HHS Public Access

Author manuscript

Bone. Author manuscript; available in PMC 2021 December 01.

Published in final edited form as:

Bone. 2020 December ; 141: 115673. doi:10.1016/j.bone.2020.115673.

Trps1 transcription factor represses phosphate-induced expression of SerpinB2 in osteogenic cells

Mairobys Socorro[1], **Apurva Shinde**[1], **Hajime Yamazaki**[1], **Sana Khalid**[1], **Daisy Monier**[1], **Elia Beniash**[1,2], **Dobrawa Napierala**[1,2]

[1]Center for Craniofacial Regeneration, Department of Oral Biology, University of Pittsburgh School of Dental Medicine, Pittsburgh, PA, USA.

[2]McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA.

Abstract

Serine protease inhibitor SerpinB2 is one of the most upregulated proteins following cellular stress. This multifunctional serpin has been attributed a number of pleiotropic activities, including roles in cell survival, proliferation, differentiation, immunity and extracellular matrix (ECM) remodeling. Studies of cancer cells demonstrated that expression of SerpinB2 is directly repressed by the Trps1 transcription factor, which is a regulator of skeletal and dental tissues mineralization. In our previous studies, we identified *SerpinB2* as one of the novel genes highly upregulated by phosphate (P_i) at the initiation of the mineralization process, however SerpinB2 has never been implicated in formation nor homeostasis of mineralized tissues. The aim of this study was to establish, if SerpinB2 is involved in function of cells producing mineralized ECM and to determine the interplay between P_i signaling and Trps1 in the regulation of SerpinB2 expression specifically in cells producing mineralized ECM. Analyses of the SerpinB2 expression pattern in mouse skeletal and dental tissues detected high SerpinB2 protein levels specifically in cells producing mineralized ECM. qRT-PCR and Western blot analyses demonstrated that *SerpinB2* expression is activated by elevated P_i specifically in osteogenic cells. However, the P_i-induced *SerpinB2* expression was diminished by overexpression of Trps1. Decreased SerpinB2 levels were also detected in osteoblasts and odontoblasts of *2.3Col1a1-Trps1* transgenic mice. Chromatin immunoprecipitation assay (ChIP) revealed that the occupancy of Trps1 on regulatory elements in the *SerpinB2* gene changes in response to P_i. In vitro functional assessment of the consequences of *SerpinB2* deficiency in cells producing mineralized ECM detected impaired mineralization in

Author for Correspondence: Dobrawa Napierala, Ph.D., University of Pittsburgh School of Dental Medicine, Center for Craniofacial Regeneration, Department of Oral Biology, Phone number: +14126249225, don11@pitt.edu.

Credit Author Statemen

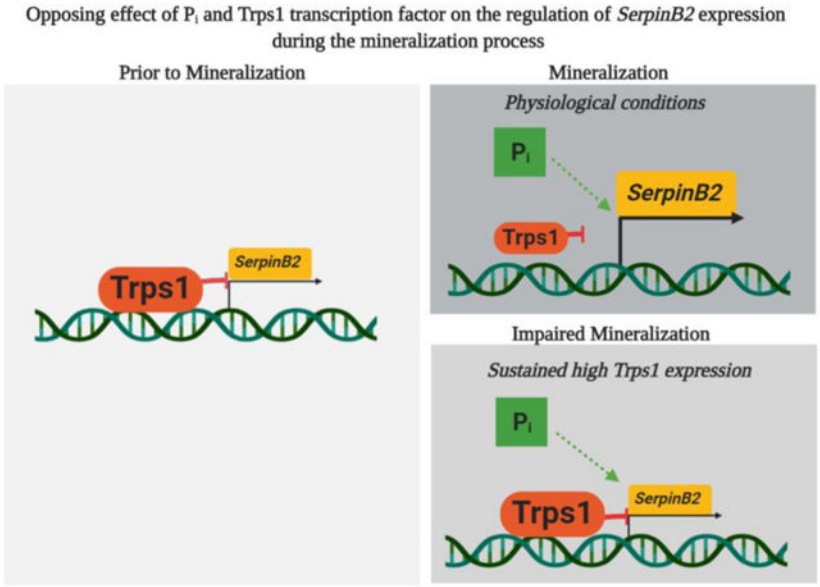
Authors' roles: Conception and design of the study: MS, EB and DN. Data acquisition: MS (qRT-PCR, Western blots, BrdU staining, mineralization assays, IHC, FTIR, ChIP), AS (IHC), HY (FTIR), and DM (generation of SerpinB2-KD cell lines, IHC). MS, AS, HY, SK, EB and DN contributed to the data analyses. MS and DN wrote the manuscript with contribution from all the authors. All authors reviewed the results and approved the final version of the manuscript. All authors take the responsibility for the integrity of the data analysis.

Disclosure

All authors state that they have no conflict of interest.

SerpinB2-deficient cells in comparison with controls. In conclusion, high and specific expression of SerpinB2 in cells producing mineralized ECM, the impaired mineralization of *SerpinB2*-deficient cells and regulation of *SerpinB2* expression by two molecules regulating formation of mineralized tissues suggest involvement of SerpinB2 in physiological mineralization.

Graphical Abstract



Keywords

Inorganic Phosphate (P_i); mineralization; gene expression; osteoblasts; odontoblasts

1. Introduction

SerpinB2/Plasminogen Activator Inhibitor-2 (PAI-2) is a serine protease inhibitor and a component of the plasminogen system [1–6]. The importance of the plasminogen system in bone development has been demonstrated using urokinase-type (uPA) and tissue-type plasminogen activator (tPA) double KO mice ($tPA^{-/-};uPA^{-/-}$ mice) [7]. These mice present the elongation of endochondral bones phenotype and increased bone mass. Histologically, accelerated mineralization of osteoid and accumulation of noncollagenous proteins in bone matrix was detected in $tPA^{-/-};uPA^{-/-}$ mice, which was attributed to defective osteoblast-driven proteolytic processing of extracellular matrix (ECM) components [7]. The involvement of the plasminogen system in bone mineralization was further supported by increased bone mineral density in plasminogen-deficient ($Plg^{-/-}$) mice [8]. Studies of Plasminogen Activator Inhibitor-1 (PAI-1) in transgenic mice also support a regulatory role of the plasminogen system in bone homeostasis, however the function of this protein in bone seems to be independent of the proteolytic activity [9]. Unlike other players of the plasminogen system, SerpinB2/PAI-2 has not been studied in the context of bone development and homeostasis, most likely due to the lack of apparent developmental abnormalities in *SerpinB2* KO mice, although bone phenotype of these mice has not been

assessed [10]. SerpinB2 has been widely studied in the context of immunity and cancer [11–15]. This multifunctional serpin has been implicated in a variety of biological processes, including immunity, proteostasis, cell proliferation and survival, and remodeling of ECM [1]. SerpinB2 is one of the most upregulated proteins following cellular stress [1, 16–19]. Interestingly, we identified *SerpinB2* as one of the novel genes highly upregulated by inorganic phosphate (P_i) in committed osteogenic cells (17IIA11 cell line) [20]. P_i is essential for the physiological mineralization of skeletal and dental tissues, and has been implicated in pathological vascular calcification in chronic hyperphosphatemic conditions [21–24]. P_i accomplishes this function, in part, by acting as a signaling molecule, which stimulates osteogenic differentiation of progenitor cells and formation of mineralized ECM by committed osteogenic cells [25–28]. However, in many other cell types elevated extracellular P_i is cytotoxic and induces a stress response [29–31]. Hence, it is not clear whether the detected P_i-activated *SerpinB2* expression is a part of the cellular stress response or SerpinB2 plays a role in physiological mineralization. An evidence indirectly supporting the latter one, was provided by a recent study of human cancer cell lines showing that *SerpinB2* expression is repressed by the GATA-type transcription factor Trps1 [32]. Since under physiological conditions, Trps1 regulates endochondral bone formation and the mineralization process [33–35], this finding supports the potential physiological function for SerpinB2 in cells producing mineralized ECM. Interestingly, it has been shown that P_i-regulated genes, specifically the ones involved in mineralization, have promoters enriched in GATA-regulatory elements [36].

The aim of this study was to characterize the expression pattern of SerpinB2 in skeletal and dental tissues in vivo and to determine the consequences of *SerpinB2* deficiency on mineralization in vitro. Additionally, we aimed to decipher molecular mechanisms regulating *SerpinB2* expression in cells producing mineralized ECM by addressing the interplay between two molecules (P_i and Trps1) involved in the formation and homeostasis of mineralized tissues. We hypothesized that SerpinB2 plays a role in physiological mineralization. This hypothesis was tested by a series of in vitro and in vivo experiments in WT and transgenic mice.

2. Materials and Methods

2.1. Mice

2.3Col1a1-Trps1 transgenic mice were generated previously [37]. Tissues from wild-type and *2.3Col1a1-Trps1* transgenic mice were harvested at postnatal day 7 and 14 (P7 and P14). Since the mice were pre-pubertal, we have not considered sex as a variable in this study. Animal studies were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, under a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.2. Cell lines and cell culture conditions

17IIA11 [38, 39], OCCM-30 [40, 41], MLO-A5 [42], MOVAS [43] and C3H10T1/2 cells [44] were used in this study. 17IIA11, OCCM-30, MOVAS and C3H10T1/2 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco; Thermo Fisher

Scientific, Logan, UT; containing 154mM total Na concentration) supplemented with 5%–10% FBS (Thermo Fisher Scientific, Logan, UT), 100 units/ml penicillin and 100 μg/ml streptomycin (Cellgro, Manassas, VA) at 37 °C and 5% CO_2. MLO-A5 cells were maintained in alpha Modified Eagle Medium (aMEM, Gibco, Thermo Fisher Scientific, Logan, UT; containing 143mM total Na concentration) supplemented with 5% FBS, 5% FCS, 100 units/ml penicillin,100 μg/ml streptomycin and 1X L-Glutamine at 37°C and 5% CO_2. Two *SerpinB2*-deficient 17IIA11 cell lines (SB2-KD16 and SB2-KD17) were generated using lentivirus-mediated delivery of two different shRNAs-expressing constructs as described previously [20]. SB2-KD16 and SB2-KD17 cell lines were generated with TRCN0000080316 and TRCN0000080317 vector (mature antisense: TATGCGGAAGTTCTAGGATCT) (Dharmacon). Appropriate 17IIA11 control stable cell lines (generated with scrambled shRNA), *Trps1*-overexpressing (Trps1-OE) and *Trps1*-deficient (Trps1-KD) stable cell lines were described before [20]. The 17IIA11 shScr, SerpinB2-KD, Trps1-OE and Trps1-KD stable cell lines were maintained in growth medium supplemented with 10ug/ml of puromycin. For P_i responsive osteogenic gene expression, confluent cells after one day of seeding were stimulated with 5 mM Na-P_i for 12, 24 and 48 h. Mineralization was induced by supplementing growth medium with 5 mM Na-P_i buffer (pH=7.4) and 50 μg/mL ascorbic acid. Osteogenic medium was changed every 48h.

2.3. Cell proliferation assay (BrdU labeling)

Cells (1.0×10^5) were plated on coverslips. After reaching confluency, cells were serum starved (used 0.5% FBS in the medium) overnight and immediately exposed to 5 mM P_i for 24h in standard growth medium (Supplementary Fig. 1A). After 24h, cells were incubated with BrdU labeling reagent (Invitrogen Cat#00–0103) for 6h, stained with AlexaFluor 488-conjugated anti-BrdU antibody (Life Technologies, Cat# B35130, 1:200 dilution), according to the manufacturer's instructions and counterstained with DAPI. Cells were visualized under a fluorescent microscope (Nikon Eclipse 90i microscope) and BrdU positive cells from three randomly chosen fields from each independent slide were counted using NIS Elements software. Percentage of BrdU positive cells was then calculated, and the results are presented as the mean ± SD.

2.4. Histology

Femurs and mandibles from P7 and P14 wild-type and *2.3Col1a1-Trps1* mice were fixed in 10% buffered formalin for 24h and tissues were demineralized in 10% ethylenediaminetetraacetic acid (EDTA) solution (pH 7.4) prior to paraffin embedding. Serial 7 μm sagittal sections were collected and deparaffinized for hematoxylin and eosin (H&E) staining and immunohistochemistry.

