HHS Public Access

Author manuscript

Bone. Author manuscript; available in PMC 2019 June 01.

Published in final edited form as:

Bone. 2018 June ; 111: 28–35. doi:10.1016/j.bone.2018.03.009.

Rescue of a cherubism bone marrow stromal culture phenotype by reducing TGFβ signaling

Yaling Liu[1], **Tulika Sharma**[1], **I-Ping Chen**[2], **Ernst Reichenberger**[1], **Yasuyoshi Ueki**[3], **Arif Yumna**[1], **Daniel Parisi**[1], and **Peter Maye**[1,*]

[1]Department of Reconstructive Sciences, School of Dental Medicine, University of Connecticut Health

[2]Department of Oral Health and Diagnostic Sciences, School of Dental Medicine

[3]Department of Oral and Craniofacial Sciences, School of Dentistry, University of Missouri

Abstract

We utilized a bone marrow stromal culture system to investigate changes in TGFβ signaling in a mouse model for cherubism *(Sh3bp2$^{KI/KI}$)*. Interestingly, bone marrow cultures derived from cherubism mice not only displayed impaired osteoblast differentiation, but also had spontaneous osteoclast formation. *PAI1*, a target gene of TGFβ signaling, was elevated 2-fold in cherubism CD11b^{-},CD45^{-} cells compared to wild type cells, while the expression of BAMBI, an inhibitor of TGFβ signaling, was down-regulated. We also discovered that treatment of cherubism cultures with antagonists of the TGFβ signaling pathway could largely rescue osteoblast differentiation and markedly reduce spontaneous osteoclast formation. Treatment with the type I TGFβ receptor small molecule inhibitor SB505124 increased osteoblast reporter gene *Col1a1*-2.3 expression 24-fold and increased the expression of osteoblast gene markers *Osterix* (*Sp7*) 25-fold, *Bone Sialoprotein* (*BSP)* 7-fold, *Osteocalcin* (*Bglap1*) 100-fold, and *Dentin Matrix Protein 1* (*DMP1)* 35-fold. In contrast, SB505124 treatment resulted in a significant reductions in osteoclast number and size. Gene expression analyses for RANKL, a positive regulator of osteoclast formation was 2.5-fold higher in osteoblast cultures derived from *Sh3bp2$^{KI/KI}$* mice compared to wild type cultures, whereas OPG, an inhibitor of RANKL was 5-fold lower. However, SB505124 treatment reduced RANKL almost back down to wild type levels, while increasing OPG expression. Our studies also implicate a role for TGFβ ligands in the etiology of cherubism. Blocking of TGFβ ligands with the monoclonal antibody 1D11 increased *Col1a1*-2.3 reporter expression 4-fold and 13-fold in cultures derived from *Sh3bp2$^{KI/+}$* and *Sh3bp2$^{KI/KI}$* mice, respectively. Serum levels of latent TGFβ1 were also 2-fold higher in *SH3BP2*$^{KI/KI}$ mice compared to wild type littermates. Taken together, these studies provide evidence that elevated levels of TGFβ signaling may contribute to

*Corresponding Author: Peter Maye, Department of Reconstructive Sciences, MC3705, L7007, School of Dental Medicine, University of Connecticut Health, 263 Farmington Avenue, Farmington, CT 06030, Phone: 860-679-7347, Fax: 860-679-2910, pmaye@uchc.edu, Website:http://regenerativemedicine.uchc.edu/faculty/bios/maye.html.

Disclaimers: None

the disease phenotype of cherubism and a reduction in pathway activity may be an effective therapeutic approach to treat this rare disease.

Keywords

Cherubism; Stromal Cell; Osteoblast; Osteoclast; Bone; TGFβ

Introduction

Cherubism is a rare congenital disease where the onset of symptoms can occur in children as early as 2 years of age. Characteristic features of the disease are enlargement of jaw bones because of the expansion of fibrous-osseous tissue, which often contains giant multinucleated cells that are TRAP positive [1, 2]. The range in disease severity is quite broad and the progression unpredictable. For most patients, treatment is limited to monitoring because, for reasons that remain unclear, facial lesions typically regress after puberty. However, patients retaining more aggressive forms of the disease often require surgery when the severe facial dysmorphology affects the function of jaw bones. Mandibular and maxillary tissue expansion also lead to disruption or loss of primary and secondary dentition, respiratory difficulties including nasal airway obstruction, and vision problems due to expansion of fibrous tissue into the orbital walls [3].

The gene for cherubism was mapped to *SH3BP2* [4], which encodes for an adaptor protein containing an N-terminal pleckstrin homology (PH) domain, a central SH3 binding domain, and a C-terminal SH2 domain. Mutations associated with cherubism are often located within a small region of the protein upstream of the SH2 domain. The most common mutation found in cherubism patients, a proline to arginine (P416R) mutation was engineered into the mouse *Sh3bp2* gene to create an animal model for cherubism ($Sh3bp2^{KI/KI}$) [5]. There is strong evidence that cherubic mutations in SH3BP2 prevent its interaction with Tankyrase 2, a poly ADP-ribose polymerase that normally targets the protein for degradation [6, 7]. Thus, excessive protein levels of the mutant form of SH3BP2 are thought to be a contributing factor of the disease.

Many characteristics of cherubism such as fibrous tissue expansion, impaired osteoblast maturation, and excessive osteoclast formation, are consistent with phenotypes that occur when TGFβ signaling is elevated. TGFβ signaling has long been associated with tissue fibrosis (reviewed in [8]). Furthermore, animal models with elevated levels of TGFβ signaling have a higher rate of bone turnover, display poor osteoblast maturation and increased osteoclast formation [9–11]. *In vitro* studies also have shown how treatment with TGFβ ligands can inhibit osteoblast differentiation and promote osteoclast formation [12–14]. In contrast, inhibition of TGFβ signaling in mice via a ligand blocking antibody (1D11) or a small molecule inhibitor (SD-208) has been shown to promote bone formation and repress bone resorption [15, 16].

As an initial way to investigate a possible connection between cherubism and TGFβ signaling, we used a bone marrow stromal cell culture model. Here we show that bone marrow cultures derived from $Sh3bp2^{KI/KI}$ mice have impaired osteoblast differentiation

and spontaneous osteoclast formation. We also provide evidence that TGFβ signaling is elevated in *Sh3bp2* $^{KI/KI}$ derived bone marrow cells and a reduction in TGFβ signaling via the use of a small molecule antagonist (SB505124) or blocking ligand antibody (1D11) can rescue this cell culture phenotype. We discuss the significance of our data with regard to understanding the biology and treatment of cherubism.

Materials and Methods

Animals

Sh3bp2$^{KI/KI}$ mice were intercrossed with *Col1a1*-2.3 EGFP mice to generate *Sh3bp2*$^{KI/+}$, *Col1a1*-2.3 EGFP mice. Male and female *Sh3bp2*$^{KI/+}$, *Col1a1*-2.3 EGFP were intercrossed to generated experimental animals. Offspring were genotyped to distinguish between wild type and the *Sh3bp2* knock-in allele using PCR primers (forward primer: 5′-CCACTATATGACAATACCTG-3′ and reverse primer: 5′-CATAGTCTTCATCTGAGTCC-3′. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Animal Care Committee of the University of Connecticut Health approved protocol 101060-0518.

