Published in final edited form as:

Connect Tissue Res. 2018 December ; 59(SUP1): 6–12. doi:10.1080/03008207.2017.1408601.

The ER Ca²⁺ sensor STIM1 can activate osteoblast and odontoblast differentiation in mineralized tissues

Yinghua Chen, Amsaveni Ramachandran, Youbin Zhang, Rahul Koshy, and Anne George*

Brodie Tooth Development Genetics & Regenerative Medicine Research Laboratory, Department of Oral Biology, University of Illinois at Chicago, Chicago, IL 60612

Abstract

Bone and dentin development requires temporal and spatial deposition of calcium phosphate mineral. A host of proteins works in concert to contribute to this tightly regulated process while malfunction in this scheme often leads to pathological defects. We have reported earlier that DMP1 stimulation of preosteoblasts leads to calcium release from internal Ca²⁺ stores and this store depletion is sensed by the ER Ca²⁺ sensor STIM1 (stromal interaction molecule 1). In this study, we first assessed the temporal and spatial localization of STIM1 protein during the development of bone and dentin by immunohistochemical methods. We further analyzed the function of STIM1 by establishing a stable MC3T3-E1 cell-line by overexpressing STIM1 (MC3T3-E1/STIM1 OE). Under mineralizing conditions, STIM1 overexpressing cells showed increased calcium deposits with higher expression of key osteogenic markers, such as Runx2 and type I collagen, BMP4 when compared with the control cells. Our results demonstrate that during mineralized matrix formation STIM1, the key ER sensor protein, can promote cellular differentiation in the presence of extracellular calcium.

Keywords

STIM1; mineralization; extracellular matrix; osteoblast; odontoblast

Introduction

Bone and dentin mineralization is a biologically controlled process orchestrated in space and time by osteoblasts and odontoblasts respectively. These cells secrete the organic matrix proteins, which self-assemble and dictate the specificity of precipitation of calcium phosphate polymorphs and their ultimate transformation to crystalline hydroxyapatite. The deposition of the organic matrix is the result of events orchestrated by the coordinated effort of a complex array of intracellular signaling pathways, cell-cell and cell-matrix interactions. The extracellular matrix of bone and dentin contains a complex network of macromolecules with distinctive physical, biochemical and biomechanical properties. Although the organic matrix is predominantly comprised of collagen fibrils, it also contains a diverse array of

*To whom correspondence should be addressed. Anne George, 312-413-0738, FAX: 312-996-6044, anneg@uic.edu.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

noncollagenous proteins. Together the organic matrix comprising the noncollagenous proteins dictate the deposition and orientation of the calcium phosphate polymorphs.

In the process of assembling a calcified matrix, osteoblasts and odontoblasts handle a large amount of calcium, however the mechanisms by which these cells maintain physiological intracellular calcium levels are not known. STIM1 and STIM2 are key modulators of store-operated calcium entry. We have reported earlier that stimulation of osteoblasts by DMP1 (dentin matrix protein 1), a key regulatory protein during matrix mineralization, results in a biphasic increase in cytosolic Ca^{2+} levels (1, 2). The initial transient increase is due to inositol triphosphate (IP_3)-mediated release of ER store Ca^{2+} , while the addition of extracellular Ca^{2+} causes an influx through the plasma membrane. Various pathways have been implicated in this process including store-operated Ca^{2+} channels (SOCC), voltage-operated Ca^{2+} channels, purinergic receptors, transient receptor membrane potential channels (TRPC) and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger among several others(3).

In this study, we demonstrated that STIM1 is highly expressed in osteoblasts and odontoblasts. We then focused on the role of STIM1 in osteoblasts and its effect on matrix mineralization.

Material and Methods

Immunohistochemistry.

Mouse heads at postnatal day 3 were fixed in 10 % neutral buffered formalin at 4 °C for 3 days and then processed for paraffin embedding. The femurs of postnatal 45 day old mice were fixed in 10 % neutral buffered formalin for 3 days at 4 °C, and decalcified with 10% EDTA, pH 7.4. Decalcification was confirmed by Faxitron image analysis and the specimens were paraffin embedded. 5 μm serial sections were used for immunohistochemistry with anti-STIM1 antibody (SC68897, Santa Cruz Biotechnology) and developed by using the Vectastain ABC Kit (Vector Labs). Sections were imaged using a Zeiss light microscope. All mice related studies were performed as per UIC protocol Animal Assurance number 16-178.