2.5. Immunohistochemistry (IHC)

Sections used for analysis by IHC were subjected to antigen retrieval at 95°C in a citrate buffer (10mM, pH 6.0) for 10 minutes. Blocking was performed by incubating the sections with 10% BSA in 1XPBS/Triton X100 at room temperature for 1h. Sections were incubated with anti-SerpinB2 antibody (Abcam Cat# ab137588, 1:100 dilution) overnight at 4°C, followed by incubation with AlexaFluor 488-conjugated secondary antibody (Life Technologies Cat# A11008, 1:400 dilution) for 1h at room temperature. Nuclear

counterstaining was performed using DAPI. Images were captured by a Nikon Eclipse 90i microscope.

2.6. Mineralization Assay

Mineralization was assessed using alizarin red (for calcium deposits) and von Kossa staining (for phosphate deposits), according to standard protocols in undifferentiated cells (day 0) and on days 5, 8, 12 and 15 of culture in osteogenic medium. Stained cells were imaged using Nikon TS100 microscope in a bright field. To quantify calcium deposits, alizarin red was extracted from stained cells and quantified by measuring the absorbance by spectrophotometry at 405 nm.

2.7. Fourier-transform infrared spectroscopy (FTIR)

FTIR spectroscopy was used to analyze mineralization in *SerpinB2*-deficient and 17IIA11 control cell lines on day 0, 5, and 8 of osteogenic differentiation. Four replicates from each group at each time point were collected. The cell sheets were scraped from the cell culture dishes, frozen in liquid N_2 and lyophilized. Five mg of lyophilized material from each sample were ground and mixed with 135 mg of potassium bromide (KBr), and pressed into a pellet at 1100 kg/cm^2 for 30 s using a hydraulic press. FTIR spectra of each sample was acquired at room temperature in the transmission mode, at a resolution of 4 cm^{-1}, and 64 scans per spectrum, using Bruker Vertex 70 FTIR spectrometer and Opus 6.5 software. Analyses were performed using Spectrum 10.02 software package (Perkin Elmer) and Origin Pro 2016 (OriginLab) to determine the mineral/protein ratio and the degree of mineral crystallinity for each sample. The details of the analyses are described in supplementary materials.

2.8. Western blot

Whole protein extracts were prepared by cell lysis in RIPA buffer supplemented with cOmplete™, EDTA-free Protease Inhibitor Cocktail (Sigma). Protein extract (10μg) was subjected to electrophoresis in 4–12% Bis Tris gels (Invitrogen) and transferred to a PVDF membrane (Fisher). Antibodies against SerpinB2 (Abcam Cat # ab137588) and V5-tag (Invitrogen Cat# R960–25) were used at 1:1000 dilution, and α-tubulin (Sigma Cat # T5168) was used at 1:10,000. Horseradish peroxidase-conjugated secondary antibodies (R&D, GE Healthcare) were used at 1:5000. The blots were developed with the Pierce™ ECL Western Blotting Substrate (Fisher, Cat# 32106). The intensity of the bands was measured by ImageJ (http://imagej.nih.gov/ij) and normalized by α-tubulin.

2.9. Quantitative RT-PCR (qRT-PCR)

RNA was extracted using RNeasy mini kit (Qiagen). Total RNA (1 μg), after DNase I treatment (Life Technologies, Grand Island, NY, USA), was converted to cDNA with SuperScript III Reverse Transcriptase kit (Life Technologies, Grand Island, NY, USA). Gene expression analyses were performed using AB Biosystems 7500 Fast Real-Time PCR System and Fast SYBR Green reaction mix (Roche, Indianapolis, IN, USA). The relative expression of genes of interest were calculated from the average Ct values of three independent experiments and normalized against *Gapdh*. Transcript abundances relative to

Gapdh were calculated by using the $2^{-\Delta C_T}$ method. All primer sequences are listed in Supplementary Table 1.

2.10. Chromatin immunoprecipitation (ChIP)

A ChIP assay was performed as previously described [45], with some modifications. Briefly, cells were incubated for 10 min in 1% formaldehyde, followed by addition of 0.125 M glycine, washed with ice-cold PBS, pelleted, and lysed in SDS Lysis Buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 7.5). Samples were sonicated to generate DNA fragments of 200 to 500 bp using a Q800R2 sonicator (QSONICA). Chromatin from 17IIA11 stable cell lines expressing V5-tagged Trps1 (Trps1-OE1 and Trps1-OE2 lines) and 17IIA11 control (17A Ctrl1 and 17A Ctrl2) cells maintained in standard growth medium and upon exposure to 5 mM P_i for 24h was analyzed. The V5 ChIP was carried out with V5 beads (Sigma A7345). Normal mouse IgG (Upstate Biotechnology, Lake Placid, NY) was used as control. V5-agarose and normal mouse agarose beads were previously washed once in 1X PBS and blocked with 1.5% fish skin gelatin (Sigma G7765) and 200 μg salmon sperm DNA in 1X PBS for 1h rotating at room temperature and washed with PBS. Diluted chromatin was taken up to 1 ml with ChIP dilution buffer (0.01% SDS, 1.1% Triton X100, 1.2 mM EDTA, 16.7 mM Tris/HCl pH8.1, 167 mM NaCl) and 1ml of the dilution was used for each chromatin immunoprecipitation. 1% of the chromatin used for one ChIP was preserved as an input sample and stored at −20°C. The lysate was pre-cleared by rotation at 4°C for 2h using 1ml of the dilution with protein G beads (Sigma Cat# 16–201). After centrifugation at 4°C for 1min at 700 × g, the supernatant was incubated with the corresponding antibody rotating overnight at 4°C on a rotating wheel. Washing was carried out as previously described [45]. Elution was done in HENG buffer (10 mM HEPES-KOH pH 9.0, 1.5 mM $MgCl_2$, 0.25 mM EDTA, 20% glycerol), 250 mM KCl, 0.3% NP40 and 0.5 mg/ml V5 peptide repeated 3 times, 20 min each [46]. DNA was purified and amplified by qPCR using the primers shown in Supplementary Table 1.

2.11. Statistical Analysis

Experiments were repeated 3 times or otherwise stated. Values are expressed as mean ± standard deviation (SD). Statistical significant differences were determined using the Student's *t*-test. A p-value of <0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Phosphate increases SerpinB2 expression specifically in committed osteogenic cells

To determine whether the initially observed activation of *SerpinB2* expression by P_i [20] is unique for the 17IIA11 committed osteogenic cell line or this is a uniform cellular response to P_i, we analyzed expression of *SerpinB2* in multiple cell lines. For that, we selected cell lines representing different types of osteogenic cells capable of producing mineralized ECM: 17IIA11 preodontoblasts [38, 39], MLO-A5 osteoblasts [42] and OCCM-30 cementoblasts [40, 41]. For comparison, MOVAS vascular smooth muscle cells (a model of pathologic vascular calcification) [43], which undergo rapid mineralization under standard osteogenic conditions, and non-mineralizing C3H10T1/2 pluripotent mesenchymal cells [44] were

analyzed. Considering that elevated P_i activates some genes within a few hours (early-response genes), while activation of other genes is delayed (late-response genes) [36], expression of *SerpinB2* was analyzed in cells exposed to 5 mM P_i for 12h, 24h and 48h (Fig. 1). qRT-PCR analyses did not detect *SerpinB2* expression in any of the analyzed cells maintained in standard growth condition (C_t values were similar to a "no template" control). Significantly upregulated expression of *SerpinB2* was detected in 17IIA11, MLO-A5 and OCCM-30 osteogenic cells treated with P_i for 24h in comparison with cells grown without additional P_i (Fig. 1A–C). This effect of P_i on *SerpinB2* expression was transient, and only 17IIA11 cells maintained significantly increased *SerpinB2* expression at 48h. Contrary to osteogenic cells, no change of *SerpinB2* expression was detected in MOVAS nor C3H10T1/2 cells exposed to elevated P_i (Fig. 1D, E). This cell type-specific activation of *SerpinB2* expression in response to P_i, considered together with a previous study that did not detect upregulation of *SerpinB2* in P_i-treated MC3T3-E1 osteogenic precursor cells [36], suggest that P_i stimulates *SerpinB2* expression specifically in committed osteogenic cells.

3.2. Inverse correlation between expression of Trps1 and SerpinB2

The Trps1 transcription factor has been shown to directly repress *SerpinB2* expression in cancer cell lines [32]. Therefore, after establishing that P_i signaling molecule is a positive regulator of *SerpinB2* in osteogenic cells, we analyzed the effect of Trps1 on *SerpinB2* expression in these cells. For that, we used previously generated *Trps1*-deficient (Trps1-KD), *Trps1*-overexpressing (Trps1-OE) and appropriate control 17IIA11 stable cell lines [20]. Analyses of *SerpinB2* mRNA levels by qRT-PCR demonstrated that P_i-stimulated *SerpinB2* expression was dampened by Trps1 overexpression (Fig. 2A). This effect of Trps1 overexpression was also apparent on the protein level, as demonstrated by Western blot analyses showing reduced levels of SerpinB2 protein in P_i-stimulated Trps1-OE 17IIA11 stable cell lines in comparison with P_i-stimulated 17IIA11 control cells (Fig. 2B). These results suggest that increased levels of Trps1 prevent upregulation of *SerpinB2* expression by P_i.

On the contrary, 17IIA11 cells deficient in Trps1 demonstrated increased basal expression of *SerpinB2* and higher levels of SerpinB2 protein in comparison with control 17IIA11 cell lines (Fig. 2C, D). This suggests that Trps1 downregulates *SerpinB2* and loss of this repressor activity is sufficient to increase *SerpinB2* expression without additional extracellular stimuli. Of note, because *Trps1*-deficient cells do not respond to elevated extracellular P_i (data not shown), we did not analyze the effect of P_i on *SerpinB2* expression in Trps1-KD cells.

3.3. SerpinB2 deficiency does not affect P_i-stimulated proliferation of osteogenic cells

To understand the role of SerpinB2 in osteogenic cells, we analyzed the consequences of *SerpinB2* deficiency on the physiology of 17IIA11 cells. For that, we generated two *SerpinB2*-deficient (SB2-KD16 and SB2-KD17) 17IIA11 stable cell lines using two different shRNAs. Efficient depletion of SerpinB2 was confirmed on the protein level (Supplementary Fig. S2).

Considering that P_i and Trps1 are regulators of cell proliferation, in addition to being regulators of the mineralization process [25, 47–54], and that SerpinB2 has also been implicated in the regulation of proliferation in vitro [55–57]; first, we analyzed the effect of *SerpinB2* deficiency on cell proliferation. Results of the BrdU incorporation assay demonstrated that there is no difference in proliferation of control and SerpinB2-KD 17IIA11 cell lines grown in standard conditions and that P_i increases proliferation of both control and *SerpinB2*-deficient cells (Fig. 3 and Supplementary Fig. S1). These results suggest that *SerpinB2* deficiency does not affect P_i-stimulated cell proliferation.

SerpinB2 has been reported to protect cells from stress, in part by limiting Caspase-3 activation [16, 58], while P_i can induce stress response in non-osteogenic cells [29–31]. Therefore, we addressed a potential role of SerpinB2 in protecting osteogenic cells from P_i-induced stress. Western blot analyses (Supplementary Fig. S3) of apoptotic markers, cleaved Caspase-3 and cleaved PARP, did not detect significant levels of these proteins in cells treated with P_i in comparison with cells treated with DTT (dithiothreitol, a positive control for apoptosis). Importantly, levels of cleaved Caspase-3 and cleaved PARP in P_i-treated *SerpinB2*-deficient cells were as low as in P_i-treated control cells (Supplementary Fig. S3). These results suggest that *SerpinB2* deficiency does not trigger osteogenic cell sensitivity to P_i-induced stress.

3.4. SerpinB2 deficiency results in delayed and decreased mineralization of 17IIA11 cells

Next, we analyzed the effect of *SerpinB2* deficiency on mineralization of 17IIA11 cells. Mineralization of control and *SerpinB2*-deficient cells was compared at day 5 and day 8 of culture in osteogenic medium, which represent ongoing mineralization and the mineralization plateau, respectively [20, 38]. At day 5, mineralization of control 17IIA11 cells was well advanced as demonstrated by alizarin red and von Kossa staining, while very few calcium and phosphate deposits were detected in *SerpinB2*-deficient cells (Fig. 4A–C and Supplementary Fig. 4). The decreased mineralization, although to a lesser degree, was also detected at day 8, 12 and 15 in *SerpinB2*-deficient cells (Fig. 4A–C and Supplementary Fig. 4). FTIR spectroscopic analyses revealed differences in the mineral/protein ratios, which are used to determine the degree of mineralization in tissues and cell cultures, between control 17IIA11 and *SerpinB2*-deficient cells (Fig. 4D,E and Supplementary Fig. 5). Specifically, the ν_4/Amide I ratio in the control at day 5 was 0.287 while in the SerpinB2-KD it was 0.253. A greater difference was observed on day 8 with a ν_4/Amide I ratio of 0.310 in the control and 0.234 in the SerpinB2-KD. These differences were highly significant only at day 8 (Fig. 4E). We have also assessed the crystallinity of the mineral in the control and SerpinB2-KD 17IIA11 cells. The mineral crystallinity in the cultures of *SerpinB2*-deficient cells was significantly lower than in the controls on both time points (Fig 4F). These results indicate that deficiency of SerpinB2 in committed osteogenic cells negatively affects mineralization, as evidenced by the decrease in mineral deposition and crystallinity, providing further evidence that SerpinB2 supports the mineralization process.

