrombospondin-1 (TSP1) is a major regulator of latent TGF-β activation and TSP1 control of TGF-β activation is critical for regulation of TGF-β activity in multiple diseases. Bone marrow-derived mesenchymal stem cells (MSCs) have osteogenic potential and they participate in bone remodeling in injury and in response to tumor metastasis. Since both TSP1 and TGF-β inhibit osteoblast differentiation, we asked whether TSP1 blocks osteoblast differentiation of MSCs through its ability to stimulate TGF-β activation. TSP1 added to human bone marrow-derived MSCs under growth conditions increases active TGF-β. Cultured MSCs express TSP1 and both TSP1 expression and TGF-β activity decrease during osteoblast differentiation. TSP1 and active TGF-β block osteoblast differentiation of MSCs grown in osteogenic media as measured by decreased Runx2 and alkaline phosphatase expression. The inhibitory effect of TSP1 on osteoblast differentiation is due to its ability to activate latent TGF-β, since a peptide which blocks TSP1 TGF-β activation reduced TGF-β activity and restored osteoblast differentiation as measured by increased Runx2 and alkaline phosphatase expression. Anti-TGF-β neutralizing antibody also increased alkaline phosphatase expression in the presence of TSP1. These studies show that TSP1 regulated TGF-β activity is a critical determinant of osteoblast differentiation.



## 1. Introduction

Transforming growth factor-β (TGF-β) is a cytokine that influences cell growth, differentiation, apoptosis, and inflammation in development, tissue repair, and in fibrosis [1]. TGF-β is a significant factor in bone remodeling and in the tumor-bone microenvironment. TGF-β stimulates early osteoblast proliferation, while inhibiting late stage osteoblast differentiation and mineralization to reduce bone formation [2–4]. TGF-β also inhibits osteogenic differentiation of mesenchymal stem cells (MSCs) [5–7]. TGF-β promotes bone catabolism through stimulation of RANKL secretion and enhancement of osteoclast survival [8,9]. These actions make TGF-β a critical factor and a therapeutic target in osteolytic bone disease in metastatic cancer [10–13]. TGF-β inhibition can restore terminal osteoblast differentiation to suppress tumor growth [14,15].

One of the major points of TGF-β regulation occurs at the step at which the latent precursor is converted to the biologically active molecule: binding of the N-terminal latency associated peptide (LAP) prevents TGF-β binding to its receptors and this interaction must be disrupted for TGF-β signaling to occur. Latent TGF-β can be converted from its latent precursor to the active form through multiple mechanisms that include proteolysis, binding to integrins, mechanical forces involving cytoskeletal-extracellular matrix interactions, modifications of the latent complex by reactive oxygen species or viral enzymes, or by binding to the secreted and extracellular matrix protein, thrombospondin-1 (TSP1) [16–18]. The specific mechanism that regulates latent TGF-β activation can vary with tissue, cell type, and with the specific disease milieu.

TSP1 is a complex, multi-functional extracellular matrix and secreted protein, abundant in platelet α-granules and widely expressed by most cell types, especially those that participate in tissue injury and repair responses [19,20]. TSP1 is a major regulator of latent TGF-β activation in a number of disease states [18,21]. TSP1 binds to the latent complex to stimulate TGF-β activation at the cell surface or in the extracellular milieu [18]. Activation occurs through binding of the KRFK sequence in the TSP1 type 1 repeats to

*Abbreviations:* TGF-β, transforming growth factor-β; TSP1, thrombospondin-1; MSC, mesenchymal stem cell; LAP, latency associated peptide.

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.05.020

LSKL in the LAP region of the latent complex, which disrupts LAP-mature domain interactions to expose the receptor binding sited on the mature domain, rendering TGF-β capable of signaling [22,23]. Peptides of these sequences can be used to either antagonize TSP1-mediated latent TGF-β activation (LSKL, GGWSHW) or alternately, to stimulate activation (KRFK, RKPK) [21].