Generation of STIM1 overexpressing MC3T3-E1 and T3 cells.

The pSTIM1-CFP, was used to construct a vector expressing STIM1-CFP for lenti-viral packaging. Briefly the STIM1-CFP coding fragments from pSTIM1-CFP were released by restriction digestion using EcoRI and NotI. The resulting DNA fragments were inserted into the same sites of pLenti-EF1a-GFP-2A-Puro to generate pEF1a-STIM1-CFP, in which EF1a promoter drives the expression of STIM1-CFP.

Lenti-viral packaging and viral transduction were conducted as previously described (4). The transduced cells with ectopic STIM1 expression were selected at 10 $\mu\text{g}/\text{ml}$ puromycin. The puromycin resistance cells were pooled and used as stable STIM1 overexpressing cells for further experiments.

Isolation of total cellular proteins and western blot analysis.

Cells were harvested by trypsin digestion and centrifugation at 500 rpm at 4 °C for 5 min. After washing with PBS, protein pellets were stored at -80 °C until use. The cells were

lysed in RIPA buffer (Cell signaling) containing 1% proteinase inhibitor cocktail (Calbiochem). The mixture was incubated on ice for 30 min with gentle shaking and then centrifuged at 14,200 rpm at 4 °C for 30 min. The supernatant was used as the total cellular proteins. The concentration was measured by BIO-RAD protein assay agent (BIO-RAD) with BSA (Sigma) used as a standard. 50µg of the proteins were separated by a 10 % SDS-PAGE gel, and were subsequently electro-transferred to a PVDF membrane (BIO-RAD). The membranes were blocked with 5% fat-free milk in PBS and probed with anti-STIM1 antibody (SC68897). HRP-conjugated goat anti-rabbit IgG was used as secondary antibody. Protein bands were detected using SuperSignal™ West Pico chemiluminescent Substrate (Thermo Scientific) according to the manufacturer's protocol. The membranes were then washed 3 times with water and incubated with a stripping buffer (Thermo Scientific) for 30 min. After extensive washing with PBS, the membranes were processed as above with anti-tubulin antibody (t9026, Sigma) and HRP-conjugated goat anti-mouse IgG. The films were scanned as color images with a HP scanner (hp scanjet 8250). To obtain target protein band densities, these images were transformed into grey scale images and analyzed by ImageJ (1.50c).

Quantitative real time PCR (q-PCR).

Total RNA was extracted from harvested cells using RNeasy Plus Mini Kit (QIAGEN) according to the manufacture's protocol. cDNA was synthesized with Superscript III Reverse Transcriptase and Oligo-dT primer (Invitrogen) for 60 min at 50 °C. qPCR was carried out using FastStart Universal SYBR Green Master reagent (Roche) and primer pairs as needed on an ABI StepOnePlus instrument. The gene expression levels were estimated by the 2^{-CT} method with GAPDH gene expression level as an internal control. Primers were synthesized by IDT (Integrated DNA Technologies, Inc.) and the primer sequences are presented in Table 1.

Mineralization induction.

MC3T3-E1 or T3 control and transgenic cells were grown in alpha MEM or DMEM/F12 with 10 % FBS and 1% Antibiotic-Antimycotic (Invitrogen), respectively. Fresh media was added every other day, and the cells were passaged at 90% confluency. For mineralization induction, 10 mM β -glycerophosphate, 100 µg/ml ascorbic acid (Sigma-Aldrich), and 10 nM dexamethasone (Sigma-Aldrich) were added to the growth medium.

Alizarin Red Staining to assess calcium deposits in mineralized Nodule.

The previously published alizarin red staining protocol was followed (2,5). Briefly, the cells were incubated with osteogenic media for 7, 14, and 21 days. The cells were fixed in formalin for 20 min at room temperature and washed with distilled water. Two percent of Alizarin Red (Sigma) solution was added to fixed cells and incubated for 10–20 min. The cells were then rinsed with distilled water and imaged.

Statistical Analyses.