Primary bone marrow stromal cell culture

Male and female mice 6 to 9 weeks of age were sacrificed by carbon dioxide asphyxiation followed by cervical dislocation. Femurs and tibia were dissected from the surrounding tissues. The epiphyseal growth plates were removed and the bone marrow was collected by flushing with αMEM culture medium containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS (Hyclone) with a 27-gauge needle. Single cell suspensions were prepared by gently mixing the cells with a pipette followed by filtration through a 70-μm strainer. Cells were centrifuged at 350 g for 10 min and plated at a density of 1.2×10^6 cells/cm^2 and grown in a 6 well dish. For assessing the impact of SB505124 (Cayman Chemical) and 1D11 (R&D Systems), reagents were added starting at day 3 of culture and were refreshed every other day with media changes. For osteogenic differentiation, cells were grown in αMEM medium with 10% FCS, 100 μ/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, and 8 mM 2-glycerol phosphate starting at day 7 of culture. Cultures were stopped at the indicated time points for gene expression analyses, FACS sorting, staining for mineral deposition using the von Kossa method, measuring calcium concentration, or for TRAP staining. Osteoclasts were detected using a Leukocyte (TRAP) Acid Phosphatase staining kit (Sigma-Aldrich) and defined as TRAP$^+$ cells with three or more nuclei. Calcium concentration was measured using a calcium assay kit (Cayman Chemical). Statistical significance was determined by comparing non-treated and vehicle treated groups to the SB505124 or 1D11 treated groups using a one-tailed t-test.

Gene expression analyses

RNA purification was carried out according to manufacturer's recommendations (Macherey-Nagel and Zymo Research). cDNA was prepared from 500ng of RNA/sample using the SuperScript II Reverse Transcriptase kit or Protoscript II kit (Life Technologies, NEB). QPCR was carried out using I-Taq Universal SybrGreen Supermix (BioRad) in a BioRad

CFX real time PCR machine. PCR primer sequences for gene expression analyses were: *Sp7* sense 5-GAGGAGTCCATTGGTGCTTGAGA-3, antisense 5-GGATGGCGTCCTCTCTGCTTGAG-3, *BGLAP1* sense 5-TCCAAGCAGGAGGGCAATAAG-3, antisense 5-GCGTTTGTAGGCGGTCTTCAAG-3, *BSP* sense 5-GAGAACTCCACACTTTCCACACT-3 antisense 5-CCACTTCTGCTTCTTCGTTCTCA-3, *DMP1* sense 5-CGCATCCCAATATGAAGACTG-3, antisense 5-GCTTGACTTTCTTCTGATGACTCA-3, *GAPDH* sense 5-AGGTCGGTGTGAACGGATTTG-3, antisense 5-TGTAGACCATGTAGTTGAGGTCA-3, *RANKL* sense 5-CAGAAGACAGCACTCACTGCT-3, antisense 5-CATTGATGGTGAGGTGTGCAA-3, *OPG* sense 5-GCAGAGACGCACCTAGCACTG-3, antisense 5-GCCAGCTGTCCGTATAAGAGT-3, *TGFβ1* sense 5-GGAGAGCCCTGGATACCAAC-3, antisense 5-CAACCCAGGTCCTTCCTAAA-3, *TGFβ2* sense 5-ATAAAATCGACATGCCGTCC-3, antisense 5-TTGTTGAGACATCAAAGCGG-3, *TGFβ3* sense 5-ATTCGACATGATCCAGGGAC-3, antisense 5-TCTCCACTGAGGACACATTGA-3, *BAMBI* sense 5-TCGCCACTCCAGCTACTTCT-3, antisense AAGCAGGCACTAAGCTCAGACT, *PAI1* sense 5-GCCTCCTCATCCTGCCTAA-3, antisense 5-TGCTCTTGGTCGGAAAGACT-3. *ACP5* sense 5-CTGGAGTGCACGATGCCAGCGACA-3, antisense 5-TCCGTGCTCGGCGATGGACCAGA-3.

FACS Sorting

Bone marrow stromal cultures derived from wild type and Sh3bp2$^{KI/KI}$ mice were grown for 7 days in culture. On day 7 adherent cells were washed twice with PBS, digested with Accutase (Innovative Cell Technologies) for 5 minutes at 37°C, gently scraped and pipetted up and down to generate a single cell suspension. An equal volume of FACS staining media (1x HBSS, 10mM HEPES, 2% FCS, 2 mM EDTA, pH 7.4) was added and cells were centrifuged and resuspended in FACS staining buffer. Cell surface staining was carried out with anti-Sca1-APC and anti-CD45-FITC or anti-CD11B-APC and anti-CD45-FITC according to manufacturer's recommendations (Miltenyi Biotech). FACS sorting was carried out on a Becton-Dickinson FACSARIA II (UCH Flow Cytometry Core).

Microscopy, Imaging, and Quantitation

Cells in culture were imaged using a Zeiss Observer Z.1 inverted microscope. EGFP fluorescence was detected using ET525/50x, ET470/40m and T495LP filter set (Chroma Technology). Images were captured using an Axiocam MRc digital camera and Zen software (Zeiss). EGFP pixel area measurements were carried out using ImageJ software or Zen.

ELISA for TGFβ1

Immediately after euthanization, whole blood was collected by cardiac puncture by inserting a 25G needle perpendicular into the chest wall. Blood was collected into a capillary collecting tube with separating gel (Becton Dickinson) and allowed to clot for 30 minutes followed by centrifugation for 2 minutes at 10,000 X g to separate clotted blood components from serum. Serum was pipetted into a new tube and frozen at −80° C. TGFβ1 levels were

measured using the LEGEND MAX™ Mouse Latent TGF-β ELISA Kit (BioLegend) according to manufacturer's protocol. Samples were read on a Safire^2 plate reader (Tecan) and values were grouped based on genotype to calculate the average and standard error. Statistical significance was calculated using a two-tailed t-test.

Results

Bone marrow stromal cultures derived from Sh3bp2$^{KI/KI}$ display impaired osteoblast differentiation and spontaneous osteoclast formation

The overgrowth of fibrous tissue and presence of Tartrate Resistant Acid Phosphatase (TRAP) positive multi-nucleated cells in the facial bones of cherubism patients suggests a complex cross-talk exists between mesenchymal and hematopoietic lineages. Therefore, using a mouse model for cherubism (*Sh3bp2$^{KI/KI}$*), we decided to exploit the features of a bone marrow stromal culture to investigate differences in crosstalk between mesenchymal and hematopoietic cell types because this culture system contains an abundant mix of both cell lineages.