3.5. SerpinB2 is highly expressed in skeletal and dental cells producing mineralized ECM

To determine if the observed effect of *SerpinB2* deficiency on mineralization is physiologically relevant, we analyzed expression of SerpinB2 in bone and developing teeth.

Considering that SerpinB2 protein exists in two forms, intracellular and extracellular [2, 59–62], we used immunohistochemistry (IHC) to be able to determine the localization of SerpinB2 protein.

Analyses of femurs and mandibles of P7 mice detected high SerpinB2 protein levels in hypertrophic chondrocytes and osteoblasts of primary spongiosa (Fig. 5A). SerpinB2 protein was also detected in resting and proliferating chondrocytes, although at much lower levels than in hypertrophic chondrocytes and osteoblasts. Of note, SerpinB2 was predominantly localized in the cytoplasm of chondrocytes and osteoblasts, but not in the cartilage nor bone ECM at P7. Analyses of alveolar bone of P7 mice confirmed high levels of SerpinB2 protein in cytoplasm of osteoblasts but not in ECM.

In the developing mouse incisor, high levels of SerpinB2 protein were detected in odontoblasts, but not in dental pulp cells (Fig. 5A). More detailed analyses of SerpinB2 at the later stage of tooth development when enamel is formed, revealed that ameloblasts are also highly positive for SerpinB2 in incisors of P14 mice (Fig. 5B). In both odontoblasts and ameloblasts, levels of SerpinB2 increased as the cells progressed in their differentiation with no or very little SerpinB2 detected in preodontoblasts and preameloblasts (Fig. 5B). Unlike in bone, extracellular SerpinB2 was detected in predentin and forming enamel ECM (Fig. 5B). In summary, we determined that SerpinB2 is highly and specifically expressed by cells producing mineralized ECM in both skeletal and dental tissues. This and the presence of SerpinB2 in predentin and enamel suggests its involvement in physiological mineralization.

3.6. Trps1 represses SerpinB2 expression in osteogenic cells

Interestingly, the SerpinB2 expression pattern in mineralizing tissues is opposite to the expression of the Trps1 transcription factor, which is highly expressed before mineralization occurs, but its expression declines after the initiation of mineralization [35, 37]. Therefore, our next step was to determine if *SerpinB2* expression can be repressed by Trps1 in cells producing mineralizing ECM in vivo. We analyzed skeletal and dental tissues of *2.3Col1a1-Trps1* transgenic mice [37], which maintain high expression of Trps1 in mature osteoblasts and odontoblasts during mineralization of bone and dentin, respectively. IHC analyses of femurs and mandibles at P7 revealed that overexpression of Trps1 in osteoblasts results in decreased levels of SerpinB2 in osteoblasts lining trabecular bone (Fig. 6A), but no apparent difference was observed between osteoblasts in the alveolar bone of *2.3Col1a1-Trps1* and WT mice (Fig. 6B). In P14 incisors, lower levels of SerpinB2 were apparent in *2.3Col1a1-Trps1* odontoblasts in comparison with odontoblasts of WT mice (Fig. 6C). There was no difference in levels of SerpinB2 in WT and *2.3Col1a1-Trps1* ameloblasts. These results indicate that Trps1 represses *SerpinB2* expression in vivo, in a cell-autonomous manner.

3.7. Trps1 binding to the SerpinB2 regulatory elements is regulated by P_i

Next, we used ChIP assay to determine if the observed downregulation of *SerpinB2* in osteoblasts and odontoblasts overexpressing Trps1 is due to direct interaction of the Trps1 transcription factor with the *SerpinB2* gene. To identify potential Trps1 binding sites that may serve as gene expression regulatory elements, we screened 2kb fragment upstream of the *SerpinB2* transcriptional start site and the entire 4kb of the *SerpinB2* first intron using

TRANSFAC (TRANScription FACtor database). We identified 8 candidate GATA consensus sequences (BS1–8; Fig. 7A, Supplementary Table S1). Due to the lack of efficient anti-Trps1 antibodies, ChIP experiments were done in 17IIA11 stable cell lines expressing V5-tagged Trps1 (Trps1-OE1 and Trps1-OE2 lines) and 17IIA11 control (17A Ctrl1 and 17A Ctrl2) cell lines generated with an empty vector [20, 37]. As a positive control for the Trps1 binding, we used a previously identified fragment of the *Dspp* promoter [37]. The fold increase of the *Dspp* fragment in protein-DNA complexes immunoprecipitated with V5 antibodies from Trps1-OE cells over 17IIA11 control cells was used as a reference to evaluate the enrichment in DNA fragments from the *SerpinB2* gene. A fragment of the *Gapdh* gene, which does not contain GATA consensus elements, served as a "non-binding" control. Immunoprecipitation of protein-DNA complexes using V5 antibodies, followed by qPCR, revealed enrichment of binding sites BS5 and BS7 in the DNA immunoprecipitated from cell lines expressing V5-Trps1 in comparison with the control cell lines (Fig. 7B, left panel). Interestingly, the Trps1 binding to BS5 site was detected only in Trps1-OE cells maintained in standard growth medium, but not in cells exposed to 5 mM P_i (P_i) for 24h (Fig. 7B). Also, the Trps1 binding to the BS7 site was decreased by P_i, however this effect was less prominent and was detected only in the Trps1-OE2 cell line. In contrast, Trps1 binding to BS6 was increased by elevated P_i levels in comparison with the standard growth conditions. ChIP experiments with control antibodies (normal mouse IgG) showed no differences for any of the analyzed DNA fragments (Supplementary Fig. S7A). In summary, we determined that Trps1 directly binds to GATA consensus sequences in the first intron of the *SerpinB2* gene, and this binding is affected by the extracellular P_i levels.

4. Discussion

SerpinB2/PAI-2 is an intriguing but enigmatic protein, which on one side, has been attributed a plethora of molecular and cellular functions, while, on the other side, its major physiological function remains to be unveiled. Although SerpinB2/PAI-2 has been often described as a component of the plasminogen system [1–6], functional assays, analyses of *SerpinB2*$^{-/-}$ mice and clinical data implicate SerpinB2 in the inflammation, immune response and cancer [10–14, 63, 64]. Results of our studies expand the array of SerpinB2 functions by showing that SerpinB2 is also directly involved in the physiological mineralization process occurring in skeletal and dental tissues by regulating this function of osteogenic cells in a cell-autonomous manner. First, we determined that the SerpinB2 is highly and specifically expressed in skeletal and dental tissues (Fig. 5). We showed that upregulation of SerpinB2 expression coincides with chondrocytes maturation and the initiation of mineralization in vivo. Consistently with this expression pattern, in vitro experiments uncovered that *SerpinB2* expression is activated by elevated P_i (Fig. 1), which is one of the major stimulators of mineralization. Importantly, this effect of P_i on expression of *SerpinB2* is specific to osteogenic cells. We have also shown that *SerpinB2* expression is directly regulated by another mineralization-related molecule, the Trps1 transcription factor. However, in contrast to the activation by P_i, *SerpinB2* expression is repressed by Trps1 (Fig. 2 and 6). We have also uncovered that P_i changes the occupancy of Trps1 on cis-elements in the *SerpinB2* gene, providing mechanistic insights into P_i-regulated gene expression (Fig.7). Finally, the involvement of SerpinB2 in the mineralization process was supported by

analyses of consequences of *SerpinB2* deficiency on the function of committed osteogenic cells, which demonstrated impaired mineralization of the *SerpinB2*-deficient cells (Fig. 4).

The combination of in vitro and in vivo approaches used in this study indicates that the expression of *SerpinB2* in osteogenic cells contributes to proper physiological mineralization, hence SerpinB2 regulates this process in a cell-autonomous manner. First, the functional assays comparing formation of mineralized ECM by control and *SerpinB2*-deficient 17IIA11 committed osteogenic cells revealed delayed initiation of the deposition of mineral in the ECM, and reduced extent of the ECM mineralization (Fig. 4, Suppl. Fig. 4). Second, the IHC detection of high levels of SerpinB2 in all cell types producing mineralized ECM suggests that SerpinB2 role in mineralization is not limited to one cell type, but it is common to all skeletal and dental tissues that produce mineralized ECM (Fig. 5).

We detected SerpinB2 in both intracellular and ECM compartments of mineralizing tissues (Fig. 5, 6 and Suppl. Fig. 8). Previous reports have shown that intracellular and extracellular SerpinB2 isoforms execute different functions [13]. The intracellular 47kDa SerpinB2 isoform has a cytoprotective role and it is involved in the protein folding [19, 58, 60], while the extracellular 60kDa isoform participates in ECM remodeling [3, 12]. High levels of SerpinB2 protein in predentin (a counterpart of bone osteoid) and unmineralized enamel matrix (Fig. 5B, Suppl. Fig. 8) suggest that this serine protease inhibitor is involved in remodeling of ECM components to allow for proper deposition of hydroxyapatite. For example, serine proteinase KLK-4 is strongly expressed by ameloblasts during the maturation stage of amelogenesis [65], when the enamel organic matrix is completely degraded and replaced by the mineral. Although, no studies of SerpinB2 KO enamel have been conducted to date, one can surmise that the role of SerpinB2 in the enamel matrix is to control the rate of proteolysis by KLK-4, which is critical for the proper enamel maturation.

It is likely that the role of SerpinB2 in cells producing mineralized ECM is not limited to ECM remodeling, as high levels of SerpinB2 were also detected within a cell body of hypertrophic chondrocytes, osteoblasts lining trabecular and alveolar bone, odontoblasts and ameloblasts (Fig. 5 and 6). Studies in neurons have demonstrated that intracellular SerpinB2 interacts with misfolded proteins and components of the ubiquitin-proteasome system [19]. Since production of ECM in skeletal and dental tissues involves high protein synthesis and upregulation of secretory machinery [66], the intracellular SerpinB2 detected in cells producing mineralized ECM may participate in proper folding and secretion of organic components of ECM. However, *SerpinB2* deficiency does not impair survival of osteogenic cells stimulated for production of mineralized ECM in our in vitro analyses (Supplementary Fig. S3). These results, together with the lack of deleterious skeletal and dental defects in *SerpinB2*$^{-/-}$ mice [10], suggest that this protein is not essential for proper formation of mineralized tissues. However, it is important to note that these tissues have not been analyzed in *SerpinB2*$^{-/-}$ mice. Hence, it is possible that these mice exhibit less apparent phenotype or they develop exacerbated phenotype under pathological conditions or have an impairment of the tissue repair process. This hypothesis is supported by clinical studies implicating SERPINB2 in periodontal disease [67, 68], as the formation of unsound mineralized tissues of the dento-alveolar complex (bone and cementum) is a strong contributing factor to this chronic inflammatory disease.

Development and homeostasis of mineralized tissues, including the dento-alveolar complex, is highly dependent on P_i [23, 27, 69–72]. This signaling molecule stimulates osteogenic cells, which, in response, form mineralized ECM. This is accomplished, in part, by changes (activation or repression) in expression of many genes involved in formation of mineralized ECM [27]. Results of our studies suggest that upregulation of *SerpinB2* by P_i is an integral component of the mineralization program activated in osteogenic cells in response to this signaling molecule. However, the mechanism, by which P_i regulates the transcription is largely unknown, and the mechanism of P_i-induced *SerpinB2* expression in osteogenic cells remains to be elucidated. Thus far, only AP-1 transcription factors have been suggested to mediate transcriptional response to P_i [36]. The same study revealed that promoters of many mineralization-related genes that are upregulated by P_i are enriched in GATA consensus sequences. Here, we identified Trps1 as a transcription factor that might be directly involved in regulation of gene expression in response to P_i in osteogenic cells. Using ChIP we detected direct Trps1 binding to the GATA consensus elements in the *SerpinB2* first intron. Interestingly, the direct interaction of Trps1 with *SerpinB2* regulatory elements was changed by elevated extracellular P_i (Fig. 7). This indicates that activation of P_i signaling leads to changes of transcription factors occupancy on their target promoters. Our results also uncovered that Trps1 is an active repressor of the *SerpinB2* gene in osteogenic cells, as demonstrated by increased expression of *SerpinB2* in *Trps1*-deficient 17IIA11 cells and decreased expression of SerpinB2 in *Trps1*-overexpressing osteogenic cells in vitro and in vivo (Fig. 2 and 6). These results together with the results of the ChIP assay (Fig. 7) suggest that Trps1 represses *SerpinB2* through direct binding to its regulatory elements.