Data were presented as the mean $\pm$ standard deviation for at least three independent experiments. Probability values were calculated using the Student's t-test. Data with p values less than 0.05 were considered significant.

Results

Expression of STIM1 and STIM2 during cellular differentiation.

Expression levels of STIM1 and STIM2 were first established in MC3T3-E1 (mouse pre-osteoblasts) and T3 (rat odontoblasts) cells during differentiation. Results in Fig.1 showed that in MC3T3-E1 cells STIM1 expression levels increased at D7, peaked at D14 and then maintained high levels at day 21; in T3 cells STIM1 expression gradually increased during differentiation. On the other hand, STIM2 showed increasing expression levels during differentiation but to a lesser extent compared to STIM1.

Localization of STIM1 in bones and teeth during development.

Sections from 3 day postnatal mouse heads were used to detect STIM1 expression in the developing craniofacial tissues by immunohistochemical analysis. Expression of STIM1 was observed in pulp, ameloblasts, odontoblasts, and alveolar bone (Fig. 2A). In addition, strong expression of STIM1 was also detected in the osteocytes of the cortical bone and osteoblasts of the trabecular bone in the femur sections from 45 day old mice (Fig. 2B).

Characterization of stable cell lines overexpressing STIM1.

Western blot analysis confirmed the presence of STIM1 overexpression in both MC3T3-E1 and T3 transgenic cells. The STIM1-CFP fusion protein showed a distinct band at ~115 kDa due to the presence of CFP when compared to endogenous STIM1 (~80kDa) as detected by the anti-STIM1 antibody. Particularly, in MC3T3-E1/STIM1 OE cells STIM1-CFP protein levels were 24 (0.48/0.02) fold higher than endogenous STIM1 expression in the control cells relative to the tubulin, while endogenous STIM1 expression in the overexpressing cells were 5.5 (0.11/0.02) fold more than that in the control MC3T3-E1 cells. Similarly, in the T3/STIM1 OE cells, expression of STIM1-CFP was 2.4 (0.56/0.23) fold higher than STIM1 expression in the control cells, and endogenous STIM1 expression was 1.3 (0.29/0.23) fold higher than that in control T3 cells (Fig 3A). The STIM1-CFP protein expression levels clearly reflected their transcript levels, as ectopic STIM1-CFP gene expression was high in STIM1 overexpressing cells. With real-time RT-PCR, STIM1-CFP mRNA expression levels were quantified in the overexpressing cell lines by normalizing to GAPDH. Results in Fig 3B show the ratio of copies of STIM1-CFP relative to GAPDH and the corresponding values determined were 0.059 in MC3T3-E1/STIM1 OE cells and 0.026 in T3/STIM1 OE cells. As human specific STIM1 primers were used to detect STIM1-CFP, therefore, no amplification was observed with cDNAs from control MC3T3-E1 or T3 cells, which are from mouse or rat, respectively (Ct>30).

STIM1 overexpression and changes in gene expression during mineralization.

Correlation between changes in STIM1 gene expression and differentiation-specific genes were examined using MC3T3-E1/STIM1 OE cells, because MC3T3-E1 cells had traceable endogenous STIM1 protein and MC3T3-E1/STIM1 OE cells achieved higher increase of STIM1-CFP protein (24 folds as described above). Quantitative PCR analysis demonstrated that overexpression of STIM1 stimulated the differentiation of pre-osteoblastic cells to differentiated osteoblasts with enhanced expression of bone morphogenetic protein 4 (BMP4), collagen I (Col1a1), Runt-related transcription factor 2 (Runx2) and cyclin D1 when compared with the control cells (Fig 4A, and 4B). In addition, at day 0 MC3T3-E1/STIM1 OE cells showed higher osteocalcin (OCN), and bone sialoprotein (BSP) gene expression and low expression of Col1a1, Runx2, BMP2 & BMP4 compared to MC3T3-E1 cells at day 0 (Fig 4C).

STIM1 overexpression and induction of mineralization.

In vitro nodule formation assay was carried out to determine if overexpression of STIM1 had an influence on mineralized matrix formation. It was observed that overexpression of STIM1 enhanced the deposition of calcium in the extracellular matrix as differentiation progressed from 7 to 21 days and correspondingly the size of the nodule increased in MC3T3-E1/STIM1-OE cells (Fig. 5B).

