Dual Role of the Trps1 Transcription Factor in Dentin Mineralization*

Maria Kuzynski,† Morgan Goss,‡ Massimo Bottini,§*†, Manisha C. Yadav,§ Callie Mobley, †Tony Winters, ‡Anne Poliard,‡ Odile Kellermann*, Brendan Lee,‡‡, Jose Luis Millan,§, and Dobrawa Napierala*

From the †Institute of Oral Health Research, Department of Oral and Maxillofacial Surgery, School of Dentistry, University of Alabama at Birmingham, Birmingham, Alabama 35294, the ‡Sanford Children’s Health Research Center, Sanford-Burnham Medical Research Institute, La Jolla, California 92037, the §Department of Experimental Medicine and Surgery, University of Rome Tor Vergata, 00133Rome, Italy, the ††EA2496 UFR d’Odontologie, Université Paris Descartes, 92120 Montrouge, France, **INSERM UMR-S 1124, Université René Descartes Paris 5, Centre Universitaire des Saints-Pères, 75270 Paris Cedex 06, France, the ‡‡Department of Molecular and Human Genetics, Baylor College of Medicine, and the *Howard Hughes Medical Institute, Houston, Texas 77030

Background: Regulation of dentin mineralization at the gene expression level is poorly understood.

Results: Trps1 supports expression of osteogenic genes Alpl, Phospho1, Runx2, and Sp7 in preodontoblastic cells, and in mature cells Trps1 represses phosphate metabolism genes Phex and Vdr.

Conclusion: The role of Trps1 in mineralization depends on odontoblastic differentiation stage.

Significance: These findings provide insights into regulation of odontoblastic maturation and function.

TRPS1 (tricho-rhino-phalangeal syndrome) is a unique GATA-type transcription factor that acts as a transcriptional repressor. TRPS1 deficiency and dysregulated TRPS1 expression result in skeletal and dental abnormalities implicating deficiency and dysregulated TRPS1 type transcription factor that acts as a transcriptional repressor.

Mineralization is an orchestrated process in which crystals of calcium phosphate, termed hydroxypatite (HA),2 are laid down in precise amounts within the fibrous extracellular matrix (ECM) (1). Physiological mineralization occurs in skeletal tissues (bone and hypertrophic cartilage) and dental tissues (dentin, cementum, and enamel). Dentin, the most abundant component of the tooth, is very similar to bone in its matrix protein composition. However, unlike bone, dentin does not undergo remodeling and does not participate in calcium homeostasis (2). Cells that produce dentin, odontoblasts, differentiate from cranial neural crest-derived mesenchyme in a process that requires sequential and reciprocal mesenchymal-epithelial interactions. Mature odontoblasts secrete organic and mineral components of the dentin ECM; however, the transcriptional regulation of this process is not well understood (2).

Initiation of mineralization takes place within matrix vesicles (MVs), which bud off from the plasma membrane of cells producing the mineralizing matrix (3–5). The unique protein and lipid composition of MVs supports accumulation of high concentrations of Ca2+ and PO43− ions facilitating initial HA crystal formation (6). In particular, mineralization-competent MVs are enriched in tissue-nonspecific alkaline phosphatase (TNAP) and PHOSPHO1 phosphatase that provide P1, (7, 8). A series of genetic experiments with knock-out and transgenic

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† To whom the correspondence should be addressed: University of Alabama at Birmingham, School of Dentistry, Dept. of Oral and Maxillofacial Surgery, 707 SDB, 1919 7th Ave. South, Birmingham, AL 35294-0007. Tel.: 205-975-4298; Fax: 205-996-5109; E-mail: dobrawan@uab.edu.