In our previous studies, we have detected decreased levels of P_i-stimulated proteins Dmp1 and Phex in cells overexpressing Trps1, although the mechanism has not been studied [20, 73]. Thus far, only a few mineralization-related genes have been demonstrated to be directly regulated by Trps1: *Runx2*, *Bglap* (osteocalcin) and *Dspp* [37, 74, 75]- none of these have been reported as a P_i-regulated gene. However, the high abundance of GATA elements in many mineralization-related genes regulated by P_i [36], strongly suggest that Trps1 is involved in the transcriptional regulation of gene expression in response to P_i. Of note, the FGF pathway, which has been implicated in P_i signaling [76, 77], has not been connected to Trps1 yet. Thus, the mechanisms of the Trps1-P_i signaling interaction remains to be elucidated.

In summary, this study identified the serine protease inhibitor SerpinB2/PAI-2 as a novel protein supporting mineralization. Furthermore, we have shown that P_i activates *SerpinB2* expression in osteogenic cells, however the overexpression of Trps1 inhibits this effect (Fig. 8). To the best of our knowledge, *SerpinB2* is the first example of P_i-stimulated gene that is also regulated by Trps1. Data presented here suggest a potential mechanism of transcriptional regulation of osteogenic cells physiology in response to P_i, whereby the presence of high levels of Trps1 prevents mineralization-related genes (those with GATA regulatory elements) from activation by P_i. Our future studies will investigate the interplay between P_i signaling and Trps1 transcription factor in the regulation of expression of mineralization-related genes as a potential tool for regulation of the mineralization process in pathological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to thank Drs. Odile Kellermann and Anne Poliard for providing the 17IIA11 cell line, and Dr. Masum Pandey for selecting potential GATA binding sites for ChIP analyses. Research reported in this publication was supported by National Institute of Dental and Craniofacial Research, and National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under awards number DE023083 and AR074981, respectively (to D.N.), National Institute of Dental and Craniofacial Research award F32DE029096 (to M.S.) and the School of Dental Medicine Dean's Summer Research Scholarship Program (to A.S.).

References

1. Johnson TA, Buzza MS, Riley EAU, and Antalis TM, Plasminogen Activator Inhibitor Type-2 (PAI-2)/SerpinB2: A Unique Multifunctional Serpin, in , Geiger M, Wahlmüller F, and Furtmüller M, Editors. 2015, Springer International Publishing: Cham p. 107–126. doi: 10.1007/978-3-319-22711-5_8.
2. Kruithof E, Baker M, and Bunn C. Biological and clinical aspects of plasminogen activator inhibitor type 2. Blood. 1995;86(11):4007–4024. doi: 10.1182/blood.V86.11.4007.bloodjournal86114007. [PubMed: 7492756]
3. Medcalf RL and Stasinopoulos SJ. The undecided serpin: the ins and outs of plasminogen activator inhibitor type 2. FEBS J. 2005;272(19):4858–4867. doi: 10.1111/j.1742-4658.2005.04879.x. [PubMed: 16176260]
4. Irving JA, Pike RN, Lesk AM, and Whisstock JC. Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. Genome Res. 2000;10(12):1845–1864. doi: 10.1101/gr.gr-1478r. [PubMed: 11116082]
5. Kawano T, MORIMOTO K, and UEMURA Y. Partial purification and properties of urokinase inhibitor from human placenta. J Biochem. 1970;67(3):333–342. doi: 10.1093/oxfordjournals.jbchem.a129257. [PubMed: 5424370]
6. Law RHP, Zhang Q, McGowan S, Buckle AM, Silverman GA, Wong W, Rosado CJ, Langendorf CG, Pike RN, Bird PI, and Whisstock JC. An overview of the serpin superfamily. Genome Biol. . 2006;7(5):216–216. Epub 2006/05/30. doi: 10.1186/gb-2006-7-5-216. [PubMed: 16737556]
7. Daci E, Everts V, Torrekens S, Van Herck E, Tigchelaar- Gutterr W, Bouillon R, and Carmeliet G. Increased bone formation in mice lacking plasminogen activators. J Bone Miner Res. . 2003;18(7):1167–1176. doi: 10.1359/jbmr.2003.18.7.1167. [PubMed: 12854826]
8. Kanno Y, Ishisaki A, Kawashita E, Chosa N, Nakajima K, Nishihara T, Toyoshima K, Okada K, Ueshima S, Matsushita K, Matsuo O, and Matsuno H. Plasminogen/plasmin modulates bone metabolism by regulating the osteoblast and osteoclast function. Journal Biol Chem. 2011;286(11):8952–8960. Epub 2011/01/14. doi: 10.1074/jbc.M110.152181. [PubMed: 21239499]
9. Nordstrom SM, Carleton SM, Carson WL, Eren M, Phillips CL, and Vaughan DE. Transgenic over-expression of plasminogen activator inhibitor-1 results in age-dependent and gender-specific increases in bone strength and mineralization. Bone. 2007;41(6):995–1004. Epub 2007/08/17. doi: 10.1016/j.bone.2007.08.020. [PubMed: 17888748]
10. Dougherty KM, Pearson JM, Yang AY, Westrick RJ, Baker MS, and Ginsburg D. The plasminogen activator inhibitor-2 gene is not required for normal murine development or survival. Proc Natl Acad Sci U S A. 1999;96(2):686–691. doi: 10.1073/pnas.96.2.686. [PubMed: 9892694]
11. Schroder WA, Le TTT, Major L, Street S, Gardner J, Lambley E, Markey K, MacDonald KP, Fish RJ, Thomas R, and Suhrbier A. A Physiological Function of Inflammation-Associated SerpinB2 Is Regulation of Adaptive Immunity. J Immunol. 2010;184(5):2663–2670. doi: 10.4049/jimmunol.0902187. [PubMed: 20130210]
12. Harris NLE, Vennin C, Conway JRW, Vine KL, Pinese M, Cowley MJ, Shearer RF, Lucas MC, Herrmann D, Allam AH, Pajic M, Morton JP, Australian I Pancreatic Cancer Genome, A.V. Biankin, M. Ranson, P. Timpson, and D.N. Saunders. SerpinB2 regulates stromal remodelling and

local invasion in pancreatic cancer. Oncogene. 2017;36(30):4288–4298. Epub 2017/03/27. doi: 10.1038/onc.2017.63. [PubMed: 28346421]

13. Schroder WA, Major L, and Suhrbier A. The Role of SerpinB2 in Immunity. Crit Rev Immunol. . 2011;31(1):15–30. doi: 10.1615/CritRevImmunol.v31.i1.20. [PubMed: 21395508]
14. Croucher DR, Saunders DN, Lobov S, and Ranson M. Revisiting the biological roles of PAI2 (SERPINB2) in cancer. Nat Rev Cancer. 2008;8(7):535–545. doi: 10.1038/nrc2400. [PubMed: 18548086]
15. Valiente M, Obenauf AC, Jin X, Chen Q, Zhang XHF, Lee DJ, Chaft JE, Kris MG, Huse JT, Brogi E, and Massagué J. Serpins promote cancer cell survival and vascular co-option in brain metastasis. Cell. 2014;156(5):1002–1016. doi: 10.1016/j.cell.2014.01.040. [PubMed: 24581498]
16. Lee JA, Cochran BJ, Lobov S, and Ranson M. Forty Years Later and the Role of Plasminogen Activator Inhibitor Type 2/SERPINB2 Is Still an Enigma. Semin Thromb Hemost. 2011;37(04):395–407. doi: 10.1055/s-0031-1276589. [PubMed: 21805446]
17. Lee JA. Characterisation of SerpinB2 as a stress response modulator. 2015.
18. Riley EAU, The Regulation and Cytoprotective Function of Intracellular Plasminogen Activator Inhibitor Type 2 in Response to Cellular Stress. 2014.
19. Lee JA, Yerbury JJ, Farrawell N, Shearer RF, Constantinescu P, Hatters DM, Schroder WA, Suhrbier A, Wilson MR, Saunders DN, and Ranson M. SerpinB2 (PAI-2) Modulates Proteostasis via Binding Misfolded Proteins and Promotion of Cytoprotective Inclusion Formation. PloS one. 2015;10(6):e0130136–e0130136. doi: 10.1371/journal.pone.0130136. [PubMed: 26083412]
20. Kuzynski M, Goss M, Bottini M, Yadav MC, Mobley C, Winters T, Poliard A, Kellermann O, Lee B, Millan JL, and Napierala D. Dual role of the Trps1 transcription factor in dentin mineralization. J Biol Chem. 2014;289(40):27481–27493. Epub 2014/08/15. doi: 10.1074/jbc.M114.550129. [PubMed: 25128529]
21. Jono S, McKee MD, Murry CE, Shioi A, Nishizawa Y, Mori K, Morii H, and Giachelli CM. Phosphate Regulation of Vascular Smooth Muscle Cell Calcification. Circ Res. 2000;87(7):e10–e17. doi: doi:10.1161/01.RES.87.7.e10. [PubMed: 11009570]
22. Giachelli CM, Speer MY, Li X, Rajachar RM, and Yang H. Regulation of Vascular Calcification. Circ Res. 2005;96(7):717–722. doi: doi:10.1161/01.RES.0000161997.24797.c0. [PubMed: 15831823]
23. Bergwitz C and Jüppner H, Disorders of phosphate homeostasis and tissue mineralisation, in Calcium and bone disorders in children and adolescents. 2009, Karger Publishers p. 133–156. doi: 10.1159/000223693.
24. Farrow EG and White KE. Recent advances in renal phosphate handling. Nat Rev Nephrol. 2010;6(4):207. [PubMed: 20177401]
25. Beck GR, Moran E, and Knecht N. Inorganic phosphate regulates multiple genes during osteoblast differentiation, including Nrf2. Exp Cell Res. 2003;288(2):288–300. doi: 10.1016/S0014-4827(03)00213-1. [PubMed: 12915120]
26. Fatherazi S, Matsa-Dunn D, Foster BL, Rutherford RB, Somerman MJ, and Presland RB. Phosphate regulates osteopontin gene transcription. J Dent Res. 2009;88(1):39–44. doi: 10.1177/0022034508328072. [PubMed: 19131315]
27. Foster BL, Tompkins KA, Rutherford RB, Zhang H, Chu EY, Fong H, and Somerman MJ. Phosphate: known and potential roles during development and regeneration of teeth and supporting structures. Birth Defects Res C Embryo Today. 2008;84(4):281–314. doi: 10.1002/bdrc.20136. [PubMed: 19067423]
28. Yamamoto Y, Toyosawa S, and Sato S. Inorganic phosphate induces cementogenic/osteogenic gene expression and mineralization in dental follicle cells in vitro. Oral Science International. 2018;15(1):13–17. doi: 10.1016/s1348-8643(17)30042-3.
29. Nguyen TT, Quan X, Hwang K-H, Xu S, Das R, Choi S-K, Wiederkehr A, Wollheim CB, Cha S-K, and Park K-S. Mitochondrial oxidative stress mediates high-phosphate-induced secretory defects and apoptosis in insulin-secreting cells. Am J Physiol Endocrinol Metab. 2015;308(11):E933–E941. doi: 10.1152/ajpendo.00009.2015. [PubMed: 25852001]
30. Marco GSD, Hausberg M, Hillebrand U, Rustemeyer P, Wittkowski W, Lang D, and Pavenstädt H. Increased inorganic phosphate induces human endothelial cell apoptosis in vitro. Am J Physiol

Renal Physiol. 2008;294(6):F1381–F1387. doi: 10.1152/ajprenal.00003.2008. [PubMed: 18385273]