DISCUSSION

One of the pathways responsible for extracellular Ca^{2+} influx depends on depletion of Ca^{2+} from ER stores, which causes activation of plasma membrane Ca^{2+} channels to refill internal stores. ORAI1 is a plasma membrane Ca^{2+} channel that is activated by STIM proteins. The mechanism by which ER acts as a capacitor has led to the term SOCE (Store-operated calcium entry) (6).

The molecular constituents of SOCE signaling identified STIM as a key player in SOCE by RNAi screening(7,8). As there are 2 vertebrate homologs of STIM, namely STIM1 and 2, therefore we first demonstrated that STIM1 is the predominant homolog present in osteoblasts and odontoblasts during early and late differentiation. Previous studies from our lab have demonstrated that when stimulated with DMP1, preosteoblasts show SOCE dependent-depletion of ER luminal Ca^{2+} store and enhanced mineralization formation. In current study, we showed that STIM1 is involved in SOCE and promotes cellular differentiation.

Immunohistochemical analysis confirmed that the mineralizing tissues of the craniofacial complex and the endochondral bone expressed STIM1. Specifically, STIM1 was localized in the odontoblasts, ameloblasts and the osteoblasts of the alveolar bone. Interestingly, STIM1 is also localized in the mineralized matrices of bone, dentin and enamel suggesting an unidentified function of STIM1 in the extracellular matrix. In the long bone, STIM1 is abundantly expressed in the periosteum, osteoblasts, osteocytes and in the matrix. Localization within the osteoblasts and odontoblasts suggests that STIM1 might be involved in SOCE during matrix mineralization.

Recent reports have shown the role of store-operated channels in providing the Ca^{2+} flux that activates downstream effectors (9–11). To demonstrate the role of STIM1 in osteoblasts and odontoblasts, we established stable cell lines overexpressing STIM1 in preosteoblast MC3T3-E1 cells and preodontoblast T3 cells. Under differentiation conditions MC3T3-E1 cells overexpressing STIM1 showed an increase in the expression of Runx2, Col1, BSP, OPN, BMP4 and cyclin D1 expression at 7 and 14 days, while ALP, osterix and BMP2 levels increased from 7 to 21 days during differentiation. Interestingly, DMP1 levels were not affected by STIM1 overexpression. Increased levels of type I collagen expression with STIM1 overexpression suggests that during mineralization the calcium phosphate deposits could be associated with the collagenous matrix. Further, higher levels of alkaline phosphatase levels indicate a dynamic extracellular matrix that can undergo calcification. Bone sialoprotein (BSP) is normally expressed early on during mineralization and is a potent nucleator of hydroxyapatite crystal. In this study BSP expression increased up to 14 days. Therefore, we conclude that STIM1 overexpression can promote the deposition of an ECM that has the potential to mineralize in the presence of calcium and phosphate. Alizarin red staining confirmed the formation of a mineralized matrix with overexpression of STIM1.

Interestingly at day 0, MC3T3-E1/STIM1 cells had higher expression of OCN and BSP with low expression levels of the other osteogenic markers when compared with the control cells. This suggests that STIM1 along with the osteogenic medium is necessary for osteoblast differentiation.

In summary, we have identified STIM1 as the main vertebrate homolog present in osteoblasts and odontoblasts. Overexpression of STIM1 in MC3T3-E1 cells promoted osteoblast differentiation and matrix mineralization, suggesting that STIM1 can promote intracellular Ca^{2+} homeostasis and in the presence of environmental cues can provide a signal for activation of downstream effectors that promote differentiation and matrix mineralization. Future studies would identify the signaling pathway activated by STIM1 that promotes osteoblast and odontoblast differentiation and matrix mineralization.

Acknowledgments:

The STIM1-CFP overexpression vector, pSTIM1-CFP, was kindly provided by Dr. Prakriya (Northwestern University).

Funding: The National Institutes of Health DE 11657 and the Brodie Endowment Fund.