When stromal progenitor cells derived from the marrow of 6 to 8-week-old *Sh3bp2$^{KI/KI}$* mice were grown under osteogenic conditions, severe defects in osteoblast differentiation were apparent compared to their wild type (WT) littermates (Fig. 1). For these studies, osteoblast differentiation was examined in three different ways. First, a *Collagen 1a1* 2.3-EGFP (*Col1a1*-2.3) transgenic mouse line was used to visualize and quantify mature osteoblasts [17, 18] by intercrossing with *Sh3bp2$^{KI/+}$* mice (Fig. 1A–D). Second, mineral deposition was qualitatively evaluated by von Kossa and quantitatively measured using a calcium content assay (Fig. 1E,F). Finally, the expression of osteoblast marker genes *Osterix* (*Sp7*), *Bone Sialoprotein* (*BSP*), *Dentin Matrix Protein 1* (*DMP1*) and *Osteocalcin* (*BGLAP1*) were examined by quantitative RT-PCR (Fig. 1G).

Quantification of *Collagen 1a1* reporter expression at day 13 of osteoblast differentiation showed that mature osteoblast formation was 8-fold lower in cultures derived from *Sh3bp2$^{KI/KI}$* mice compared to wild type mice (Fig. 1A and B). It is worth noting that the extent of fold difference in comparing *Sh3bp2$^{KI/KI}$* to wild type can be correlated with the stage of differentiation as later time points typically showed greater fold differences than earlier timepoints (data not shown). Having said that, obtaining data from *Sh3bp2$^{KI/KI}$* cultures past 15 days of osteogenic differentiation was often difficult because cell detachment from the tissue culture surface was a frequent occurrence.

At higher magnification, the viewing of Col1a1-2.3$^+$ cells from *Sh3bp2$^{KI/KI}$* cultures revealed significant differences in cellular morphology (Fig. 1C,D). While Col1a1-2.3$^+$ osteoblasts derived from wild type mice displayed a more cuboidal shape and robust reporter expression (Fig. 1C), the much fewer *Sh3bp2$^{KI/KI}$* Col1a1-2.3$^+$ osteoblasts that formed often exhibited a fibroblast-like spindle shaped morphology and exhibited decreased reporter expression (Fig. 1D, white arrow). While quantitation of osteoblast reporter expression was dramatically reduced, examination of mineral deposition was less dramatic, but still noticeably reduced (Fig. 1E,F). Cherubism cultures consistently showed reduced mineral deposition as assayed by von Kossa (Fig. 1E). Quantitative assessment of mineral deposition

using a calcium content assay varied with *Sh3bp2*$^{KI/KI}$ cultures roughly having half the calcium content compared to wild type cultures (Fig. 1F). Again, the ability to assay for mineral content of cherubism cultures was challenged by the frequent occurrence of cell detachment with more pronounced deficits in cellular differentiation being more likely to detach. Thus, the measured outcomes for mineral content represent the best performing cherubism cultures.

Consistent with decreased osteoblast reporter expression and decreased mineral deposition, gene markers of osteoblast differentiation were also substantially lower (Fig. 1G). Levels of *Osteocalcin* (*BGLAP1*) and *Bone Sialoprotein* (*IBSP*) were >14-fold lower in cherubism cultures, while *Dentin Matrix Protein 1* (*DMP1*) and *Osterix* (*Sp7*), were >28-fold lower.

While it has been shown that myeloid cells derived from cherubism mice are more sensitive to levels of Rankl in promoting osteoclast formation [5], no one has reported the spontaneous formation of osteoclasts in mixed primary cultures. Unexpectedly, we observed osteoclast formation in stromal cultures derived from *Sh3bp2*$^{KI/KI}$ mice without the addition of specific inducers (Fig. 1H,I). To more accurately quantify osteoclast formation, TRAP staining was carried out after seven days in culture. Osteoclasts were defined as TRAP^{+} cells with three or more nuclei. While bone marrow cultures derived from wild type mice showed no signs of osteoclast formation, numerous osteoclasts were present in cultures derived from *Sh3bp2*$^{KI/KI}$ mice (Fig. 1J). Finally, examination of the hematopoietic (CD45^{+},Sca1^{+}) and mesenchymal (CD45^{-}, Sca1^{+}) cell fractions at day 7 of culture indicated no significant differences between wild type versus cherubism samples suggesting that the deficits in osteoblast differentiation and, in turn, robust osteoclast formation that occurred in cherubism cultures was not due to detectable differences in cell numbers (Fig. 1K). Although a more detailed characterization of mesenchymal and hematopoetic cell types present in culture is warranted.

Evidence for Increased TGFβ Signaling in Sh3bp2$^{KI/KI}$ bone marrow stromal cultures

The decreased osteoblast differentiation and spontaneous osteoclast formation observed in *Sh3bp2*$^{KI/KI}$ bone marrow cultures suggested to us that the cross-talk between mesenchymal hematopoietic cell types has markedly changed relative to cultures derived from wild type mice. Work by others have shown how TGFβ signaling can repress osteoblast formation and promote osteoclast formation [9, 12, 19, 20]. Along these lines, other studies have shown how antagonism of TGFβ signaling can promote bone formation and repress bone resorption [11, 15, 16]. Given the phenotype of the *Sh3bp2*$^{KI/KI}$ bone marrow stromal culture and understanding the effect of TGFβ signaling on these processes, we sought to determine whether TGFβ signaling was elevated in *Sh3bp2*$^{KI/KI}$ cells. To assay for levels of TGFβ signaling, we evaluated the expression of *Plasminogen Activator Inhibitor 1* (*PAI1*), a known target gene of TGFβ signaling. However, in unsorted cultures the level of *PAI1* was only slightly higher in *Sh3bp2*$^{KI/KI}$ cells compared to wild type cells (Fig. 2A, compare red bars to black). To provide evidence that *PAI1* is positively regulated by TGFβ signaling in this culture model, we added SB505124 (SB), a small molecule type I TGFβ receptor inhibitor [21]. Upon treatment with SB, levels of PAI1 decreased by more than half compared to no treatment (NT) and vehicle (V) treatment (Fig. 1A); thus, substantiating the positive

regulation of *PAI1* expression by TGFβ signaling. Therefore, knowing the cellular makeup of bone marrow cultures is highly heterogeneous, we decided to examine *PAI1* expression in both hematopoietic and mesenchymal cell fractions by FACS sorting for CD11b and CD45 (Fig. 2B). FACS sorting revealed that *PAI1* was 2-fold higher in the $Sh3bp2^{KI/KI}$ CD11b^{-},CD45^{-} mesenchymal cell fraction compared to wild type mesenchymal cells (Fig. 2B). Complementary to the increase in *PAI1* expression, we also discovered that *BAMBI*, a negative regulator of TGFβ signaling was down-regulated in $Sh3bp2^{KI/KI}$ derived cells (Fig. 2C). Taken together, these gene expression studies suggested that TGFβ signaling was elevated at least in $Sh3bp2^{KI/KI}$ mesenchymal cells, if not both populations.