31. Sekiguchi S, Suzuki A, Asano S, Nishiwaki-Yasuda K, Shibata M, Nagao S, Yamamoto N, Matsuyama M, Sato Y, Yan K, Yaoita E, and Itoh M. Phosphate overload induces podocyte injury via type III Na-dependent phosphate transporter. Am J Physiol Renal Physiol. 2011;300(4):F848–F856. doi: 10.1152/ajprenal.00334.2010. [PubMed: 21307129]
32. Rangel R, Lee S-C, Hon-Kim Ban K, Guzman-Rojas L, Mann MB, Newberg JY, Kodama T, McNoe LA, Selvanesan L, Ward JM, Rust AG, Chin K-Y, Black MA, Jenkins NA, and Copeland NG. Transposon mutagenesis identifies genes that cooperate with mutant Pten in breast cancer progression. Proc Natl Acad Sci U S A. 2016;113(48):E7749–E7758. Epub 2016/11/14. doi: 10.1073/pnas.1613859113. [PubMed: 27849608]
33. Lüdecke HJ, Schaper J, Meinecke P, Momeni P, Gross S, von Holtum D, Hirche H, Abramowicz MJ, Albrecht B, Apacik C, Christen HJ, Claussen U, Devriendt K, Fastnacht E, Forderer A, Friedrich U, Goodship TH, Greiwe M, Hamm H, Hennekam RC, Hinkel GK, Hoeltzenbein M, Kayserili H, Majewski F, Mathieu M, McLeod R, Midro AT, Moog U, Nagai T, Niikawa N, Orstavik KH, Plöchl E, Seitz C, Schmidtke J, Tranebjaerg L, Tsukahara M, Wittwer B, Zabel B, Gillessen-Kaesbach G, and Horsthemke B. Genotypic and phenotypic spectrum in tricho-rhino-phalangeal syndrome types I and III. Am J Hum Genet. 2001;68(1):81–91. Epub 2000/12/07. doi: 10.1086/316926. [PubMed: 11112658]
34. Malik TH, Von Stechow D, Bronson RT, and Shivdasani RA. Deletion of the GATA domain of TRPS1 causes an absence of facial hair and provides new insights into the bone disorder in inherited tricho-rhino-phalangeal syndromes. Mol Cel Biol. 2002;22(24):8592–8600. doi: 10.1128/mcb.22.24.8592-8600.2002.
35. Kantaputra P, Miletich I, Lüdecke H-J, Suzuki EY, Praphanphoj V, Shivdasani R, Wuelling M, Vortkamp A, Napierala D, and Sharpe PT. Tricho-Rhino-Phalangeal Syndrome with Supernumerary Teeth. J Dent Res. 2008;87(11):1027–1031. doi: 10.1177/154405910808701102. [PubMed: 18946009]
36. Camalier CE, Yi M, Yu L-R, Hood BL, Conrads KA, Lee YJ, Lin Y, Garneys LM, Bouloux GF, Young MR, Veenstra TD, Stephens RM, Colburn NH, Conrads TP, and Beck GR Jr. An integrated understanding of the physiological response to elevated extracellular phosphate. J Cell Physiol. 2013;228(7):1536–1550. doi: 10.1002/jcp.24312. [PubMed: 23280476]
37. Napierala D, Sun Y, Maciejewska I, Bertin TK, Dawson B, D'Souza R, Qin C, and Lee B. Transcriptional repression of the Dspp gene leads to dentinogenesis imperfecta phenotype in Col1a1-Trps1 transgenic mice. J Bone Miner Res. . 2012;27(8):1735–1745. doi: 10.1002/jbmr.1636. [PubMed: 22508542]
38. Priam F, Ronco V, Locker M, Bourd K, Bonnefoix M, Duchêne T, Bitard J, Wurtz T, Kellermann O, Goldberg M, and Poliard A. New cellular models for tracking the odontoblast phenotype. Arch Oral Biol. 2005;50(2):271–277. doi: 10.1016/j.archoralbio.2004.10.007. [PubMed: 15721161]
39. Lacerda-Pinheiro S, Dimitrova-Nakov S, Harichane Y, Souyri M, Petit-Cocault L, Legrès L, Marchadier A, Baudry A, Ribes S, and Goldberg M. Concomitant multipotent and unipotent dental pulp progenitors and their respective contribution to mineralised tissue formation. Eur Cell Mater. 2012;23:371–386. doi: 10.22203/ecm.v023a29. [PubMed: 22623164]
40. D'Errico JA, Ouyang H, Berry JE, MacNeil RL, Strayhorn C, Imperiale MJ, Harris NL, Goldberg H, and Somerman MJ. Immortalized cementoblasts and periodontal ligament cells in culture. Bone. 1999;25(1):39–47. doi: 10.1016/S8756-3282(99)00096-4. [PubMed: 10423020]
41. D'Errico JA, Berry JE, Ouyang H, Strayhorn CL, Windle JJ, and Somerman MJ. Employing a Transgenic Animal Model to Obtain Cementoblasts In Vitro. J Periodontol. 2000;71(1):63–72. doi: 10.1902/jop.2000.71.1.63. [PubMed: 10695940]
42. Kato Y, Boskey A, Spevak L, Dallas M, Hori M, and Bonewald LF. Establishment of an Osteoid Preosteocyte-like Cell MLO-A5 That Spontaneously Mineralizes in Culture. J Bone Miner Res. . 2001;16(9):1622–1633. doi: 10.1359/jbmr.2001.16.9.1622. [PubMed: 11547831]
43. Afroze T, Yang LL, Wang C, Gros R, Kalair W, Hoque AN, Mungrue IN, Zhu Z, and Husain M. Calcineurin-independent regulation of plasma membrane Ca2+ ATPase-4 in the vascular smooth muscle cell cycle. Am J Physiol Cell Physiol. 2003;285(1):C88–C95. doi: 10.1152/ajpcell.00518.2002. [PubMed: 12660151]

44. Reznikoff CA, Brankow DW, and Heidelberger C. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. Cancer Res. 1973;33(12):3231–3238. [PubMed: 4357355]
45. Günther K, Rust M, Leers J, Boettger T, Scharfe M, Jarek M, Bartkuhn M, and Renkawitz R. Differential roles for MBD2 and MBD3 at methylated CpG islands, active promoters and binding to exon sequences. Nucleic Acids Res. 2013;41(5):3010–3021. Epub 2013/01/29. doi: 10.1093/nar/gkt035. [PubMed: 23361464]
46. Kolodziej KE, Pourfarzad F, de Boer E, Krpic S, Grosveld F, and Strouboulis J. Optimal use of tandem biotin and V5 tags in ChIP assays. BMC molecular biology. 2009;10:6–6. doi: 10.1186/1471-2199-10-6. [PubMed: 19196479]
47. Ju-Rong Y, Ke-Hong C, Kun H, Bi-Qiong F, Li-Rong L, Jian-Guo Z, Kai-Long L, and Ya-Ni H. Transcription Factor Trps1 Promotes Tubular Cell Proliferation after Ischemia-Reperfusion Injury through cAMP-Specific 3',5'-Cyclic Phosphodiesterase 4D and AKT. J Am Soc Nephrol. 2017;28(2):532–544. Epub 2016/07/27. doi: 10.1681/ASN.2016010009. [PubMed: 27466160]
48. Goss M, Socorro M, Monier D, Verdelis K, and Napierala D. Trps1 transcription factor regulates mineralization of dental tissues and proliferation of tooth organ cells. Mol Genet Metab. 2019;126(4):504–512. Epub 2019/01/23. doi: 10.1016/j.ymgme.2019.01.014. [PubMed: 30691926]
49. Suemoto H, Muragaki Y, Nishioka K, Sato M, Ooshima A, Itoh S, Hatamura I, Ozaki M, Braun A, Gustafsson E, and Fässler R. Trps1 regulates proliferation and apoptosis of chondrocytes through Stat3 signaling. Dev Biol. 2007;312(2):572–581. doi: 10.1016/j.ydbio.2007.10.001. [PubMed: 17997399]
50. Wuelling M, Kaiser FJ, Buelens LA, Braunholz D, Shivdasani RA, Depping R, and Vortkamp A. Trps1, a regulator of chondrocyte proliferation and differentiation, interacts with the activator form of Gli3. Dev Biol. 2009;328(1):40–53. doi: 10.1016/j.ydbio.2009.01.012. [PubMed: 19389374]
51. Michigami T and Ozono K. Roles of Phosphate in Skeleton. Front Endocrinol. 2019;10:180–180. doi: 10.3389/fendo.2019.00180.
52. Kimata M, Michigami T, Tachikawa K, Okada T, Koshimizu T, Yamazaki M, Kogo M, and Ozono K. Signaling of extracellular inorganic phosphate up-regulates cyclin D1 expression in proliferating chondrocytes via the Na+/Pi cotransporter Pit-1 and Raf/MEK/ERK pathway. Bone. 2010;47(5):938–947. doi: 10.1016/j.bone.2010.08.006. [PubMed: 20709201]
53. Julien M, Khoshniat S, Lacreusette A, Gatius M, Bozec A, Wagner EF, Wittrant Y, Masson M, Weiss P, Beck L, Magne D, and Guicheux J. Phosphate-Dependent Regulation of MGP in Osteoblasts: Role of ERK1/2 and Fra-1. J Bone Miner Res. . 2009;24(11):1856–1868. doi: 10.1359/jbmr.090508. [PubMed: 19419315]
54. Conrads KA, Yi M, Simpson KA, Lucas DA, Camalier CE, Yu L-R, Veenstra TD, Stephens RM, Conrads TP, and Beck GR. A Combined Proteome and Microarray Investigation of Inorganic Phosphate-induced Pre-osteoblast Cells. Mol Cell Proteomics. 2005;4(9):1284. doi: 10.1074/mcp.M500082-MCP200. [PubMed: 15958391]
55. Yu H, Maurer F, and Medcalf RL. Plasminogen activator inhibitor type 2: a regulator of monocyte proliferation and differentiation. Blood. 2002;99(8):2810–2818. doi: 10.1182/blood.V99.8.2810. [PubMed: 11929770]
56. Hibino T, Matsuda Y, Takahashi T, and Goetinck PF. Suppression of Keratinocyte Proliferation by Plasminogen Activator Inhibitor-2. J Invest Dermatol. 1999;112(1):85–90. doi: 10.1046/j.1523-1747.1999.00466.x. [PubMed: 9886269]
57. Wang Y and Jensen PJ. Regulation of the level and glycosylation state of plasminogen activator inhibitor type 2 during human keratinocyte differentiation. Differentiation. 1998;63(2):93–99. doi: 10.1046/j.1432-0436.1998.6320093.x. [PubMed: 9674118]
58. Delhase M, Kim S-Y, Lee H, Naiki-Ito A, Chen Y, Ahn E-R, Murata K, Kim S-J, Lautsch N, Kobayashi KS, Shirai T, Karin M, and Nakanishi M. TANK-binding kinase 1 (TBK1) controls cell survival through PAI-2/serpinB2 and transglutaminase 2. Proc Natl Acad Sci U S A. 2012;109(4):E177–E186. Epub 2011/12/27. doi: 10.1073/pnas.1119296109. [PubMed: 22203995]
59. Wohlwend A, Belin D, and Vassalli JD. Plasminogen activator-specific inhibitors produced by human monocytes/macrophages. J Exp Med. 1987;165(2):320–339. doi: 10.1084/jem.165.2.320. [PubMed: 2434595]