References

- (1). Narayanan K, Ramachandran A, Hao J, He G, Park K, Cho M, George A. Dual functional roles of dentin matrix protein 1 - Implications in biomineralization and gene transcription by activation of intracellular Ca^{2+} store. *J Biol Chem* 2003 5 9;278(19):17500–17508. [PubMed: 12615915]
- (2). Eapen A, Sundivakkam P, Song Y, Ravindran S, Ramachandran A, Tiruppathi C, George A. Calcium-mediated Stress Kinase Activation by DMP1 Promotes Osteoblast Differentiation. *J Biol Chem* 2010 11 19;285(47):36339–36351. [PubMed: 20841352]
- (3). Berridge M, Bootman M, Roderick H. Calcium signalling: Dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 2003 7;4(7):517–529. [PubMed: 12838335]

- (4). Zhang Y, Song Y, Ravindran S, Gao Q, Huang CC, Ramachandran A, Kulkarni A, George A. DSPP Contains an IRES Element Responsible for the Translation of Dentin Phosphophoryn. *J Dent Res* 2014 2;93(2):155–161. [PubMed: 24352500]
- (5). Eapen A, Ramachandran A, George A. Dentin Phosphoprotein (DPP) Activates Integrin-mediated Anchorage-dependent Signals in Undifferentiated Mesenchymal Cells. *J Biol Chem* 2012 2 17;287(8):5211–5224. [PubMed: 22134916]
- (6). Parekh A, Putney J. Store-operated calcium channels. *Physiol Rev* 2005 4;85(2):757–810. [PubMed: 15788710]
- (7). Liou J, Kim M, Heo W, Jones J, Myers J, Ferrell J, et al. STIM is a Ca²⁺ sensor essential for Ca²⁺-store-depletion-triggered Ca²⁺ influx. *Curr Biol* 2005 7 12;15(13):1235–1241. [PubMed: 16005298]
- (8). Roos J, DiGregorio P, Yeromin A, Ohlsen K, Lioudyno M, Zhang S, Safrina O, Kozak JA, Wagner SL, Cahalan MD, Velicelebi G, Stauderman KA. STIM1, an essential and conserved component of store-operated Ca²⁺ channel function. *J Cell Biol* 2005 5 9;169(3):435–445. [PubMed: 15866891]
- (9). Ng SW, Di Capite J, Singaravelu K, Parekh AB. Sustained Activation of the Tyrosine Kinase Syk by Antigen in Mast Cells Requires Local Ca²⁺ Influx through Ca²⁺ Release-activated Ca²⁺ Channels. *J Biol Chem* 2008 11 14;283(46):31348–31355. [PubMed: 18806259]
- (10). Chang W, Di Capite J, Singaravelu K, Nelson C, Halse V, Parekh AB. Local Ca²⁺ influx through Ca²⁺ release-activated Ca²⁺ (CRAC) channels stimulates production of an intracellular messenger and an intercellular pro-inflammatory signal. *J Biol Chem* 2008 2 22;283(8):4622–4631. [PubMed: 18156181]
- (11). Di Capite J, Ng SW, Parekh AB. Decoding of Cytoplasmic Ca²⁺ Oscillations through the Spatial Signature Drives Gene Expression. *Curr Biol* 2009 5 26;19(10):853–858 [PubMed: 19375314]

Fig. 1: STIM1 is the predominant vertebrate homologue present during the differentiation of MC3T3-E1 and T3 cells

MC3T3-E1 cells (A) and T3 cells (B) were cultured in differentiation media and total RNA was isolated at 0,7,14 & 21 days and real-time PCR analysis was performed. The expression of STIM1 increased significantly from 7 to 14 days and decreased slightly at 21 days while STIM2 expression levels changed very slightly with differentiation in MC3T3-E1 cells (A). In T3 odontoblast cells STIM1 expression levels progressively increased with differentiation while STIM2 had a low expression. Gene expression were normalized to GAPDH expression and the expression levels at day 1 were set as 1 to calculate the fold changes. *: $p < 0.05$.

Fig. 2: Localization of STIM1 during the development of bone and teeth

(A). Localization of STIM1 in the osteoblasts of alveolar bone and odontoblasts of postnatal day 3 mouse head section. The images (2) and (3) were zoomed insets in (1). Arrows indicate representative cells. Am = ameloblasts; Od = odontoblasts; P = dental pulp cells. Bars: 200 μ m (1); 50 μ m (2, 3).