Impaired osteoblast differentiation from Sh3bp2$^{KI/KI}$ bone marrow stromal cultures is rescued by antagonizing type I TGFβ receptor signaling

Understanding the negative impact elevated TGFβ signaling can have on osteoblast differentiation, we reduced levels of TGFβ signaling by treating cultures with the small molecule type I TβR1 inhibitor, SB505124. Initial studies sought to determine whether a specific dosage range of SB505124 existed that could optimally rescue osteoblast differentiation (Fig. 3). These repeated dosage studies revealed that a concentration between 0.1–0.25μM of SB505124 resulted in an optimal increase in osteoblast differentiation in $Sh3bp2^{KI/KI}$ cells based on *Col1a1*-2.3 reporter expression. which showed 23-fold higher over control samples at day 15 of differentiation (Fig. 3A (green fluorescence) and B). Examination of mineral deposition by von Kossa staining (Fig. 3A, top panel) and calcium content (sFig. 1) also indicated a dosage range between 0.1–0.25uM was optimal. The rescue of osteoblast formation could also be appreciated at higher magnification, where cultures treated with SB505124 had Col1a1-2.3^{+} cells with a cuboidal cell morphology and strong reporter expression (Fig. 3C). Comparatively, $Sh3bp2^{KI/KI}$ cells with vehicle or without treatment retained few *Col1a1*-2.3^{+} cells that appeared fibroblast-like and had weak reporter expression (Fig. 3C). The ability of SB505124 to rescue osteoblast differentiation was also substantiated through gene expression analyses (Fig. 3D). By day 12 of differentiation, SB505124 treatment resulted in remarkable increases in the expression of osteoblast marker genes in $Sh3bp2^{KI/KI}$ samples (Fig. 3D). Relative to vehicle, SB505124 treated samples increased the expression of *Sp7* 25-fold, *BSP* 7-fold, *Bglap1* 100-fold, and *DMP1* 35-fold. Wild type samples also showed significant increases in response to treatment, but understandably not as much as $Sh3bp2^{KI/KI}$ cells.

Evidence that TGFβ ligands contribute to the cherubism phenotype

Type I TGFβ receptors (TβR1/ALK5, ACVR1b/ALK4, and ACVR1c/ALK7) can be activated by several different ligands including TGFβs 1, 2, and 3, Activins and certain GDFs [22–25]. Thus, inhibition of type I TGFβ receptors with SB505124, does not provide evidence for which ligand(s) may be involved. However, based on work by others [16, 26, 27], we speculated that TGFβ 1 is likely to be the primary ligand contributing to the cherubism phenotype. Additionally, the dosage of SB505124 used in our studies has been shown to be twice as effective at repressing TGFβ signaling compared to pathway stimulation by Activin [21]. Therefore, we examined the gene expression of TGFβ ligands and found that all three TGFβ ligands were expressed (Fig. 4A–C). *TGFβ1* was expressed in both the CD11b^{+},CD45^{+} and CD11b^{-},CD45^{-} cell fraction, while *TGFβ2* and *TGFβ3* were

selectively expressed in the CD11b^{-},CD45^{-} mesenchymal cell fraction. Interestingly, mRNA expression levels of *TGFβ1* were noticeably lower in *Sh3bp2*$^{KI/KI}$ cells. Understanding that this data potentially contradicted our *PAI1* and *BAMBI* expression data (Fig. 2), we investigated the possibility of whether a negative feedback loop exists where levels of TGFβ signaling can negatively influence the level of *TGFβ1* expression. To test this idea, we examined *TGFβ1* expression in the presence and absence of SB505124 (SB) treatment (Fig. 4D). These studies revealed that SB treatment resulted in a 2-fold increase in *TGFβ1* expression over vehicle (v) treatment in both wild type and *Sh3bp2*$^{KI/KI}$ cells (Fig. 4D). Thus, in the bone marrow stromal culture model, levels of TGFβ signaling can negatively feedback on the level of *TGFβ1* expression.

To functionally examine the role of TGFβ ligands, we decided to treat cherubism stromal cultures with 1D11, a pan-TGFβ functional blocking monoclonal antibody (Fig. 4E,F). These studies revealed that 1D11 at a concentration of 4ug/ml resulted in significant rescue of osteoblast differentiation as revealed by *Col1a1*-2.3 reporter expression at day11 of osteoblast differentiation (Fig. 4E, bright green regions). *Sh3bp2*$^{KI/+}$ and *Sh3bp2*$^{KI/KI}$ cultures showed a >7.5-fold and >5.5-fold increase in *Col1a1*-2.3 reporter expression, while wild type cultures only showed an increase of <3-fold (Fig. 4F). Examination of calcium content also indicated that 1D11 treatment increased osteoblast differentiation of cherubism cultures (sFig. 1). Because 1D11 was effective at rescuing osteoblast differentiation of cherubism stromal cultures, we decided to examine the *in vivo* levels of TGFβ1 in the circulation of *Sh3bp2*$^{KI/KI}$ mice relative to wild type mice. These studies revealed that levels of TGFβ1 were almost 2-fold higher in *Sh3bp2*$^{KI/KI}$ mutants (n=9) compared to wild type littermates (n=8) (Fig. 4G), further implicating a role for TGFβ ligands in cherubism.

Antagonism of TGFβ signaling markedly reduced osteoclast formation in *Sh3bp2*$^{KI/KI}$ bone marrow stromal cultures

While the formation of osteoclasts is not normally observed in bone marrow cultures, robust osteoclast formation was apparent in bone marrow cells derived from *Sh3bp2*$^{KI/KI}$ mice (Fig. 1F,G). Work by others have shown that TGFβ signaling can promote osteoclast formation either by directly signaling to osteoclasts and/or indirectly signaling through mesenchymal cells [13, 14, 19, 20, 28, 29]. In agreement with these studies, antagonism of TGFβ signaling resulted dramatic reductions in osteoclast formation and size. Treatment with 0.25μM SB505124 on days 3, 6, and 9 and assayed at day 10 of culture resulted in a 5-fold reduction in the number of osteoclasts formed per well compared to vehicle and control samples (Fig. 5A). Treatment with SB505124 and 1D11 at days 3 and 5 of culture and assessed at day 7 also reduced osteoclast numbers by more than half (Fig. 5B). The 3+ nuclei criteria for counting osteoclasts under-represented the noticeable impact TGFβ pathway antagonism had on osteoclast formation as viewed in culture (Fig. 5 compare C to D,E). Further, while SB505124 and 1D11 were both effective in reducing osteoclast number and size, qualitatively, SB505124 appeared to be slightly more potent than 1D11.

Further substantiating TRAP staining, we examined the expression of *ACP5*, the gene that encodes for TRAP in cultures treated with SB505124 and 1D11 (Fig. 5F,G). *ACP5* expression was highly expressed in cherubism derived cultures and SB505124 and 1D11

treatment reduced *ACP5* expression by ~3.5 and ~3.0 fold, respectively. Interestingly, gene expression analyses for *RANKL*, a critical regulator of osteoclast formation, was 2.5-fold higher in cultures derived from *Sh3bp2*$^{KI/KI}$ mice compared to wild type cultures (Fig. 5H). In contrast, expression of *OPG*, an inhibitor of *RANKL* was 5-fold lower in cultures derived from *Sh3bp2*$^{KI/KI}$ mice compared to wild type littermates (Fig. 5I). However, treatment with SB505124 reduced *RANKL* expression almost back down to wild type levels (Fig. 5G), while resulting in increased *OPG* expression (Fig. 5I), thereby providing at least one explanation for how antagonizing TGFβ signaling reduced osteoclast formation.