60. Genton C, Kruithof EK, and Schleuning WD. Phorbol ester induces the biosynthesis of glycosylated and nonglycosylated plasminogen activator inhibitor 2 in high excess over urokinase-type plasminogen activator in human U-937 lymphoma cells. Journal Cell Biol. 1987;104(3):705–712. doi: 10.1083/jcb.104.3.705. [PubMed: 3102504]
61. Kruithof E, Vassalli J-D, Schleuning W, Mattaliano R, and Bachmann F. Purification and characterization of a plasminogen activator inhibitor from the histiocytic lymphoma cell line U-937. J Biol Chem. 1986;261(24):11207–11213. [PubMed: 3090045]
62. Belin D, Wohlwend A, Schleuning WD, Kruithof EK, and Vassalli JD. Facultative polypeptide translocation allows a single mRNA to encode the secreted and cytosolic forms of plasminogen activators inhibitor 2. EMBO J. 1989;8(11):3287–3294. [PubMed: 2583099]
63. Lee N-H, Park S-R, Lee JW, Lim S, Lee S-H, Nam S, Kim DY, Hah SY, Hong I-S, and Lee H-Y. SERPINB2 Is a Novel Indicator of Cancer Stem Cell Tumorigenicity in Multiple Cancer Types. Cancers. 2019;11(4):499. doi: 10.3390/cancers11040499.
64. Kinnby B, Borgström MK, Granath L, Lecander I, and Sundin B. Tissue plasminogen activator (t-PA) and placental plasminogen activator inhibitor (PAI-2) in gingival fluid from 8–9-year-old children. Scand J Dent Res. 1993;101(5):279–281. doi: 10.1111/j.1600-0722.1993.tb01120.x. [PubMed: 8248729]
65. Simmer JP and Hu JCC. Expression, Structure, and Function of Enamel Proteinases. Connect Tissue Res. 2002;43(2–3):441–449. doi: 10.1080/03008200290001159. [PubMed: 12489196]
66. Bonnans C, Chou J, and Werb Z. Remodelling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol. . 2014;15(12):786–801. doi: 10.1038/nrm3904. [PubMed: 25415508]
67. Toyman U, Tüter G, Kurtiş B, Kıvrak E, Bozkurt Ş, Yücel AA, and Serdar M. Evaluation of gingival crevicular fluid levels of tissue plasminogen activator, plasminogen activator inhibitor 2, matrix metalloproteinase-3 and interleukin 1-β in patients with different periodontal diseases. J Periodontal Res. 2015;50(1):44–51. doi: 10.1111/jre.12179. [PubMed: 24690077]
68. Lindberg P, Kinnby B, Lecander I, Lang NP, and Matsson L. Increasing expression of tissue plasminogen activator and plasminogen activator inhibitor type 2 in dog gingival tissues with progressive inflammation. Arch Oral Biol. 2001;46(1):23–31. doi: 10.1016/S0003-9969(00)00098-4. [PubMed: 11163592]
69. Tiosano D and Z.e. Hochberg. Hypophosphatemia: the common denominator of all rickets. J Bone Miner Metab. 2009;27(4):392–401. doi: 10.1007/s00774-009-0079-1. [PubMed: 19504043]
70. Murshed M, Harmey D, Millán JL, McKee MD, and Karsenty G. Unique coexpression in osteoblasts of broadly expressed genes accounts for the spatial restriction of ECM mineralization to bone. Genes Dev. 2005;19(9):1093–1104. Epub 2005/04/15. doi: 10.1101/gad.1276205. [PubMed: 15833911]
71. Zhang H, Chavez MB, Kolli TN, Tan MH, Fong H, Chu EY, Li Y, Ren X, Watanabe K, Kim DG, and Foster BL. Dentoalveolar Defects in the Hyp Mouse Model of X-linked Hypophosphatemia. J Dent Res. 2020;99(4):419–428. doi: 10.1177/0022034520901719. [PubMed: 31977267]
72. Bergwitz C, Roslin NM, Tieder M, Loredo-Osti JC, Bastepe M, Abu-Zahra H, Frappier D, Burkett K, Carpenter TO, Anderson D, Garabedian M, Sermet I, Fujiwara TM, Morgan K, Tenenhouse HS, and Juppner H. SLC34A3 mutations in patients with hereditary hypophosphatemic rickets with hypercalciuria predict a key role for the sodium-phosphate cotransporter NaPi-IIc in maintaining phosphate homeostasis. Am J Hum Genet. 2006;78(2):179–192. Epub 2005/12/09. doi: 10.1086/499409. [PubMed: 16358214]
73. Mobley CG, Kuzynski M, Zhang H, Jani P, Qin C, and Napierala D. Dspp-independent Effects of Transgenic Trps1 Overexpression on Dentin Formation. J Dent Res. 2015;94(8):1128–34. Epub 2015/05/23. doi: 10.1177/0022034515586709. [PubMed: 25999324]
74. Napierala D, Sam K, Morello R, Zheng Q, Munivez E, Shivdasani RA, and Lee B. Uncoupling of chondrocyte differentiation and perichondrial mineralization underlies the skeletal dysplasia in tricho-rhino-phalangeal syndrome. Hum Mol Genet. 2008;17(14):2244–54. Epub 2008/04/22. doi: 10.1093/hmg/ddn125. [PubMed: 18424451]
75. Piscopo DM, Johansen EB, and Derynck R. Identification of the GATA factor TRPS1 as a repressor of the osteocalcin promoter. J Biol Chem. 2009;284(46):31690–703. Epub 2009/09/18. doi: 10.1074/jbc.M109.052316. [PubMed: 19759027]

76. Nishino J, Yamazaki M, Kawai M, Tachikawa K, Yamamoto K, Miyagawa K, Kogo M, Ozono K, and Michigami T. Extracellular Phosphate Induces the Expression of Dentin Matrix Protein 1 Through the FGF Receptor in Osteoblasts. J Cell Biochem. 2017;118(5):1151–1163. Epub 2016/09/18. doi: 10.1002/jcb.25742. [PubMed: 27639037]

77. Takashi Y, Kosako H, Sawatsubashi S, Kinoshita Y, Ito N, Tsoumpra MK, Nangaku M, Abe M, Matsuhisa M, Kato S, Matsumoto T, and Fukumoto S. Activation of unliganded FGF receptor by extracellular phosphate potentiates proteolytic protection of FGF23 by its O-glycosylation. Proc Natl Acad Sci U S A. 2019;116(23):11418–11427. Epub 2019/05/18. doi: 10.1073/pnas.1815166116. [PubMed: 31097591]

Highlights:

- SerpinB2 is highly expressed by cells producing mineralized ECM.
- *SerpinB2* expression is activated by phosphate, while the Trps1 transcription factor represses *SerpinB2* in committed osteogenic cells.
- SerpinB2 deficiency impairs mineralization in vitro.

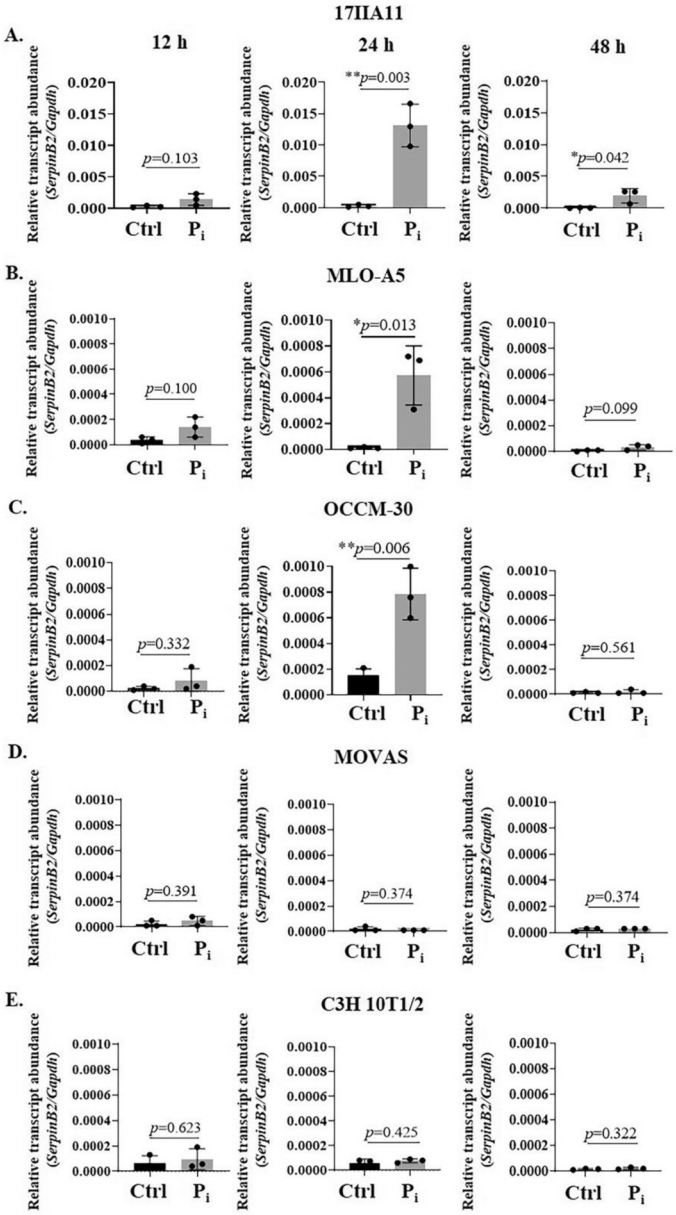


Fig. 1.

Phosphate increases *SerpinB2* expression specifically in committed osteogenic cells. qRT-PCR results showing *SerpinB2* expression in cells maintained in standard growth medium (Ctrl) in comparison with cells exposed to 5 mM P_i (P_i) for 12, 24 and 48h. (A) 17IIA11, (B) MLO-A5, (C) OCCM-30, (D) MOVAS, and (E) C3H10T1/2 cell lines (non-mineralizing control). qRT-PCR data are presented as the mean relative transcript abundance of *SerpinB2* mRNA normalized to *Gapdh* ±S.D. from three independent RNA preparations per cell line; *p<0.05, and **p<0.01.

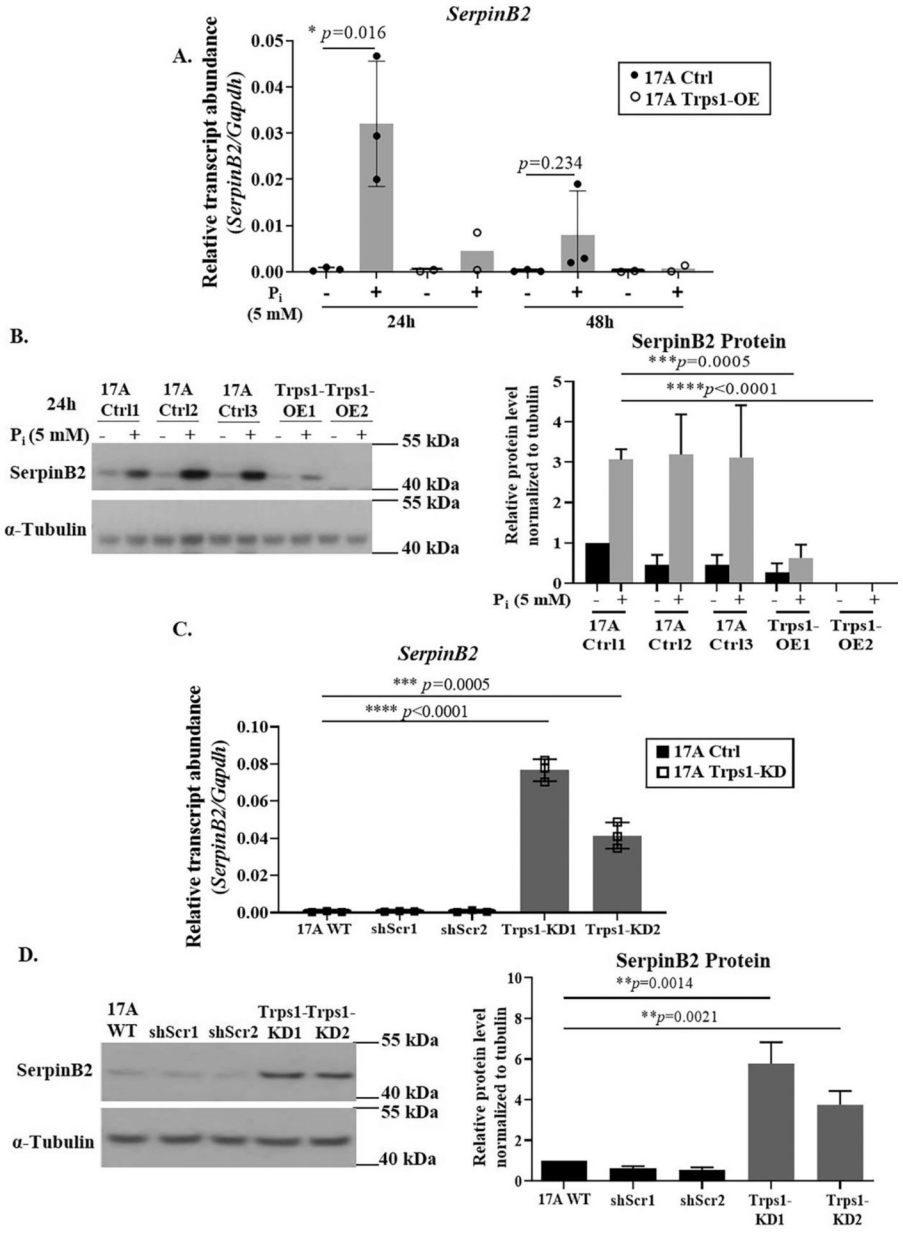


Fig. 2.