TSP1 is expressed in long and calvarial bones [24]. TSP1 inhibits matrix mineralization of MC3T3-E1 osteoblast-like cells and it is highly expressed during early osteogenesis [25–28]. TSP1 in a mixture of platelet proteins inhibits bone nodule formation and mineralization [29,30]. TSP1 can also prevent endochondral ossification [31]. TSP1 null mice have mild lordosis of the spine and craniofacial dysmorphism [32]. Despite these observations, the mechanisms by which TSP1 inhibits osteoblast differentiation are not clear [33].

Given that TGF-β inhibits osteoblast differentiation and that TSP1 can activate latent TGF-β, we asked whether TSP1-dependent activation of TGF-β influenced osteoblast differentiation of bone marrow-derived mesenchymal stem cells (MSCs). Our results show that TSP1 blocks osteoblast differentiation of MSCs through activation of latent TGF-β while maintaining the myofibroblast phenotype, suggesting a novel role for TSP1 in the bone microenvironment.

## 2. Materials and methods

### 2.1. Human bone marrow mesenchymal stem cells (MSC)

Human bone marrow-derived mesenchymal stem cells (MSCs) were purchased from BioWhitaker/Cambrex/Lonza. MSCs obtained from the UAB Center for Metabolic Bone Disease Core Facility were used in Fig. 1. Use of these cells is in accord with the UAB Institutional Review Board for Use of Human Subjects.

### 2.2. Growth factors and other reagents

Mesenchymal cell growth serum was purchased from Cambrex BioScience, Inc. (Walkersville, MD). The following items were purchased: recombinant human TGF-β (R&D Systems, Minneapolis, MN); neomycin sulfate (G418) (ICN Biomedicals, Aurora, OH); Luciferase 1000 assay system (Promega, Madison, WI); Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Science, Boston, MA).

### 2.3. Proteins, antibodies, and peptides

TSP1 depleted of associated TGF-β was purified from human platelets as previously described [34]. Peptides were synthesized and purified by AnaSpec, Inc. (San Jose, CA). The following antibodies were purchased: anti-α-SMA (Biocarta, San Diego, CA), anti-ED-A fibronectin and anti-collagen type I (AbCam Inc., Cambridge, MA), anti-β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-TGF-β neutralizing antibody (R&D Systems, Minneapolis, MN). Mouse monoclonal antibody 133 to human platelet TSP1 was produced in our laboratory.

### 2.4. Cell culture and differentiation

MSCs were maintained in Dulbecco's Modified Eagles medium (DMEM) with 10% mesenchymal cell growth serum. For basal conditions, cells at 50% confluence were rendered quiescent for 48 h in serum-free DMEM. Quiescent cultures were then treated with 1 ml of serum-free DMEM in the presence or absence of TGF-β (5 ng/ml), TSP1 (1 nM), anti-TGF-β neutralizing antibody (5 μg/ml), LSKL (1 μM), or SLLK (1 μM) for 48 h with re-feeding at 24 h. Conditioned media and cell lysates were collected for analyses.

Osteoblast differentiation was induced in MSC cultures using the following conditions: cells were grown in 6-well plates to confluence in DMEM with 10% mesenchymal cell growth serum. At confluence, cultures were switched to osteogenic medium (DMEM with 10% mesenchymal growth serum supplemented with 10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid-2-phosphate) [35,36]. Medium was changed every 2 days for 20–30 days. TGF-β (5 ng/ml), TSP1 (10 nM), anti-TGF-β neutralizing antibody (5 μg/ml), LSKL (25 μM), or SLLK (25 μM) was added at the initial feeding of osteogenic medium and every 2 days until termination of the experiment.