‡‡ Howard Hughes Medical Institute, Houston, Texas 77030

§§ INSERM UMR-S 1124, Université René Descartes Paris 5, Centre Universitaire des Saints-Pères, 75270 Paris Cedex 06, France

The abbreviations used are: HA, hydroxyapatite; ECM, extracellular matrix; MV, matrix vesicle; TNAP, tissue-nonspecific alkaline phosphatase; Opn, osteopontin; MGP, matrix Gla protein; qRT-PCR, quantitative RT-PCR; AFM, atomic force microscopy; BisTris, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxymethyl)propane-1,3-diol; F, forward; R, reverse.
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mice demonstrated that TNAP and PHOSPHO1 have nonredundant functions in supporting mineralization (9, 10). PHOSPHO1 acts inside MVs to initiate deposition of HA in this compartment, whereas TNAP regulates mineralization by modifying the ECM environment (9, 11–13). The release of HA from MVs to the ECM supports further tissue mineralization. The extent of tissue mineralization depends on the composition and modifications of ECM proteins, the availability of Ca\(^{2+}\) and P\(_{i}\), and concentration of mineralization inhibitors, such as osteopontin (Opn), matrix Gla protein (MGP), and inorganic pyrophosphate (PPi) (14–16). Activity of TNAP and transporters of P\(_{i}\) (PiT1 and PiT2) and PPi (Ank) establishes a P\(_{i}\)/PPi ratio that either supports or represses mineralization (11). Although phosphate homeostasis is controlled at the systemic level, many of the proteins involved in this process are highly expressed in cells producing mineralizing matrix, suggesting that they participate in the locally regulated phosphate availability at the sites of mineralization (17–21). In humans, mutations in genes coding for major matrix proteins as well as in genes involved in phosphate metabolism result in skeletal and dental defects, underscoring the critical role of matrix composition and phosphate homeostasis for tissue mineralization (17, 22–26). For example, a genetically heterogeneous disorder, hypophosphatemia rickets, manifests as defective mineralization of skeletal and dental tissues due to dysregulated phosphate homeostasis (27, 28). TNAP deficiency (hypophosphatemia) also results in impaired mineralization associated with increased levels of mineralization inhibitors (29, 30).

A growing body of evidence implicates the Trps1 transcription factor in mineralization, although its role in this process and the mechanism whereby Trps1 regulates mineralization are not well understood. Trps1 is a unique zinc finger protein that belongs to the GATA family of transcription factors (31). Although a majority of studies have demonstrated that Trps1 is a transcriptional repressor, recently it has been shown that during hair formation Trps1 can activate expression of Wnt pathway genes (32–36). Mutations of the TRPS1 gene in humans cause the craniofacial and skeletal dysplasia tricho-rhino-phalangeal syndrome (TRPS) and Ambras syndrome (37, 38). Although these two diseases have distinct clinical presentations, abnormalities observed in patients with TRPS and Ambras indicate that TRPS1 is involved in the development of endochondral bones and teeth. We and others have shown that in perichondrial cells of endochondral bones, as well as in developing odontoblasts, Trps1 is highly expressed prior to mineralization, and the onset of mineralization coincides with down-regulation of Trps1 (32, 39, 40). This expression pattern suggests that Trps1 is involved in the maturation of cells destined to produce mineralizing matrix or that it prevents premature mineralization. The latter function has been demonstrated in our previous studies of a mouse model of TRPS (Trps1\(^{AGT}\) mice), where we uncovered that Trps1 deficiency leads to premature mineralization of the perichondrium of developing endochondral bones (32). In those studies, we did not address mineralization of dentin, because this occurs postnatally and Trps1\(^{AGT}/AGT\) mice die at birth. To determine whether Trps1 is sufficient to inhibit osteoblast and/or odontoblast-driven mineralization, we generated Col1a1-Trps1 transgenic mice expressing Trps1 from a cell type-specific 2.3-kb fragment of the Col1a1 promoter. Analyses of Col1a1-Trps1 mice demonstrated that Trps1 has a strong dominant negative effect on dentin but little effect on bone mineralization. The impairment in dentin formation in Col1a1-Trps1 mice is associated with repression of the Dsp gene, coding for major dentin matrix proteins required for dentin formation (41). Collectively, results of the studies of Trps1-deficient mice and mice overexpressing Trps1 in osteoblasts and odontoblasts suggest a context-dependent function of Trps1 in the mineralization process. This context may be determined by the type of cell that is driving mineralization or by the cell differentiation stage.