Discussion

The histological features of cherubism suggest a complex cross-talk exists between hematopoietic and mesenchymal cell types that results in excessive bone erosion and fibrous tissue overgrowth. While not a source of tissue from the craniofacial region, we decided to use a bone marrow stromal culture model to study cherubism because both hematopoietic and mesenchymal lineages abundantly exist thereby providing a way to study cross-talk amongst these two cell populations. Interestingly, our studies have yielded striking differences of bone marrow cultures derived from *Sh3bp2*$^{KI/KI}$ mice compared to their wild type littermates. Bone marrow cultures derived from *Sh3bp2*$^{KI/KI}$ mice showed impaired osteoblast differentiation and spontaneous osteoclast formation. Furthermore, our studies have indicated that TGFβ signaling is elevated in *Sh3bp2*$^{KI/KI}$ cells and that a reduction in TGFβ pathway activity markedly improved osteoblast differentiation while reducing osteoclast formation. Below we discuss this work and its significance to the biology and treatment of cherubism.

Impaired osteogenesis and spontaneous osteoclast formation reveals differences in cross-talk between mesenchymal and hematopoietic cell types

An increasing body of work has shown how the mechanisms regulating osteoblast and osteoclast differentiation are highly interconnected [30–32]. While the dysregulation of *RANKL* and *OPG* gene expression (Fig. 5H,I) explained the spontaneous formation of osteoclasts, it did not explain how osteogenesis was impaired. However, work by others have shown how TGFβ signaling can repress osteoblast differentiation and enhance osteoclast formation [9, 11, 12, 14–16]. This led us to consider the TGFβ signaling pathway as a possible contributor to the phenotype displayed in cherubism cultures. Interestingly, expression of *PAI1*, a target gene of TGFβ signaling was 2-fold higher in the mesenchymal cell fraction, while *BAMBI,* an inhibitor to TGFβ signaling was down regulated in both the mesenchymal and hematopoietic cell fractions (Fig. 2). We also noted that levels of *TGFβ1* mRNA were also lower in cherubism cultures (Fig. 4A). However, with pathway repression, *TGFβ1* expression increased (Fig. 4D) further reinforcing the evidence that TGFβ signaling is elevated in *Sh3bp2*$^{KI/KI}$ cells and also revealing that negative feedback mechanisms exist within this culture system.

In contrast to the detection of lower levels of TGFβ1 mRNA in cherubism cells *in vitro*, elisa data showed that protein levels of TGFβ1 were higher in serum derived from cherubism mice. While this contradicts our *in vitro* mRNA expression data for TGFβ1, the

correlation of mRNA levels to protein levels is not always consistent. Particularly, when you consider the complex regulation and storage of TGFβ ligands within the extracellular matrix of soft tissues and in bone tissue, which are rich depots of stored TGFβ ligands. With that in mind, it is a tempting idea to consider bone tissue as a potential source of TGFβ ligands in a disease like cherubism where excessive bone resorption liberates TGFβs into the local bone environment to promote fibrous tissue over growth and greater osteoclast formation. However, TGFβ ligands are expressed in a variety of cell types. Therefore, it is possible that other cell types within the microenvironment of fibro-osseous lesions are also a source of TGFβ ligands.

Contemplating the Therapeutic Targeting of TGFβ Signaling

The possibility of targeting TGFβ signaling as a therapeutic approach to treat cherubism would be different than the current focus which has been on inflammation. Homozygous *Sh3bp2*$^{KI/KI}$ mice have systemically elevated levels of TNFα, which has prompted research and treatments to concentrate on inflammation [5]. Unfortunately, animal studies and a small pilot study with two patients with cherubism indicated that treatment with TNFα antagonists after the onset of cherubic symptoms did not result in the regression of existing fibrous lesions nor prevent their further expansion [33, 34]. Therefore, alternative and/or combinatorial therapies to treat cherubism are needed.

The development of drugs to selectively target components of the TGFβ pathway has been an active area of research because TGFβ signaling is thought to be a major contributor to a variety of diseases. Most of these diseases occur at a much higher incidence than cherubism and include different fibrotic conditions and cancers, Marfan syndrome, osteogenesis imperfecta [35], neurofibromatosis [36], Camurati-Engelmann [26], muscular dystrophy [37], and renal osteodystrophy [27]. Therefore, the safety and efficacy of anti-TGFβ drugs will likely be learned through clinical trials to combat these diseases. Using TGFβ as a key word search in clinicaltrials.gov turns up 326 registered studies. The range of pharmacological approaches being tested include small molecule inhibitors, monoclonal antibodies, antisense oligonucleotides and competitive peptides [38]. The ubiquitous role of TGFβ in numerous physiological processes, no doubt, will make it extremely challenging to target this pathway for therapy. However, outcomes from some of the phase I studies have been encouraging indicating that many anti-TGFβ drugs are reasonably tolerated. Future clinical studies will determine the efficacy of different TGFβ therapies and preclinical studies in Sh3bp2$^{KI/KI}$ mice should be pursued to provide some evidence of whether anti-TGFβ therapy may be an effective approach to treat cherubism.

Acknowledgments

This work was supported by a grant from NIAMS 1R21 DE025933 and a stimulus grant from the University of Connecticut.