### 2.5. SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting

Conditioned media were collected in siliconized tubes on ice. Cell layers were lysed with 4× Laemmli sample buffer, scraped, and sonicated to shear DNA. Equal volumes of conditioned media or cell layer lysates were loaded onto SDS–polyacrylamide gels under reducing conditions and proteins electrophoretically transferred to nitrocellulose. Equal loading and transfer of protein samples were verified by Ponceau S staining. Membranes were

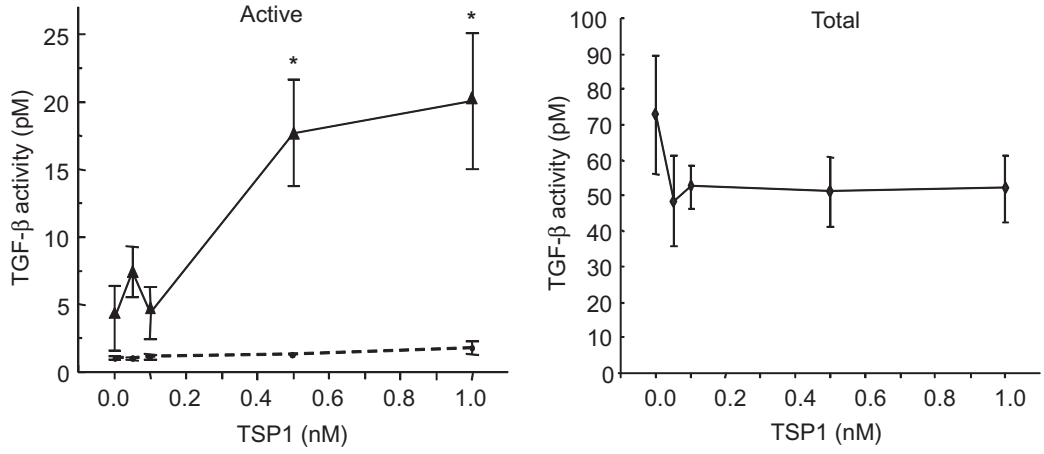


**Fig. 1.** TSP1 increases TGF-β activity in MSCs. MSC were plated in 6-well plates in growth media until 50% confluent. Cells were serum starved for 48 h and then treated daily with increasing concentrations of TSP1 for 48 h. Levels of active and total TGF-β in the conditioned media were measured using the PAI-1 promoter reporter luciferase assay. The dashed line represents residual TGF-β activity in the purified TSP1 added to the reporter cells. Data are the means ± SD of triplicate determinations.

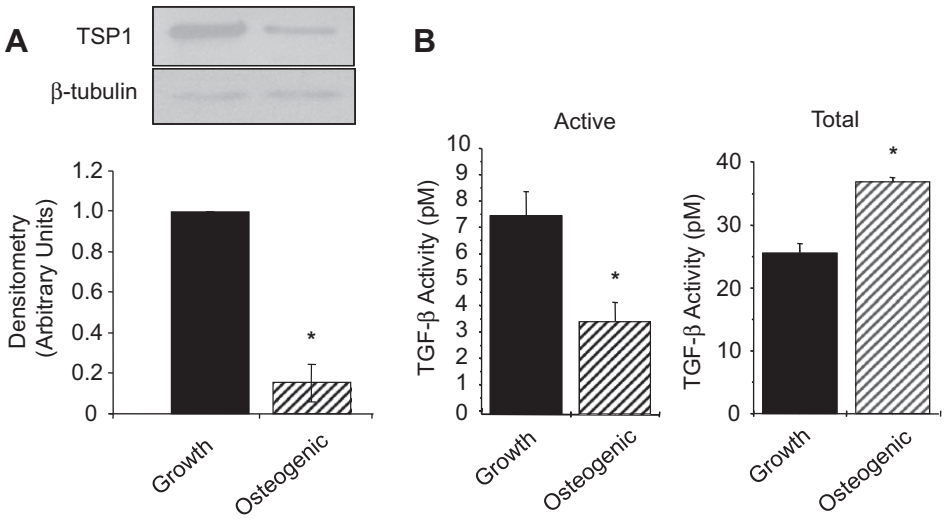


**Fig. 2.** TSP1 and active TGF-β are decreased in the MSCs under osteogenic conditions. Cells were grown to confluence and then treated every 2 days for 30 days with osteogenic media or maintained in growth media. After 30 days, the media and cell lysates were collected. (A) TSP1 in the conditioned media was determined by immunoblotting. In separate gels, cell lysates were resolved and immunoblotted for β-tubulin. Results were normalized to β-tubulin. Results of densitometric analysis of bands are expressed as the mean ± SD of triplicate samples. (B) Active and total TGF-β in the conditioned media were measured by PAI-1 promoter reporter luciferase assay To obtain total TGF-β levels, samples were heat activated for 3 min at 100 °C. $^{*}p < 0.05$.