The dental phenotype of TRPS and Ambras patients clearly indicates that TRPS1 is involved in tooth development. On the molecular level, the dynamic and specific expression pattern of Trps1 in developing odontoblasts suggests its role in dentinogenesis. In these studies, we address the role of Trps1 in odontoblast-driven mineralization. We analyzed the consequences of both Trps1 deficiency and up-regulation on the mineralization process and the expression of genes involved in it. Results of these studies demonstrate for the first time that Trps1 regulates mineralization through different mechanisms in preodontoblasts and mature odontoblasts, and thus the role of Trps1 in the mineralization process depends on the odontoblast differentiation stage.

EXPERIMENTAL PROCEDURES

Cell Culture—Preodontoblastic 17IIA11 cells (42, 43) were maintained in standard DMEM (Invitrogen) with 5% FBS (Thermo Fisher Scientific, Logan, UT) and 100 units/ml penicillin and 100 µg/ml streptomycin (Cellgro, Manassas, VA) at 37 °C and 8% CO\(_2\). For the osteo-odontogenic differentiation experiments, cells were plated at 5 × 10\(^4\) cells per well of a 6-well plate. Once cells reached 85–95% confluency, osteo-odontogenic differentiation was induced by osteogenic medium (standard medium supplemented with 7 mM β-glycerophosphate and 50 µg/ml ascorbic acid). Osteogenic medium was changed every 48 h. Trps1-deficient, Trps1-overexpressing, and control stable cell lines were generated as described previously (41).

RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR (qRT-PCR)—Total RNA was extracted using GenElute Mammalian Total RNA miniprep kit (Sigma). Total RNA (1 µg), after DNase I treatment (Invitrogen), was converted to cDNA with SuperScript III reverse transcriptase kit (Invitrogen). Gene expression analyses were performed using AB Biosystems 7500 fast real time PCR system and Fast SYBR Green reaction mix (Roche Applied Science). Primer sequences are as follows: Gapdh F, GCAAGAGAGGCCCTATCCCAA, and R, CTCCC-TAGGCCCTCTCCTGTATAT; Actb F, GACGGTGACATCC-GTAAGACC, and R, CAGGAGGAGCAATGATCTTG-ATC; Trps1 F, ACAACCGCGACGAGATTATAG, and R, TAGTCAATGAAACCCGGTGCTTCTGTA; Phex F, CAGAAAG-GCCAAAATCCTCTACTCA, and R, TCCAGTCTAAGCAC-GCACTTCA; Vdr F, GCCCTCAATTCTGTGCAGACTTGAA-TACA, and R, GAGCGCTCTTCTATCATATCTCGTG; Fam20C F, AACCGTGAGAAGCGGAGAAG, and R, GGAAG-GGGACCTGCGGAAATC; Alpl F, CAGTGGGAGTGAGAAGC.
GCAGCC, and R, GACTGGGTGTGGCTTGTGTT; Phospho1 F, CCTGGGAAACAGCCGGCTGTTG, and R, CCCGGAGGAGCATAGCAAAGCGAG; Runx2 F, TGCCCGGGGAAATGTGAGAAC, and R, TGAAACTCCTCCTCGTCGG; Sp7 F, GGGCCGTTCAAGTCGCACTG, and R, ATCCGGGCGGCTGATTG; ATF4 F, GTGCGCCACTGGTAAAACC, and R, GGAAGCGCATCCTCCTTGG; M, PGF, AAGCCCCAAAGAGATCCCGG, and R, AATGACGTGTGTAGCCAGC; Opm F, ATAGGGCTCGAGTTTCTCTGG, and R, AAAGCCTCTTCTCTGAGCTGCC; Ank F, CAXCCTGATAGCCTCATCAGTAC, and R, GGAAGCGCAGCGAGATAGCAGG; Pit1 F, AGCCAGTTGCTGGCCTTT, and R, GGCACGTGCAACACACTACC; and Pit2 F, CGCGGTTCCTCCGGAGGGGAGG, and R, AGGTGTCTAATCTCCGGAGGCA. Dssp primer sequences are described in Ref. 44.