Inverse correlation between expression of *Trps1* and *SerpinB2* in 17IIA11 (17A) osteogenic cell line. (A) qRT-PCR results showing *SerpinB2* expression in three 17IIA11 control cell lines (17ACtrl1, 17ACtrl2, 17ACtrl3) and two 17IIA11 cell lines overexpressing Trps1 (Trps1-OE1, Trps1-OE2) maintained in standard growth medium and upon exposure to 5 mM P_i (P_i) for 24 and 48h. qRT-PCR data are presented as the mean relative transcript abundance of *SerpinB2* mRNA normalized to *Gapdh* ±S.D. Asterisks denote statistical significant difference: *p<0.05. (B) Representative Western blot images and a graph (showing densitometry-based quantification of the Western blot results by ImageJ) of SerpinB2 protein (47kDa) in 17IIA11 control cell lines (17ACtrl1, 17ACtrl2, 17ACtrl3) and 17IIA11 cell lines overexpressing Trps1 (Trps1-OE1, Trps1-OE2) maintained in standard

growth medium and upon exposure to 5 mM P_i for 24h. α-tubulin was used as the protein loading control. (C) qRT-PCR results showing *SerpinB2* expression in three 17IIA11 control cell lines (17A WT, shScr1, shScr2) and two 17IIA11 cell lines deficient in Trps1 (Trps1-KD1, Trps1-KD2) maintained in standard growth medium. Individual data points of 3 independent experiments per cell line, mean ± SD are shown. Asterisks denote statistical significant difference: ***p<0.001, ****p<0.0001. (D) Representative Western blot images and graph (showing densitometry-based quantification of the Western blot results by ImageJ) of SerpinB2 protein (47kDa) in 17IIA11 control cell lines (17A WT, shScr1, shScr2) and two 17IIA11 cell lines deficient in Trps1 (Trps1-KD1, Trps1-KD2) maintained in standard growth medium. α-tubulin was used as a protein loading control.

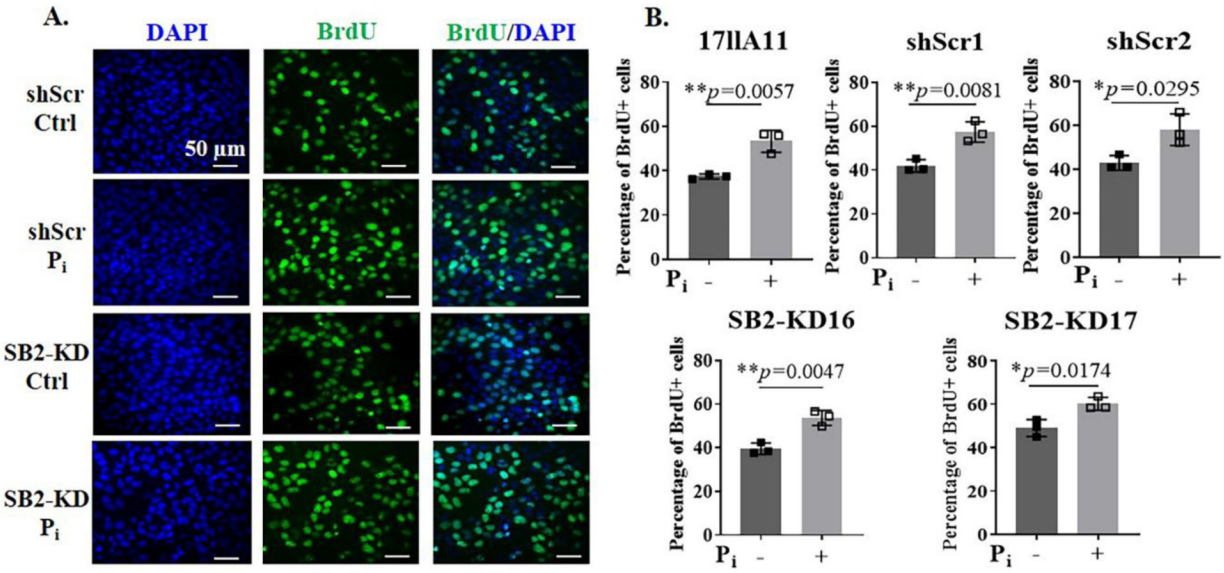


Fig. 3.

SerpinB2 deficiency does not affect P_i-stimulated proliferation of osteogenic cells. Proliferation of control (17IIA11, shScr1, shScr2) and *SerpinB2*-deficient (SB2-KD16 and SB2-KD17) 17IIA11 cell lines was compared using BrdU labeling and quantification of BrdU-positive cells. (A) Representative IF images showing BrdU-positive control (shScr2) and *SerpinB2*-deficient (SB2-KD16) 17IIA11 cell lines maintained in standard growth medium (Ctrl) and stimulated with 5 mM P_i (P_i) for 24h. (B) Quantification of cell proliferation, expressed as the percentage of BrdU-positive cells. Individual data points of 3 experiments per cell line, mean ± SD are shown. Asterisks denote statistical significant difference: *p<0.05, **p<0.01.

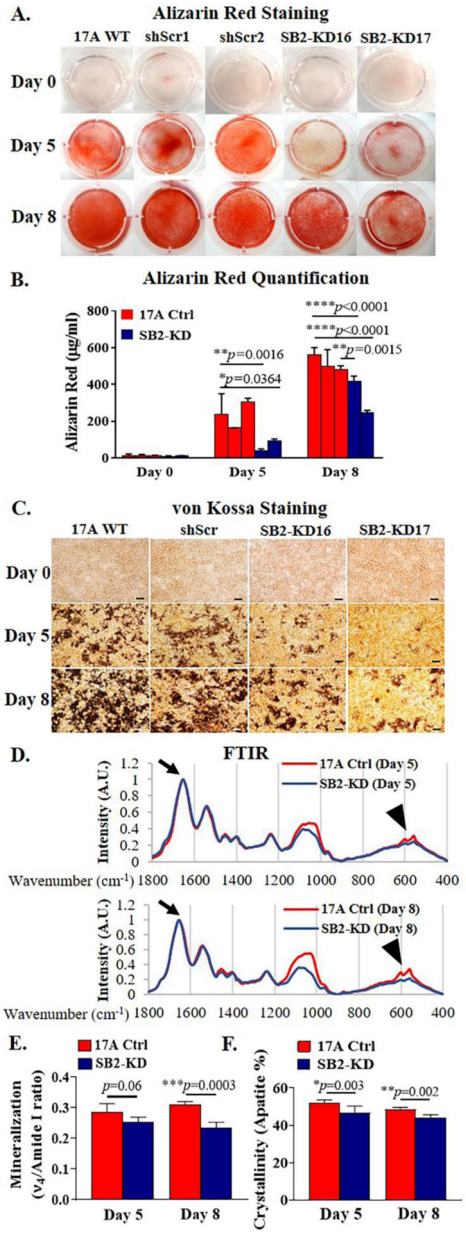


Fig. 4.

SerpinB2 deficiency results in delayed and decreased mineralization in vitro. (A) Representative images of alizarin red staining at mineralization day 0, 5 and 8 of 17IIA11 control (17A WT, shScr1, shScr2) and *SerpinB2*-deficient (SB2-KD16 and SB2-KD17) cell lines. (B) Quantification of alizarin red is presented as the mean ± SD from three independent experiments per cell line. Asterisks denote statistically significant difference: $*p<0.05$; $**p<0.01$; $****p<0.0001$. (C) Representative images of von Kossa staining at mineralization day 0, 5 and 8 of 17IIA11 control and *SerpinB2*-deficient cell lines. (D) Representative FTIR spectra (in the 1800–400 cm^{-1} region) of ECM from 17IIA11 control and *SerpinB2*-deficient cells at mineralization day 5 and 8. The solid arrow shows Amide I peak in the 1800–1500 cm^{-1} region and the arrowhead shows ν_4 peak in the 700–500 cm^{-1}

region. (E) Mineral content, expressed as mineral (ν_4 of phosphate) to organic (protein Amide I) ratios and (F) mineral crystallinity of 17IIA11 control and *SerpinB2*-deficient cells at mineralization day 5 and 8. Data represents mean ± SD of 4 individual experiments. Asterisks denote statistically significant difference: *p<0.05, **p<0.01, ***p<0.001.

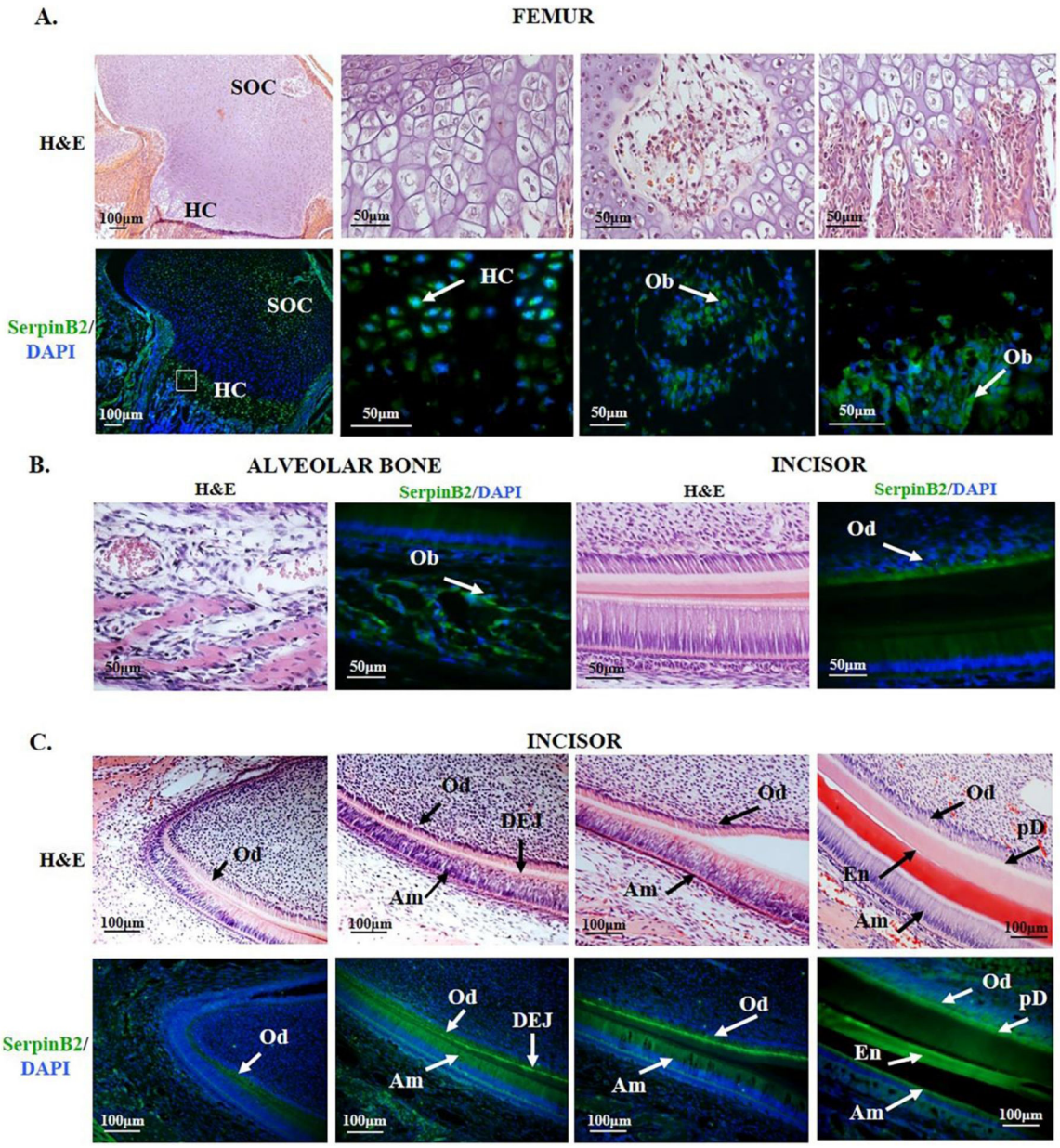


Fig. 5.

SerpinB2 is highly and specifically expressed in cells producing mineralized ECM and hypertrophic chondrocytes. (A) Representative H&E (upper row) and corresponding IHC images (lower row) of distal femur of P7 WT mice showing high expression of SerpinB2 in hypertrophic chondrocytes (HC), osteoblasts (Ob) of secondary ossification center (SOC), and osteoblasts (Ob) of primary spongiosa. (B) Representative H&E and corresponding IHC images of alveolar bone and mandibular incisor of P7 WT mice showing high expression of SerpinB2 in osteoblasts (Ob) of alveolar bone and odontoblasts (Od) in the incisor. (C) Representative H&E and corresponding IHC images of mandibular incisor of P14 WT mice showing SerpinB2 protein in the apical part of odontoblasts (Od) and ameloblasts (Am), dentino-enamel junction (DEJ), predentin (pD) and developing enamel (En).