blocked with 1% casein and then incubated with primary antibodies (anti-ED-A fibronectin at 1:5000, anti-α-SMA at 1:1000, or anti-β-tubulin at 1:500), washed, and incubated with appropriate peroxidase-conjugated secondary antibodies (0.1 μg/ml) for immunodetection by enhanced chemiluminescence. Immunoblots of cell layer lysates were stripped and reprobed with anti-β-tubulin. For conditioned media, corresponding cell layer lysates were blotted onto separate membranes and probed with anti-β-tubulin. All results were normalized to β-tubulin.

### 2.6. Bioassay for TGF-β activity

Active and total TGF-β in conditioned media were measured by bioassay using mink lung epithelial cells stably transfected with the TGF-β response element of the human plasminogen activator inhibitor-1 (PAI-1) gene promoter fused to firefly luciferase reporter gene, (gift from Dr. D.B. Rifkin, New York University Medical Center) [37]. Luciferase assay results were normalized to β-tubulin in the cell lysates of cells from which the conditioned media were collected.

### 2.7. Alkaline phosphatase staining

Histochemical analysis of alkaline phosphatase activity was performed on day 20 of osteogenic induction. Cell layers were fixed with 2% paraformaldehyde/0.1 M cacodylic buffer and then incubated with Tris–maleate buffer (pH 8.4) containing 500 μg/ml naphthol AS-MX phosphate disodium salt and 1 mg/ml Fast Red TR salt. After 30 min at 37 °C, cell layers were washed with 0.1 M cacodylic buffer.

### 2.8. RNA extraction and RT-PCR

Total RNA was extracted using TRIzol (Invitrogen Life Technologies). The yield and purity of RNA was estimated using the $A_{260}/A_{280}$ ratio. First strand cDNA synthesis was performed with the Advantage RT-for-RCR kit. RNA (1 μg) was reverse transcribed using M-MLV reverse transcriptase. The reverse cDNA preparation was used in real-time PCRs on an iCycler iQ (Bio-Rad, Hercules, CA) with iQ SybrGreen Supermix (Bio-Rad). Primers for real-time PCR were designed to amplify 100–150-bp fragments using Beacon Designer 2.1 software (Biosoft International, Palo Alto, CA). For each experiment, a standard curve was generated using fivefold dilutions of cDNA. The first dilution was arbitrarily set to copy number 1000. When PCR products were within the range of the standard curve, the amount of cDNA was calculated using the standard curve and normalized to GAPDH controls. Samples were run in duplicate and each experimental treatment in triplicate. Prior to each run, a melting curve was generated for each primer to determine optimal annealing temperatures and to ensure that no primer dimers or secondary products formed. The following primer sequences were used: Runx2 (forward 5′–3′) GATGACACTGCC ACCTCTGACTT, (reverse 5′–3′) AAAAAGGGCCCAGTT CTGAAG; GAPDH (forward 5′–3′) ATGGGGAAGATAAAGGTCG, (reverse 5′–3′) TAAAAGCAGCCCTGGTGACC.

### 2.9. Densitometry

Immunoblots were scanned and bands were quantified using Scanalytics One-Dscan Software, version 1.31. Multiple exposures were obtained for each blot and only films exhibiting linear responses were analyzed by densitometry.

### 2.10. Statistical analysis

Results were expressed as means ± the standard deviation for three separate experiments. Statistical differences among treatment conditions were determined using one-way analysis of variance. The analysis was performed with SigmaStat 3.0 software (SPSS Inc., Chicago, IL). Values of $p \leqslant 0.05$ were considered significant.