Microarray and Data Processing—RNA was isolated as described above, and its purity was assessed by gel electrophoresis (Agilent 2100 Bioanalyzer). Transcriptional profiling was carried out using the Affymetrix Mouse Gene ST 1.0 array at the University of Alabama at Birmingham Heflin Center for Genomic Science using standard methods (Affymetrix GeneChip Expression Technical Manual). The Mouse Gene ST 1.0 chip consists of 24,582 well annotated genes. Briefly, 300 ng of total RNA from each sample was used to generate double strand cDNA by linear amplification using T7-linked random primers and reverse transcriptase. Subsequently, cRNA was generated by standard methods (Affymetrix) followed by single-stranded DNA fragmentation, end label biotinylation, and preparation of hybridization mixture. The arrays were hybridized overnight at 45 °C and then washed, stained, and scanned the next day. Data acquisition software (Affymetrix GeneChip Command Console Software) was used to generate a cell intensity (CEL) file from the stored images containing a single intensity value for each probe cell on the array. The CEL files were imported into GeneSpring GX 11.5.1 (Agilent Technologies, Santa Clara, CA). Using the RMA16 summarization algorithm, GeneSpring GX summarized the CEL data files. Expression values for each mRNA were obtained by the robust multiarray analysis method of Irizarry et al. (45). This protocol adjusts for the background on the raw intensity scale, carries out a nonlinear quantile normalization of the perfect match values, log-transforms the background-adjusted perfect match values, and carries out a robust multi-chip analysis of the quantile normalized log-transformed values (45). Each sample underwent baseline transformation to the mean of the control samples. Entities were filtered based on their signal intensity values by satisfying the upper and lower percentile cutoffs 20–100%. Filtered data were further processed in GeneSpring GX by using a one-way analysis of variance and the multiple testing correction method of Benjamini Hochberg. A fold change cutoff of ≥ ± 2 was used to generate downstream datasets (45). To control for the occurrence of false discoveries in the datasets, a corrected value (q value) ≤ 0.05 was used.

Western Blot—Whole protein extracts were prepared by cell lysis in RIPA buffer supplemented with 1 mM NaF, 2 mM Na2VO4, 2 mM leupeptin, 2 mM pepstatin, and 2 mM PMSF. Protein concentration was determined by micro BCA protein assay kit (Thermo Scientific, Rockford, IL). Protein (15 μg) was subjected to electrophoresis on 4–12% precast BisTris gels (Invitrogen) and transferred onto a nitrocellulose membrane. Specific proteins were detected by fluorescence (Li-Cor Odyssey Infrared Imaging System, LI-COR Biosciences, Lincoln, NE). Primary antibodies against Trps1 (ProteinTech, Chicago) were used at 1:1500 dilution; Vdr (Thermo Scientific, Rockford, IL) and Gapdh (Cell Signaling, Danvers, MA) were used at 1:1000; Runx2 (MBL International, Woburn, MA) was used at 1:250 dilution; Sp7 (Abcam, Cambridge, MA) was used at 1:200; and tubulin (Sigma) was used at 1:10,000. All fluorescent secondary antibodies (LI-COR Biosciences, Lincoln, NE) were used at 1:20,000.

Alizarin Red Staining and Quantification of Mineralization—Cells were fixed in 4% paraformaldehyde and stained with 40 μm alizarin red-S (Sigma) for 10 min. Excess dye was removed by washing with deionized water. To quantify calcium deposits, alizarin red was extracted from stained cells with 10% acetic acid, neutralized with 10% NH4OH to pH 4.1–4.3, and quantified by colorimetric detection by spectrophotometry at 405 nm.