References

1. Papadaki ME, et al. Cherubism: best clinical practice. Orphanet J Rare Dis. 2012; 7(Suppl 1):S6. [PubMed: 22640403]

2. Reichenberger EJ, et al. The role of SH3BP2 in the pathophysiology of cherubism. Orphanet J Rare Dis. 2012; 7(Suppl 1):S5. [PubMed: 22640988]
3. Raposo-Amaral CE, et al. Two-Stage Surgical Treatment of Severe Cherubism. Annals of Plastic Surgery. 2007; 58(6):645–651. [PubMed: 17522488]
4. Ueki Y, et al. Mutations in the gene encoding c-Abl-binding protein SH3BP2 cause cherubism. Nat Genet. 2001; 28(2):125–6. [PubMed: 11381256]
5. Ueki Y, et al. Increased myeloid cell responses to M-CSF and RANKL cause bone loss and inflammation in SH3BP2 "cherubism" mice. Cell. 2007; 128(1):71–83. [PubMed: 17218256]
6. Levaot N, et al. Loss of Tankyrase-mediated destruction of 3BP2 is the underlying pathogenic mechanism of cherubism. Cell. 2011; 147(6):1324–39. [PubMed: 22153076]
7. Guettler S, et al. Structural basis and sequence rules for substrate recognition by Tankyrase explain the basis for cherubism disease. Cell. 2011; 147(6):1340–54. [PubMed: 22153077]
8. Rockey DC, Bell PD, Hill JA. Fibrosis--a common pathway to organ injury and failure. N Engl J Med. 2015; 372(12):1138–49. [PubMed: 25785971]
9. Erlebacher A, Derynck R. Increased expression of TGF-beta 2 in osteoblasts results in an osteoporosis-like phenotype. J Cell Biol. 1996; 132(1–2):195–210. [PubMed: 8567723]
10. Erlebacher A, et al. Osteoblastic responses to TGF-beta during bone remodeling. Mol Biol Cell. 1998; 9(7):1903–18. [PubMed: 9658179]
11. Filvaroff E, et al. Inhibition of TGF-beta receptor signaling in osteoblasts leads to decreased bone remodeling and increased trabecular bone mass. Development. 1999; 126(19):4267–79. [PubMed: 10477295]
12. Alliston T, et al. TGF-beta-induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. EMBO J. 2001; 20(9):2254–72. [PubMed: 11331591]
13. Karst M, et al. Roles of stromal cell RANKL, OPG, and M-CSF expression in biphasic TGF-beta regulation of osteoclast differentiation. J Cell Physiol. 2004; 200(1):99–106. [PubMed: 15137062]
14. Gingery A, et al. TGF-beta coordinately activates TAK1/MEK/AKT/NFkB and SMAD pathways to promote osteoclast survival. Exp Cell Res. 2008; 314(15):2725–38. [PubMed: 18586026]
15. Mohammad KS, et al. Pharmacologic inhibition of the TGF-beta type I receptor kinase has anabolic and anti-catabolic effects on bone. PLoS One. 2009; 4(4):e5275. [PubMed: 19357790]
16. Edwards JR, et al. Inhibition of TGF-beta signaling by 1D11 antibody treatment increases bone mass and quality in vivo. J Bone Miner Res. 2010; 25(11):2419–26. [PubMed: 20499365]
17. Dacic S, et al. Col1a1-driven transgenic markers of osteoblast lineage progression. J Bone Miner Res. 2001; 16(7):1228–36. [PubMed: 11450698]
18. Kalajzic I, et al. Stage specific inhibition of osteoblast lineage differentiation by FGF2 and noggin. J Cell Biochem. 2003; 88(6):1168–76. [PubMed: 12647299]
19. Stern PH, et al. Human transforming growth factor-alpha stimulates bone resorption in vitro. J Clin Invest. 1985; 76(5):2016–9. [PubMed: 3877079]
20. Ibbotson KJ, et al. Human recombinant transforming growth factor alpha stimulates bone resorption and inhibits formation in vitro. Proc Natl Acad Sci U S A. 1986; 83(7):2228–32. [PubMed: 3485799]
21. DaCosta Byfield S, et al. SB-505124 is a selective inhibitor of transforming growth factor-beta type I receptors ALK4, ALK5, and ALK7. Mol Pharmacol. 2004; 65(3):744–52. [PubMed: 14978253]
22. ten Dijke P, et al. Characterization of type I receptors for transforming growth factor-beta and activin. Science. 1994; 264(5155):101–4. [PubMed: 8140412]
23. Attisano L, et al. Activation of signalling by the activin receptor complex. Mol Cell Biol. 1996; 16(3):1066–73. [PubMed: 8622651]
24. Mazerbourg S, et al. Growth differentiation factor-9 signaling is mediated by the type I receptor, activin receptor-like kinase 5. Mol Endocrinol. 2004; 18(3):653–65. [PubMed: 14684852]
25. Chang HM, et al. Growth differentiation factor 8 down-regulates pentraxin 3 in human granulosa cells. Mol Cell Endocrinol. 2015; 404:82–90. [PubMed: 25641196]
26. Tang Y, et al. TGF-beta1-induced migration of bone mesenchymal stem cells couples bone resorption with formation. Nat Med. 2009; 15(7):757–65. [PubMed: 19584867]

27. Liu S, et al. Role of TGF-beta in a mouse model of high turnover renal osteodystrophy. J Bone Miner Res. 2014; 29(5):1141–57. [PubMed: 24166835]
28. Yasui T, et al. Regulation of RANKL-induced osteoclastogenesis by TGF-beta through molecular interaction between Smad3 and Traf6. J Bone Miner Res. 2011; 26(7):1447–56. [PubMed: 21305609]
29. Omata Y, et al. Genomewide comprehensive analysis reveals critical cooperation between Smad and c-Fos in RANKL-induced osteoclastogenesis. J Bone Miner Res. 2015; 30(5):869–77. [PubMed: 25431176]
30. Lian JB. Mysterious cross talk between bone cells. J Clin Invest. 1996; 98(8):1697–8. [PubMed: 8878417]
31. Rho J, Takami M, Choi Y. Osteoimmunology: interactions of the immune and skeletal systems. Mol Cells. 2004; 17(1):1–9. [PubMed: 15055519]
32. Matsuo K. Cross-talk among bone cells. Curr Opin Nephrol Hypertens. 2009; 18(4):292–7. [PubMed: 19395964]
33. Hero M, et al. Anti-tumor necrosis factor treatment in cherubism--clinical, radiological and histological findings in two children. Bone. 2013; 52(1):347–53. [PubMed: 23069372]
34. Yoshitaka T, et al. Etanercept administration to neonatal SH3BP2 knock-in cherubism mice prevents TNF-alpha-induced inflammation and bone loss. J Bone Miner Res. 2014; 29(5):1170–82. [PubMed: 24978678]
35. Grafe I, et al. Excessive transforming growth factor-beta signaling is a common mechanism in osteogenesis imperfecta. Nat Med. 2014; 20(6):670–5. [PubMed: 24793237]
36. Rhodes SD, et al. Hyperactive transforming growth factor-beta1 signaling potentiates skeletal defects in a neurofibromatosis type 1 mouse model. J Bone Miner Res. 2013; 28(12):2476–89. [PubMed: 23703870]
37. Heydemann A, et al. Latent TGF-beta-binding protein 4 modifies muscular dystrophy in mice. J Clin Invest. 2009; 119(12):3703–12. [PubMed: 19884661]
38. Akhurst RJ, Hata A. Targeting the TGFbeta signalling pathway in disease. Nat Rev Drug Discov. 2012; 11(10):790–811. [PubMed: 23000686]

Highlights

- Bone marrow stromal cultures derived from cherubism mice display deficient osteogenesis and robust osteoclast formation.
- Levels of TGFβ signaling are higher in bone marrow stromal cultures derived from cherubism mice.
- Repression of TGFβ signaling rescues osteoblast differentiation and reduces osteoclast formation.