## 3. Results

### 3.1. Thrombospondin-1 activates latent TGF-β expressed by MSCs

MSCs were treated for 48 h with TSP1 depleted of TGF-β activity which associates with TSP1 during purification from platelets. Levels of active and total TGF were assessed to determine whether TSP1 activates latent TGF-β expressed by MSCs. TSP1 induced a

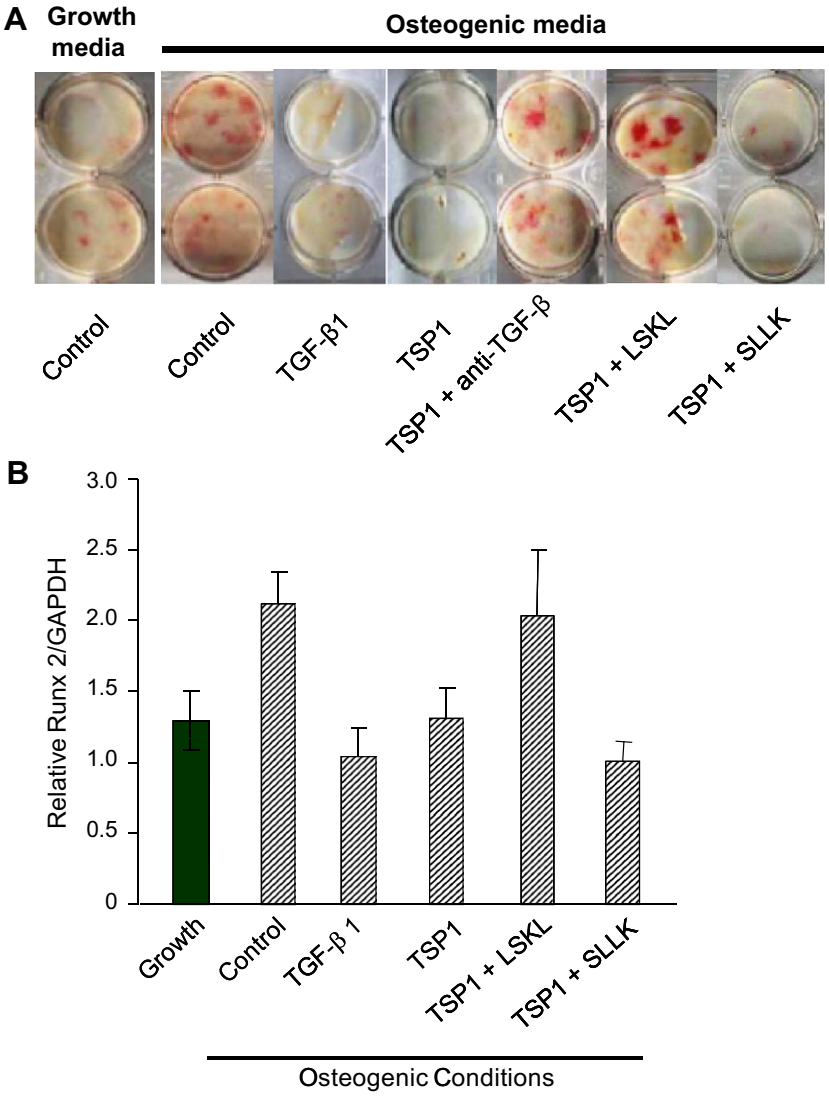


**Fig. 3.** TSP1 and TGF-β decrease osteoblast differentiation and TSP1 inhibitory peptide LSKL increases osteoblast differentiation by MSCs under osteogenic conditions. (A) MSCs were grown to confluence in basal (control) media. Cells were treated with control growth media, osteogenic media, or osteogenic media with TGF-β (5 ng/ml) or stripped TSP1 (10 nM), TSP1 + LSKL (25 μM inhibitory peptide), TSP1 + SLLK (25 μM control peptide), or TSP1 + anti-TGF-β (5 μg/ml). Cultures were fed daily for 20 days. Alkaline phosphatase staining is representative of triplicate wells. (B) Cells were grown in 6-well plates and treated every 2 days for 20 days with MSC growth media (growth), osteogenic media (control) or osteogenic media supplemented with TGF-β (5 ng/ml) or TSP1 (10 nM). Some TSP1 treated cultures were also treated daily with 25 μM LSKL or 25 μM SLLK control peptide. RNA was isolated from cells treated under these conditions and used for RT-PCR analysis of Runx2 expression. Samples were run in duplicate and each experimental treatment in triplicate.

fourfold increase in active TGF-β in the conditioned media, whereas levels of total TGF-β remained unchanged with TSP1 treatment (Fig. 1). TSP1 also increased phosphorylation of Smad 2 in MSCs (data not shown), a downstream mediator of TGF-β signaling, and induced the expression of α-smooth muscle actin and ED-A fibronectin, two TGF-β responsive genes (Supplemental Fig. 1).