TNAP Activity Assay—To detect TNAP activity, cells were fixed in 4% paraformaldehyde, and the alkaline phosphatase substrate naphthol AS-MX phosphate (0.1 mg/ml) was added in reaction buffer containing 0.5% N,N-dimethylformamide, 2 mM MgCl2, 0.6 mg/ml Fast blue BB salt, and 0.1 mM Tris-HCl, pH 8.5. The reaction was stopped by washing in water, and stained cells were imaged. Densitometry of TNAP activity staining was performed using ImageJ software. Images were formatted to the same size; image background was subtracted, and a circular area was defined to ensure measurements were taken of the same location and size, and the selected area was analyzed for each image. Measurement values were normalized by taking the inverse and multiplying by 50,000 so that the larger numbers correspond to darker staining (46). Statistics were calculated using the raw, untransformed values.

Isolation of Matrix Vesicles—MVs were isolated by collagenase digestion as described previously (13). Briefly, cells were plated at a density of 7 × 105 cells per 10-cm plate in 10 ml of osteogenic differentiation medium. An equal number of cells was used for each cell line. Plates were incubated at 37 °C with 5% CO2 for 9 days with media change every 3 days. On day 9, cells were washed and digested for 1.5 h with 2.5 mg/ml collagenase (Worthington). After collagenase digestion, cells were centrifuged at 3000 rpm for 10 min. Supernatant was collected and centrifuged at 19,000 rpm for 10 min. Supernatant was again centrifuged at 42,000 rpm for 45 min to get the MV pellet. The pellet was then resuspended in Tris-buffered saline (20 mM Tris, 0.15 mM NaCl).

AFM Images of MVs—Five microliters of MV solution was dropped onto a freshly cleaved mica substrate (Ted Pella, Redding, CA) and allowed to stand for a couple of minutes. Next, 5 μl of glutaraldehyde solution (8% in H2O) (Sigma) was spotted onto the sample drop and dried at room temperature overnight. Samples were imaged by noncontact (AAC) mode in air using a 5500 AFM (Agilent Technologies). Silicon-nitride cantilevers with a nominal resonance frequency of ~190 kHz (Nanosensors™, Neuchatel, Switzerland) were employed. Tridimen-
sional AFM images were generated by PicoView software (Agi-

Calculation of MV Diameter and Number—AFM imaging 
was used to investigate the morphology (diameter) and number 
of MVs isolated from cells. MV diameters were calculated as the 
peak value of the cross-sections of $n = 100$ vesicles for each cell 
line. Mean and standard deviation values for the diameter dis-
tributions were obtained through Gaussian fit. The number of 
MV isolates from cells was calculated by counting the globular 
features in AFM images (scan size $5 \times 5 \mu m$).

Statistics—All experiments were performed on three control 
and three $TrpS_{1}$-modified stable cell lines. Data are presented as 
the mean $\pm$ S.D. Probability values were calculated using the 
Student’s $t$ test. $p < 0.05$ (*) and 0.005 (**) were considered to be 
statistically significant and highly significant, respectively.

RESULTS

$TrpS_{1}$ Expression during Osteo-odontogenic Differentiation of 
$17IIA11$ Cells—To gain mechanistic insights into the role of 
$TrpS_{1}$ in the physiology and pathology of odontoblast-regulated 
mineralization, we employed the $17IIA11$ cell line (17A cells) 
derived from mouse odontoblasts (42, 43). 17A cells are a good 
model to study molecular mechanisms of odontoblast-mediated 
mineralization, because they express key transcription factors as 
well as enzymes and matrix proteins required for mineralization (42).