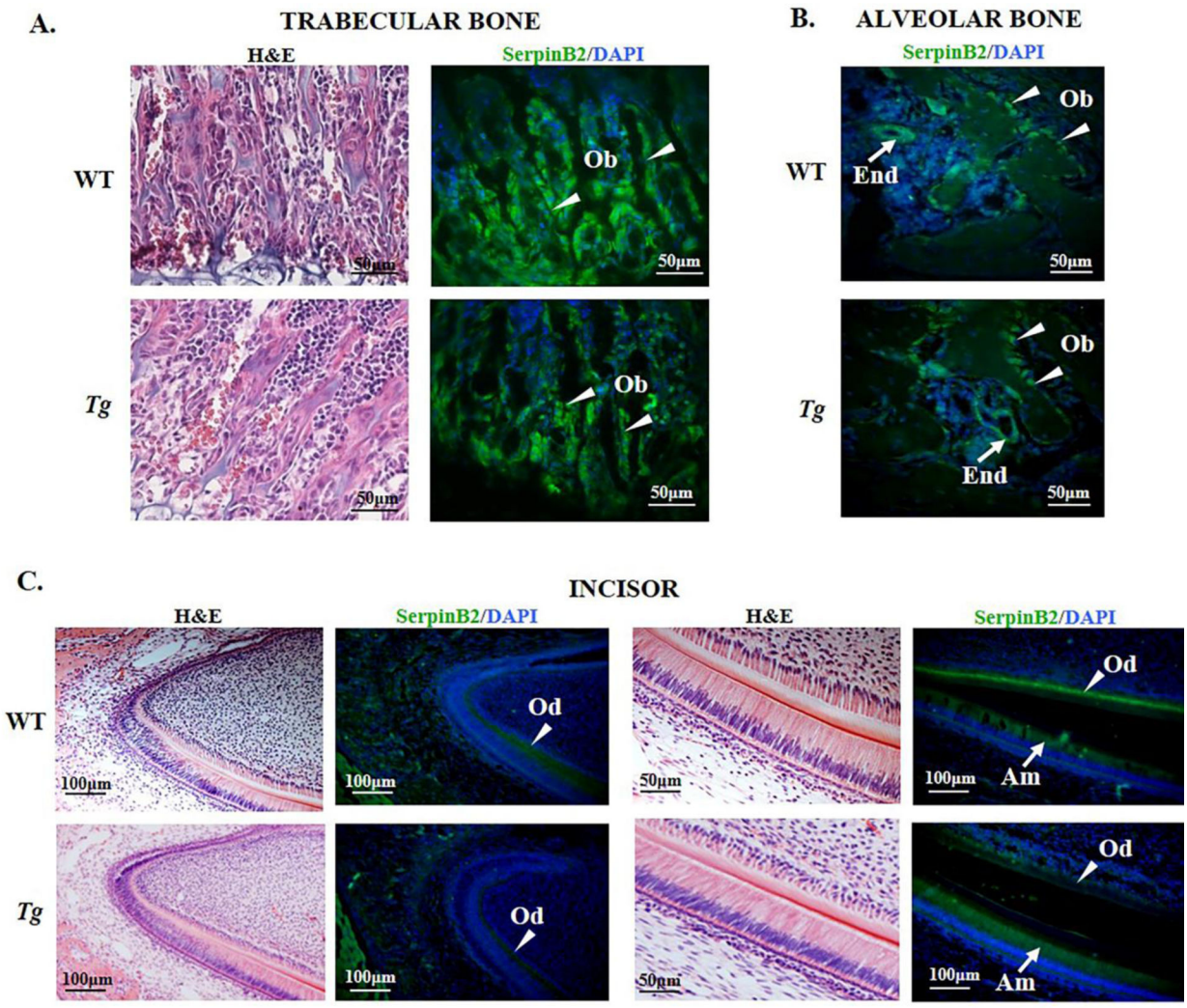


Fig. 6.

Transgenic overexpression of Trps1 results in cell type-specific downregulation of SerpinB2. (A) Representative H&E and IHC images of distal femur trabecular bone of P7 WT and *2.3Col1a1-Trps1* transgenic mice *(Tg)*. Arrowheads indicate SerpinB2 in osteoblasts (Ob). Lower levels of SerpinB2 are apparent in osteoblasts in the trabecular bone of *2.3Col1a1-Trps1* in comparison with WT mice. (B) Representative IHC images of alveolar bone of P14 WT and *2.3Col1a1-Trps1* transgenic mice. Arrowheads indicate SerpinB2 in osteoblasts (Ob); arrows point at SerpinB2 in endothelial cells (End). There is no apparent difference in SerpinB2 levels in osteoblasts lining the alveolar bone nor in endothelial cells of WT and *2.3Col1a1-Trps1* mice. (C) Representative H&E and IHC images of mandibular incisor of P14 WT and *2.3Col1a1-Trps1* transgenic mice. Arrowheads indicate SerpinB2 in odontoblasts (Od), arrows point at SerpinB2 in ameloblasts (Am). *2.3Col1a1-Trps1* odontoblasts show lower levels of SerpinB2 than WT odontoblasts. There is no apparent difference in SerpinB2 levels in ameloblasts of WT and *2.3Col1a1-Trps1* mice.

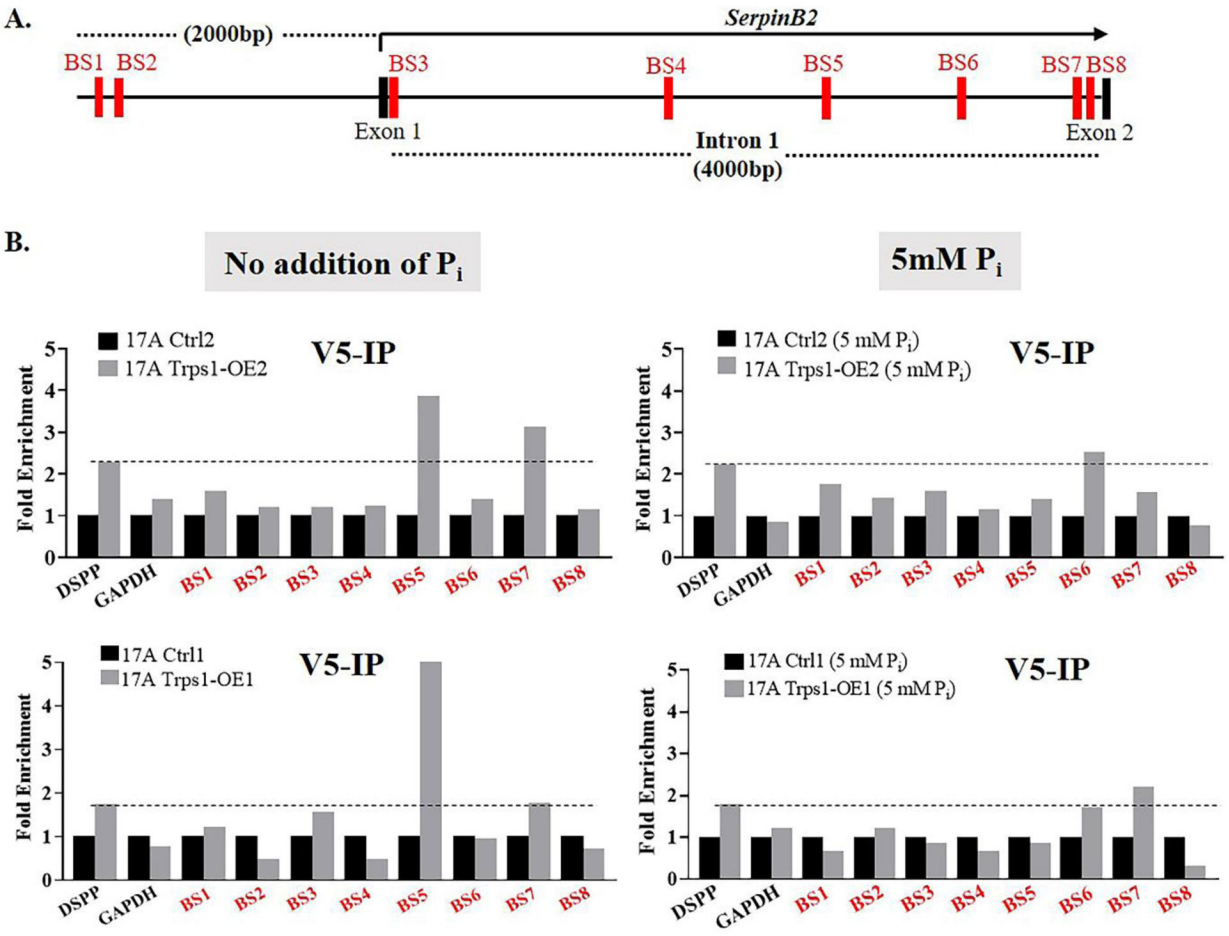


Fig. 7.

Phosphate affects Trps1 binding to the *SerpinB2* gene. (A) Approximate location of 8 putative Trps1 binding sites (BS; red bars) in the mouse *SerpinB2* promoter and intron 1 identified using TRANSFAC database. Black bars indicate position of *SerpinB2* exon 1 and 2. (B) Results of the ChIP assays expressed as fold enrichment of Trps1 binding sites in DNA-protein complexes immunoprecipitated with V5 antibody from Trps1-OE cells in comparison with control 17IIA11 cells. Left panel: results of ChIP assays performed in cells maintained in standard growth medium; right panel: results of ChIP assays performed in cells exposed to 5 mM P_i for 24h.

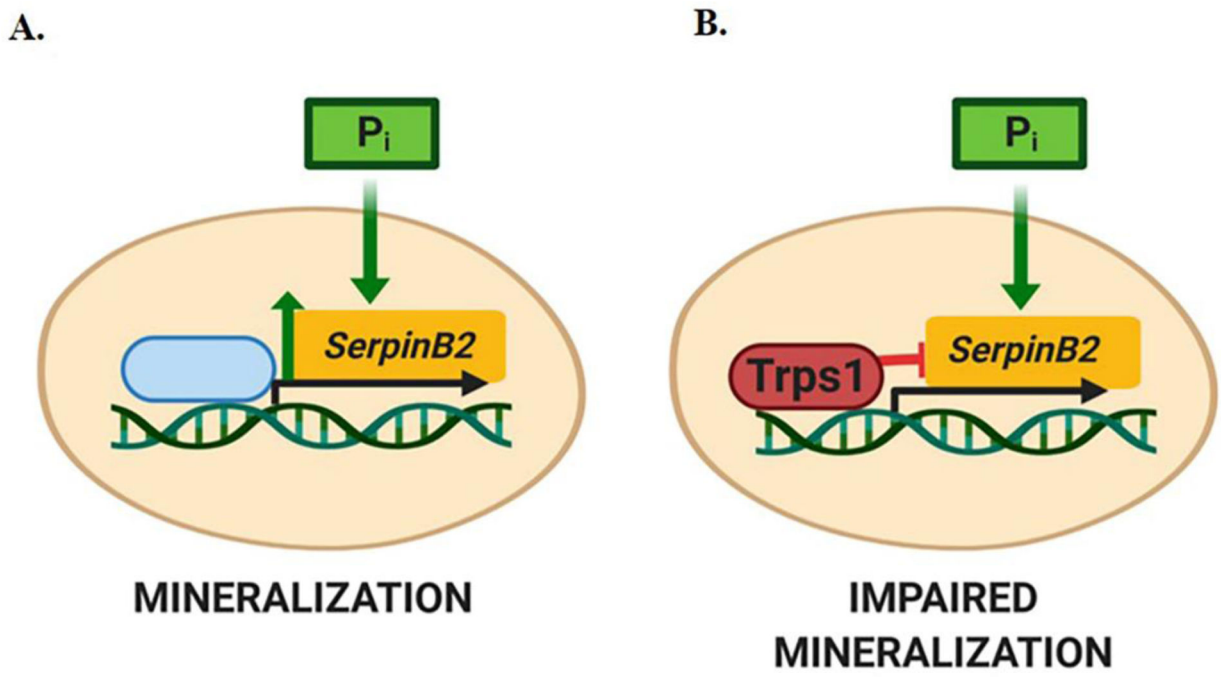


Fig. 8.

A model of regulation of *SerpinB2* expression by mineralization-regulating factors: (A) P_i signaling molecule activates *SerpinB2* expression in osteogenic cells through yet unknown transcriptional mechanism. P_i-induced expression of *SerpinB2* supports mineralization. (B) Trps1 transcription factor represses *SerpinB2* expression through direct interaction with its regulatory elements. Diminished *SerpinB2* expression in cells forming mineralized ECM impairs mineralization.