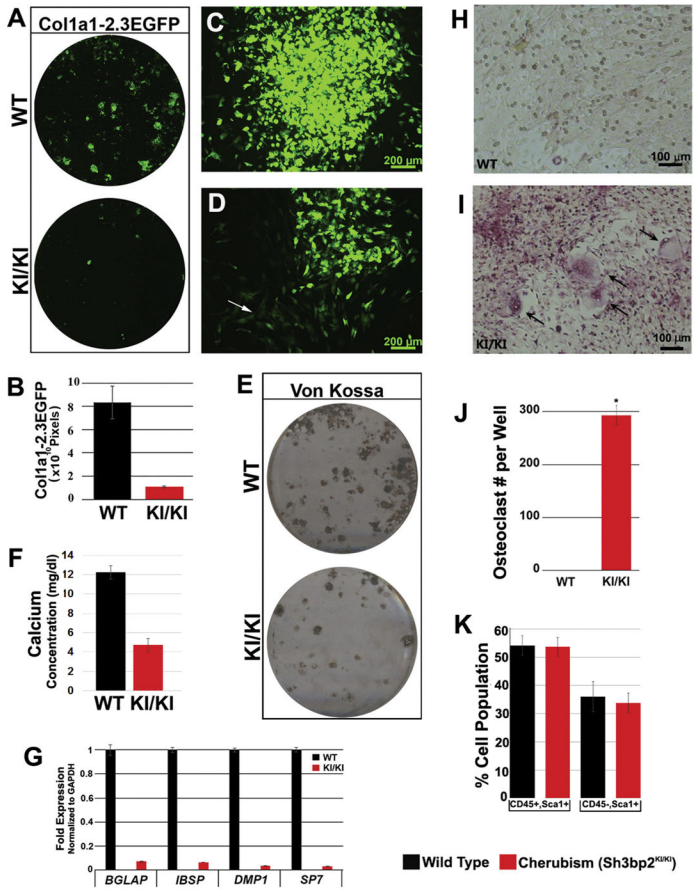

Figure 1. Sh3bp2$^{KI/KI}$ derived bone marrow stromal cultures display impaired osteoblast differentiation and spontaneous osteoclast formation

(A,B) Imaging and quantitation of osteoblast reporter gene Col1a1-2.3 EGFP expression at day 13 of differentiation. **(A)** Upper panel shows representative image of bone marrow cells derived from wild type mice. Lower panel shows representative image of bone marrow cells derived from cherubism mice. **(B)** Quantitation of osteoblast reporter expression reveals an 8-fold reduction in osteoblast formation in bone marrow cells derived from cherubism mice. **(C,D)** Comparison of wild type (upper panel) and Sh3bp2$^{KI/KI}$ (lower panel) Col1a1-2.3 reporter expression in osteoblast colonies at higher magnification revealed how many Col1a1-2.3+ cells from cherubism mice had lower levels of reporter expression and a fibroblastic-like cell morphology. **(E)** Detection of mineral deposition at day 13 of differentiation by von Kossa staining. Wild type cultures (upper panel) showed greater mineral deposition than cherubism cultures (lower panel). **(F)** Quantitative measurement of mineral deposition by assaying for calcium in cultures derived from wild type and Sh3bp2$^{KI/KI}$ mice. **(G)** Comparison of osteoblast gene marker expression between wild type and cherubism cultures at day 13 of differentiation (*Osteocalcin* (*Bglap1*), *Bone Sialoprotein* (*BSP*), *Dentin Matrix Protein 1* (*DMP1*), and *Osterix* (*Sp7*)). **(H, I)** Detection of osteoclast formation by TRAP staining and counting of TRAP+ cells with three or more nuclei **(J)**. Osteoclasts typically do not form in wild type bone marrow stromal cultures **(H**, upper panel**)**, but become readily apparent in cherubism cultures **(I**, lower panel, black arrows**).**

(K) FACS analyses of day 7 wild type and Sh3bp2$^{KI/KI}$ derived bone marrow stromal cultures stained for CD45^{+},Sca1^{+} (hematopoietic) and CD45^{-}, Sca1^{+} (mesenchymal) cell populations. For graphs, wild type data and Sh3bp2$^{KI/KI}$ data is denoted by black bars and red bars, respectively.

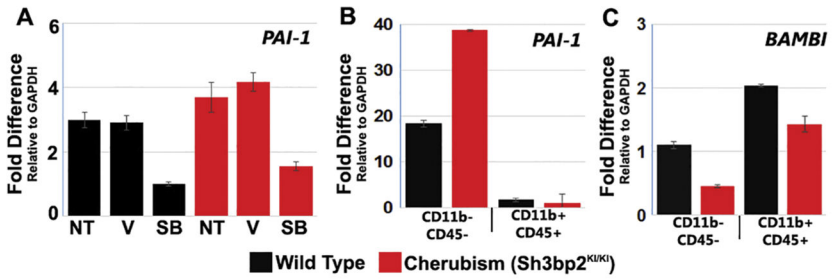

Figure 2. Increased TGFβ Signaling in Sh3bp2$^{KI/KI}$ bone marrow stromal cultures

(A) Comparison of *PAI1* expression levels in wild type (black bars) and Sh3bp2$^{KI/KI}$ (red bars) bone marrow stromal cultures. *PAI1* expression was examined in untreated (NT), vehicle treated (V) and SB505124 (SB) treated cultures. SB treatment lowered *PAI1* expression indicating its regulation by TGFβ signaling. **(B)** *PAI1* expression in CD11b^{-},CD45^{-} (mesenchymal) and CD11b^{+},CD45^{+} (hematopoietic) sorted cells. Cherubism mesenchymal cells (red bar) showed 2-fold higher levels of *PAI1* compared to wild type mesenchymal cells (black bar). **(C)** *BAMBI* expression in CD11b^{-},CD45^{-} and CD11b^{+},CD45^{+} sorted cells. BAMBI, an inhibitor of TGFβ signaling was expressed at lower levels in cherubism cells (red bars) compared to wild type cells (black bars).

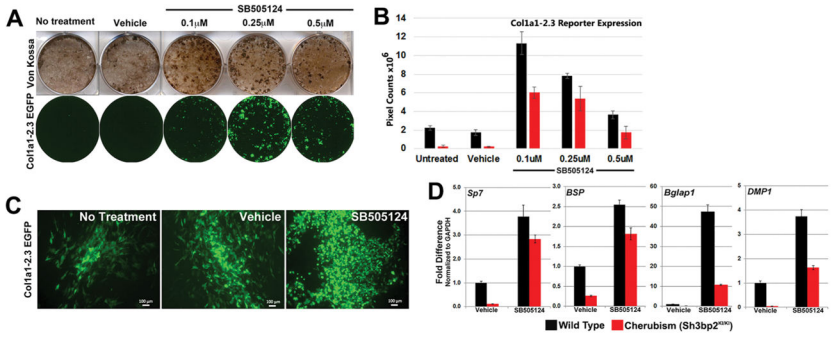

Figure 3. SB505124 treatment rescues osteoblast differentiation of cherubism stromal cells

(A) Evaluation of SB505124 at different dosages during osteoblast differentiation of Sh3bp2$^{KI/KI}$ bone marrow stromal cells. Representative images at day 15 of osteoblast differentiation show a dose of 0.25μM optimally enhanced osteoblast differentiation as determined by staining for mineral deposition by von Kossa (top row) and *Col1a1*-2.3 reporter expression (bottom row). **(B)** Quantitation of *Col1a1*-2.3 reporter expression from wild type (black bars) and cherubism (red bars) cultures grown in the presence of increasing doses of SB505124 at day 10 of differentiation. **(C)** Images of *Col1a1*-2.3^{+} cells at higher magnification under different treatment conditions reveals significant changes in cell morphology in response to SB505124 treatment. **(D)** Examination of osteoblast gene marker expression from wild type (black bars) and cherubism (red bars) cultures at day 12 of differentiation supported increased osteoblast differentiation in cherubism cultures with SB505124 treatment.