FIGURE 1. $TrpS_{1}$ expression during osteo-odontogenic differentiation of 17A cells. A, representative images of alizarin red staining showing the progression of 
17A-regulated mineralization. B, qRT-PCR results demonstrating $TrpS_{1}$ expression in 17A cells. Data are presented as the mean relative levels of $TrpS_{1}$ mRNA (normal-
ized to Gapdh) $\pm$ S.D. from three independent differentiation experiments. $TrpS_{1}$ levels in undifferentiated 17A cells (day 0 of differentiation) were arbitrarily set at 1 and 
used as a reference for the remaining time points. C, Western blot analyses of $TrpS_{1}$ protein levels. Results of densitometric analyses (top) of Western blots (bottom) 
demonstrating relative $TrpS_{1}$ protein levels. Data are presented as the mean relative density of $TrpS_{1}$ normalized to tubulin (a protein loading control) $\pm$ S.D. from 
three independent differentiation experiments. Normalized $TrpS_{1}$ levels in undifferentiated 17A cells were arbitrarily set at 1 and used as a reference for the remaining 
time points. Asterisks denote statistically significant differences compared with day 0 of differentiation (*, $p \leq 0.05$; **, $p \leq 0.005$).

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were detected in preodontoblastic cells as well as during osteo-odontogenic differentiation and mineralization of 17A cells. At the mRNA level, the highest Trps1 expression was observed in 17A preodontoblastic cells (Fig. 1B). Upon induction of differentiation with osteogenic medium, Trps1 expression transiently decreases, reaching the lowest level prior to formation of mineralization nodules. From days 6 to 9, when differentiated 17A cells support the growth of mineralization nodules, Trps1 expression is maintained at a steady level. At the protein level, the changes of Trps1 in general follow the mRNA, except from day 1 of differentiation, when transient up-regulation of Trps1 is detected (Fig. 1, B and C). Although the changes of Trps1 expression during osteo-odontogenic differentiation of 17A cells are not as dramatic as during odontoblast differentiation in vivo (39), the overall Trps1 expression pattern in 17A odontoblastic cells and odontoblasts in vivo are similar, with the highest Trps1 expression in progenitor cells and the lowest expression in newly differentiated cells. Up-regulation of Trps1 in Differentiated Odontoblastic Cells Impairs Mineralization—We have recently demonstrated that increased Trps1 expression in mature odontoblasts in vivo results in severe impairment in dentin formation and repression of the Dspp gene (41). Although there is a significant overlap in dental phenotypes of Col1a1-Trps1 and Dspp−/− mice, the onset of dentin abnormalities and extent of mineralization defects in Col1a1-Trps1 mice are more severe than the phenotype of Dspp−/− mice (41, 47). This suggests that Trps1-dependent repression of dentin formation reflects dysregulation of a number of genes involved in mineralization. 17A cells were used as a model of odontoblast-regulated mineralization to identify additional genes that contribute to impaired mineralization caused by Trps1 up-regulation in mature odontoblasts. To generate a cellular model of Trps1 up-regulation throughout odontoblastic differentiation, a transposon-mediated genomic integration approach was used. We generated three clonal stable cell lines with 3.0-, 3.5-, and 4.5-fold overexpression of V5-tagged Trps1 (Trps1-OE cells) and three control clonal cell lines generated with a “no insert” transposon (Cntr expression of V5-tagged Trps1 (Trps1-OE cells) and three control clonal stable cell lines with 3.0-, 3.5-, and 4.5-fold overexpression were detected in preodontoblastic and odontoblastic Trps1-OE and control cells (Fig. 2C).