### 3.2. Osteogenesis-inducing conditions decrease active TGF-β and, TSP1 expression by MSCs

Bone marrow derived progenitor cells contribute to bone remodeling [6,7]. Thus, we asked whether TSP1 regulates osteogenic differentiation of MSCs. The expression of TSP1 and TGF-β activity were assessed in MSCs stimulated to undergo osteoblast differentiation. After 30 days of culture, osteogenic differentiation was confirmed by increased alkaline phosphatase activity and by the appearance of mineralized nodules (Von Kossa staining) (data not shown). Upon osteoblastic differentiation, TSP1 expression is reduced approximately fivefold (Fig. 2A) and TGF-β activity is decreased by 50% as compared to cells in growth medium (Fig. 2B). The reduction in active TGF-β occurs despite a 40% increase in total TGF-β. The proportion of active TGF-β was reduced under osteogenic conditions (10% active) as compared to growth conditions (33% active). Consistent with reduced TSP1 and TGF-β activity, osteoblast-differentiated MSCs expressed less α-smooth muscle actin and ED-A fibronectin than cells maintained under growth conditions (data not shown). These results show that the TSP1–TGF-β pathway is down-regulated during late osteogenesis of MSCs and suggest that down-regulation of the TSP1–TGF-β axis might be required for terminal osteoblast differentiation of MSCs.

### 3.3. TGF-β and TSP1 inhibit osteoblast differentiation of MSCs

To address whether TSP1 inhibits osteoblastic differentiation of MSCs, cells were cultured under osteogenic conditions for 20 days

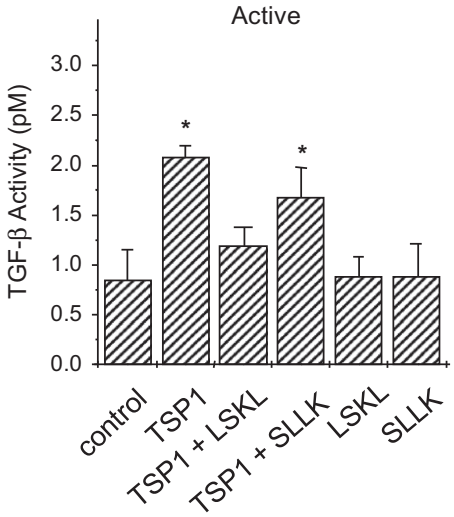


**Fig. 4.** LSKL peptide decreases active TGF-β in MSCs under osteogenic conditions. Cells were grown in 6-well plates and treated every 2 days for 20 days with osteogenic media (control) or with TGF-β (5 ng/ml) or TSP1 (10 nM). Some TSP1 treated cultures were also treated daily with 25 μM LSKL or 25 μM SLLK control peptide. Conditioned media were harvested and active TGF-β measured using the PAI-1 promoter reporter luciferase assay. Data are the means of triplicate determinations ± SD. $^{*}p < 0.05$ vs. control.