(B). Localization of STIM1 in the periosteum, osteocytes of the cortical bone (1) and osteoblasts of the trabecular bone (2) of 45 day mouse femur sections. Arrows indicate representative cells. Oc = Osteocytes; Ob = Osteoblasts. Bars: 15 μ m

Fig. 3: Overexpression of STIM1 in MC3T3-E1 and T3 cells

hSTIM1-CFP constructs were used for establishing a stable cell line overexpressing STIM1 in MC3T3-E1 and T3 cells. Western blot analysis of the total cell lysate showed the expression of STIM1-CFP in both the cell types, along with the endogenous STIM1 (A). The membranes were stripped and probed with α -tubulin which was used as loading control. The relative densities of target proteins to tubulin are shown. (B) Quantitative analysis of hSTIM1 mRNA levels in MC3T3-E1 cells overexpressing STIM1-CFP and T3 cells overexpressing STIM1-CFP. The results are expressed as the ratio of copies of STIM1-CFP gene relative to GAPDH. * indicates a significant change: $p < 0.05$.

Figure 4. Overexpression of STIM1 promotes osteogenic gene expression

MC3T3-E1 and MC3T3-E1/STIM1 OE cells were grown in the osteogenic differentiation media from 0–21 days. At the indicated time points, total RNA was extracted from cells, and were used to determine osteogenic gene expression by real time PCR. Gene expression was normalized to GAPDH expression and the expression level at day 0 was set as 1 to calculate the corresponding fold changes in MC3T3-E1 (A), and MC3T3-E1-STIM1-OE (B). Gene expression levels at day 0 in MC3T3-E1-STIM1 OE cells when compared to that of MC3T3-E1 cells (C). *: $p < 0.05$.

Fig. 5. Overexpression of STIM1 influences matrix mineralization

At the indicated time points, day 0, 7, 14 & 21 cells grown in regular and osteogenic medium were fixed and stained with Alizarin Red. Representative images of the whole plate (A) and magnified images of the mineral nodules at 40 X are shown (B). Alizarin Red staining demonstrate increased calcium deposition during the differentiation process. Bar: 100 μ m.

Table 1.

Primer list

Name Accession number	Forward primer	Reverse primer
Runx2 NM_001145920	CCT GAA CTC TGC ACC AAG TC	GAG GTG GCA GTG TCA TCA TC
Osx NM_130458	AGC GAC CAC TTG AGC AA A CAT C	CGG CTG ATT GGC TTC TTC TTC C
Alp NM_007431	GAT CAT TCC CAC GTT TTC AC	TGC GGG CTT GTG GGA CCT GC
OCN NM_007541	CTC CTG A GA GTC TGA CAA AGC CTT	GCT GTG ACA TCC ATT ACT TGC
BSP NM_008318	CAG GGA GGC AGT GAC TCT TC	AGT GTG GAA AGT GTG GCG TT
Opn NM_001204201	AGC AAG AAA CTC TTC CAA GCA A	GTG AGA TTC GTC AGA TTC ATC CG
Dmp1 NM_016779	CCC AAA GGA ACA CAA GGAGA	TTC GCT GAG GTT TTG ACC TT
Bmp2 NM_007553	CGG GAA CAG ATA CAG GAA GC	GCT GTT TGT GTT TGG CTT GA
BMP4 NM_007554	GCG AGC CAT GCT AGT TTG ATA CCT	CTC TGC CGA GGA GAT CACCTC ATT
Cyclin D1 NM_007631.2	GCG TAC CCT GAC ACC AAT CTC	CTC CTC TTC GCA CTT CTG CTC
Col1a1 NM_007742	GCT CAG CTT TGT GGA TAC GCG	TCT TGT CCT TGG GGT TCT TG
Gapdh NM_008085	AGG TCG GTG TGA ACG GAT TTG	TGT AGA CCA TGT AGT TGA GGT CA
hStim1 NM_003156	AGT CAC AGT GAG AAG GCG AC	CAA TTC GGC AAA ACT CTG CTG
mStim1 NM_009287	TGA AGA GTC TAC CGA AGC AGA	AGG TGC TAT GTT TCA CTG TTG G
mStim2 NM_001081103	CGA AGT GGA CGA GAG TGA TGA	GGA GTG TTG TTC CCT TCA CAT T