In Col1a1-Trps1 transgenic mice, dentin mineralization defects are associated with repression of the Dspp gene; therefore, expression of Dspp was compared during the differentiation of Trps1-OE and control cell lines. qRT-PCR analyses did not detect Dspp expression in undifferentiated cells. Upon initiation of osteogenic differentiation, Dspp is transiently up-regulated shortly before formation of mineralization nodules in control cells. Consistent with the mineralization defects caused by Trps1 overexpression, Dspp up-regulation is delayed and less profound in Trps1-OE cells in comparison with controls (Fig. 2D). However, it is important to note that even at the peak of expression in control cell lines, the levels of Dspp mRNA are very low. Furthermore, we were unable to detect Dsp protein by Western blot analyses (data not shown). This suggests that other mineralization-related genes are involved in the mineralization defects observed in Trps1-OE cells. Trps1 Represses Phex and Vdr during Later Stages of Mineralization—After confirming that Trps1 up-regulation in 17A odontoblastic cells results in similar mineralization defects to those observed in the dentin of Col1a1-Trps1 mice, we used Trps1-OE cells to identify mineralization-related genes that are dysregulated by Trps1-overexpression in mature odontoblasts. The Affymetrix Mouse Gene ST 1.0 array was used to compare global gene expression in Trps1-OE and Cntr cells at day 6 of differentiation. At this time point, mineralization nodules are clearly visible in Cntr but not in Trps1-OE cells, indicating delayed propagation of HA in the ECM. These analyses identified 158 genes significantly (over 2-fold) down-regulated and 188 genes significantly up-regulated in Trps1-OE cells in comparison with Cntr. Interestingly, the group of most down-regulated genes in Trps1-OE cells is enriched in mineralization-related genes that are associated with hypophosphatemic rickets and mineralization defects in dentin (Table 1). Therefore, the subsequent analyses were focused on determining the changes in Enpp1, Phex, and Vdr expression upon up-regulation of Trps1.

Results of qRT-PCR analyses demonstrated that Phex and Vdr are expressed at low levels in Cntr preodontoblasts and during the initiation phase of mineralization (Fig. 3A). Phex expression increases shortly before mineralization nodules are detectable by alizarin red staining and reaches the highest level at day 6 of osteo-odontogenic differentiation, when mineralization nodules are clearly visible (Fig. 3A). As in Cntr cells, the highest Phex mRNA levels are detected on day 6 of differentiation of Trps1-OE cells; however, the magnitude of Phex up-regulation is significantly lower in Trps1-OE cells than in Cntr cells. Western blot analyses of protein extracts isolated on day 8 of differentiation confirmed decreased levels of Phex in Trps1-OE cells (Fig. 3B). The effects of Trps1 overexpression on Vdr were similar to those observed for Phex. In Cntr cells, Vdr expression increases after day 2 of osteo-odontogenic differentiation and is further up-regulated during the mineralization phase. In contrast to up-regulation of Vdr during osteo-odontogenic differentiation of Cntr cells, Trps1-OE cells express Vdr at a steady level. As a result, Vdr is down-regulated during the mineralization nodule formation phase in Trps1-overexpressing cells (Fig. 3A). Although the differences between expression of Vdr in Cntr and Trps1-OE cells were statistically significant.
on the mRNA level, we did not detect a difference on the protein level by Western blot analyses (Fig. 3B).

During osteo-odontogenic differentiation of Cntr cells, Enpp1 expression follows the same pattern as Phex and Vdr; however, Enpp1 is expressed at very low levels. Overexpression of Trps1 resulted in a further decrease of Enpp1 expression, and we were unable to detect Enpp1 mRNA at any day of osteo-odontogenic differentiation in Trps1-OE cells (data not shown).

For comparison, we performed similar expression analyses for the Fam20C gene which, like Phex, Vdr, and Enpp1, is associated with hypophosphatemia and abnormal mineralization in humans (48). Microarray analyses did not detect significant differences in Fam20C expression between Cntr and Trps1-OE cells (data not shown), and this was confirmed by qRT-PCR and Western blot on day 6 of osteo-odontogenic differentiation (Fig. 3). Interestingly, the pattern of Fam20C expression during differentiation and mineralization of Cntr cells is different from the pattern observed for Phex and Vdr (Fig. 3A). Unlike Phex and Vdr, which are strongly up-regulated during mineralization nodule formation, expression of Fam20C increases only transiently on day 6 of osteo-odontogenic differentiation, suggesting different roles of Fam20C versus Phex and Vdr in the mineralization process. In summary, overexpression of Trps1 in odontoblastic cells results in decreased expression of Phex and Vdr specifically during the later phase of mineralization when the growth of nodules is observed.