in the presence of exogenous TSP1. Both TGF-β and TSP1 inhibited osteogenic differentiation of MSCs as demonstrated by inhibition of alkaline phosphatase activity and by a decrease in Runx2 expression in MSCs grown under osteogenic conditions (Fig. 3A, B). TSP1 increased active TGF-β in the conditioned media of MSCs grown in osteogenic media, suggesting that the TSP1-mediated inhibition of osteogenesis might be mediated by TSP1-dependent latent TGF-β activation (Fig. 4). To test this hypothesis, we used a peptide (LSKL) antagonist of TSP1-dependent TGF-β activation. Treatment of MSCs grown in osteogenic media with LSKL blocked TSP1 inhibition of alkaline phosphatase and Runx2 expression (Fig. 3A, B). The control peptide, SLLK, had no effect on levels of Runx2 or alkaline phosphatase. In addition, neutralizing antibody to TGF-β reversed TSP1-mediated suppression of alkaline phosphatase activity (Fig. 3A). Notably, TSP1 and TGF-β also maintained expression of myofibroblast markers (ED-A fibronectin, α-smooth muscle actin,) in MSCs in osteogenic media (Supplemental Fig. 2). Together, these data indicate that TSP1 prevents osteogenic differentiation of MSC through stimulation of latent TGF-β activation.

## 4. Discussion

These studies demonstrate that TSP1 regulates osteoblast differentiation of MSCs through its ability to stimulate TGF-β activation. Under conditions which induce osteogenesis, TSP1 treatment prevented expression of osteoblast markers (alkaline phosphatase and Runx2) while maintaining expression of myofibroblast markers. Inhibition of osteoblast differentiation was reversed by the TSP1 antagonist peptide, suggesting that TSP1 antagonizes osteoblast differentiation through activating latent TGF-β.

The data presented here suggest that TSP1 is an important factor in limiting osteogenesis. TSP1 has been localized in the osteoid of under mineralized bone [38]. Vascular pericytes with osteogenic potential express increased TSP1 protein and mRNA during formation of bone nodules, but TSP1 expression decreases upon nodule mineralization [39]. Constitutive expression of TSP1 in MC3T3-E1 osteoblastic cells inhibits mineralization [25]. TSP1 mRNA in MC3T3-E1 osteoblastic cells increased in the first nine days of cell conversion (closely parallel to alkaline phosphatase activity) and dropped as differentiation progressed. These findings are consistent with our observations that MSCs have reduced TSP1 expression following 30 days under osteogenic conditions. Despite these observations, the mechanism by which TSP1 inhibits bone matrix mineralization has not been established. Our data now show that TSP1 acts by stimulating increased TGF-β activation.

TGF-ß1 stimulates osteoblast progenitor recruitment and proliferation, thus expanding the pool of committed osteoblasts: however, TGF-β blocks the later phases of differentiation and mineralization [5]. In part, this occurs through TGF-β activation of Smad 3, resulting in Smad 3 binding to Runx2, which inhibits Runx2-dependent transcription of osteoblast genes and of Runx2 itself [40]. This is consistent with our data showing that exogenous TGF-β1 and TSP1 inhibit Runx2 expression at 20 days.

It is not known if TSP1 regulated TGF-β can block osteogenic differentiation when presented to MSCs at a later stage of differentiation as our studies only examined the effects of continuous exposure to TSP1 in a heterogeneous mesenchymal cell environment. The effects of TGF-β on BMP-induced osteogenesis are complex and time dependent [41]. Future studies will determine whether there is a critical time during osteoblast differentiation at which MSCs become unresponsive to the TSP1–TGF-β switch.

The studies presented here identify a novel role for TSP1-dependent regulation of TGF-β in bone remodeling. The ability of TSP1 to inhibit osteogenic differentiation of MSCs and maintain myofibroblast traits further suggests that TSP1 might be important in processes where osteogenic repair is deficient or in metastatic bone disease [42]. Blockade of the TSP1-dependent TGF-β activation pathway might present a new strategy for modifying bone catabolism in repair and in metastatic disease.

## Acknowledgments

The authors would like to thank Mr. Antonio Pallero for general lab operations and Dr. Amjad Javed, School of Dentistry, UAB, for helpful discussions regarding measurement of Runx2. This work was support in part by grants to KBD from the Southern Regional Education Board (SREB) and the UAB Comprehensive Minority Faculty and Student Development Program (CMFSDP) and by NIH HL079644 and by a UAB CCC-ADDA grant to JEM-U. MZ was supported by start-up funds from the Department of Pathology. This investigation was conducted in a facility constructed with support from Research Facilities Improvement Program Grant No. C06RR15490 from the National Center for Research Resources, NIH.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.020.

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