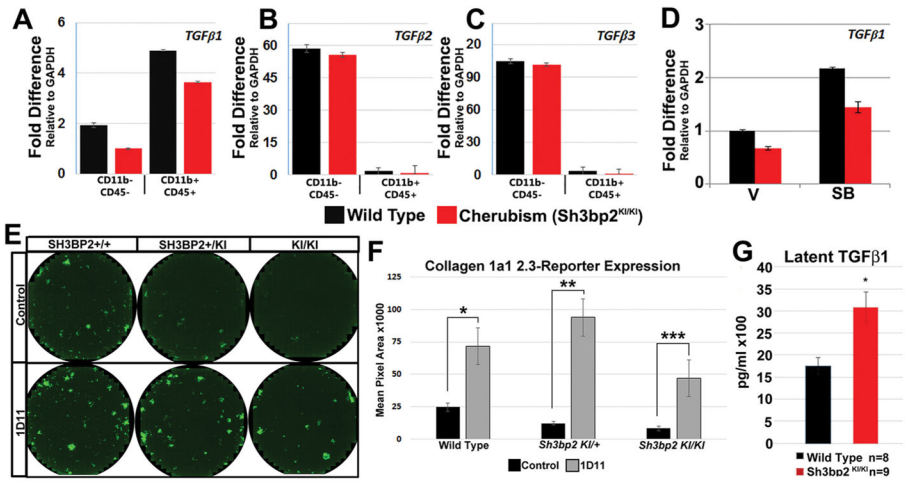

Figure 4. TGFβ ligands contribute to the cherubism phenotype

(A–C) Examination of *TGFβ 1, 2,* and *3* gene expression in wild type (black bars) and cherubism (red bars) $CD11b^{-}$,$CD45^{-}$ mesenchymal and $CD11b^{+}$,$CD45^{+}$ hematopoietic cells. **(A)** *TGFβ1* was detected in both mesenchymal and hematopoietic cell populations, while *TGFβ2* **(B)** and *TGFβ3* **(C)** were selectively expressed in the mesenchymal population. **(D)** Examination of *TGFβ1* expression in cherubism and wild type cultures in the presence of vehicle and SB505124 treatment showed increased *TGFβ1* expression with pathway repression. **(E)** Imaging of *Col1a1*-2.3 osteoblast reporter expression from cultures treated with 1D11 from days 4 to 14 of osteoblast differentiation. Top Row: vehicle treatment; Bottom Row: 1D11. **(F)** Pixel area measurements of *Col1a1*-2.3 Reporter shown in E. **(G)** Latent TGFβ1 levels are higher in *Sh3bp2*$^{KI/KI}$ mice compared to wild type littermates. Serum derived from 6–9-week-old mice showed that levels of latent TGFβ1 were almost 2-fold higher in *Sh3bp2*$^{KI/KI}$ mice compared to wild type littermates (*p=0.00628).

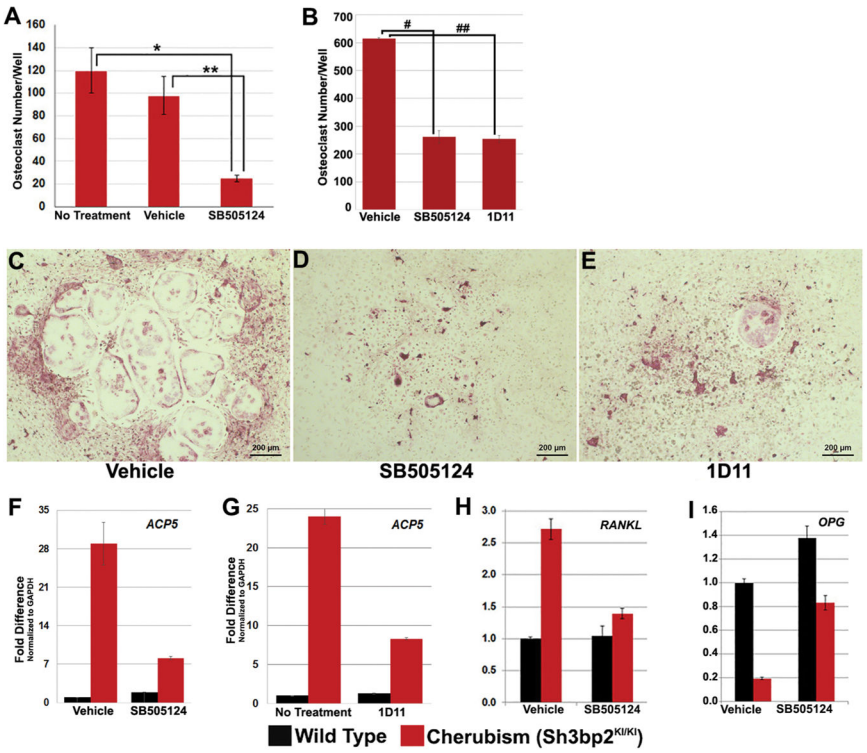

Figure 5. Antagonism of TGFβ signaling markedly reduced osteoclast formation in *Sh3bp2*$^{KI/KI}$ bone marrow stromal cultures

(A) SB505124 treatment resulted in a 5-fold reduction in osteoclast formation by day 10 of culture (*p=0.021, **p=0.025). **(B)** Two treatments of SB505124 to 1D11 on days 3 and 5 of culture and assayed at day 7 reduced the osteoclast number by over half ($^{\#}$p=0.029, $^{\#\#}$p=0.015). No osteoclasts formed in cultures derived from wild type mice. **(C–D)** Representative images of osteoclast formation at day 7 of culture from vehicle **(C)**, SB505124 **(D)** and 1D11 **(E)** treated cultures. **(F,G)** Examination of *ACP5* expression, the gene that encodes for TRAP at day 12 of osteoblast differentiation in wild type and *Sh3bp2*$^{KI/KI}$ cultures treated with SB505124 **(F)** and 1D11 **(G)**. *ACP5* expression decreased more than 3-fold in cherubism cultures treated with TGFβ antagonists. **(H,I)** Gene expression analyses of RANKL and OPG in bone marrow stromal cultures derived from cherubism and wild type mice at day 12 of osteoblast differentiation. RANKL expression was >2.5-fold higher in cherubism derived stromal cells compared to wild type cells. OPG was 5-fold lower in cherubism derived stromal cells compared to wild type cells. For graphs, wild type data and Sh3bp2$^{KI/KI}$ data is denoted by black bars and red bars, respectively.