Trps1-deficient Odontoblastic Cells Do Not Support Mineralization—As shown, overexpression of Trps1 results in decreased and delayed mineralization. Therefore, it would be expected that Trps1-deficient cells have enhanced and/or accelerated mineralization. To test this hypothesis, we generated stable cell lines deficient for Trps1 (Trps1-KD) using lentivirus-delivered shRNAs targeting the Trps1 transcript. Control cell lines were generated using lentivirus-delivered scrambled shRNA (shScr cells). Trps1 knockdown was confirmed at the mRNA level by qRT-PCR and the protein level by

FIGURE 2. Delayed and decreased mineralization of 17A odontoblastic cells overexpressing Trps1. A, qRT-PCR (top panel) and Western blot (bottom panel) results demonstrating overexpression of Trps1 in three clonal stable cell lines (Trps1-OE) and controls (Cntr, undifferentiated cells). qRT-PCR data are presented as the mean relative levels of Trps1 mRNA normalized to Gapdh ± S.D. from three independent RNA preparations per cell line. Relative Trps1 levels in one of the Cntr analyses were arbitrarily set at 1 and used as a reference for the remaining control and Trps1-OE cell lines. On the Western blot analyses, tubulin was used as a protein loading control. B, representative images of alizarin red staining (top panel) and quantification (bottom panel) of Trps1-OE and Cntr cell lines during osteo-odontogenic differentiation. Quantification of alizarin red staining is presented as the mean ± S.D. from differentiation of three stable cell lines. Asterisks denote statistically significant differences (*, p < 0.05; **, p < 0.005). C, representative images of TNAP activity assay in Trps1-OE and Cntr undifferentiated (day 0) and differentiated (day 9) cells (left panel) and results of densitometric quantification of TNAP activity (right panel). Data are presented as the mean ± S.D. from differentiation of three stable cell lines. No statistically significant difference was detected. D, qRT-PCR results demonstrating dynamic Dspp expression during osteogenic differentiation of Trps1-OE and Cntr cell lines. Data are presented as the mean relative levels of Dspp mRNA (normalized to Gapdh) ± S.D. from three stable cell lines. Dspp expression was not detected in undifferentiated controls; therefore, values for day 1 of Cntr cells were arbitrarily set at 1 and used as a reference for the remaining time points.
Western blot (Fig. 4A). To characterize the consequences of Trps1 deficiency on mineralization, we selected three stable cell lines with the most efficient knockdown of Trps1 as follows: 70, 80, and 85% decreased Trps1 mRNA levels in comparison with shScr cell lines.

The osteo-odontogenic differentiation of Trps1-KD and shScr cell lines was carried out as before, and progression of mineralization was monitored by alizarin red staining. Surprisingly, even after 9 days of culture in osteogenic medium, there was no evidence of mineralization nodule formation in any of Trps1-KD stable cell lines, whereas in shScr cells abundant mineralization nodules were present already at day 6 of differentiation (Fig. 4B). Considering that 17A cells are already committed to odontoblastic lineage, as demonstrated previously by high TNAP activity and expression of osteogenic markers (42, 43), this indicates that Trps1 deficiency results in loss of the mineralization potential. To identify the underlying cause of this loss, we first analyzed TNAP activity, which is a characteristic feature of mineralizing cells and is required for mineral formation. Comparison of Trps1-KD and shScr cell lines demonstrated that Trps1 deficiency in odontoblastic cells results in

### TABLE 1

Ten most up-regulated and down-regulated genes in 17A odontoblastic cells overexpressing Trps1 (day 6 of osteogenic differentiation)

| Gene name | Entrez ID | Accession | Log fold change (Trps1-OE vs. Cntr) |
|-----------|-----------|-----------|-----------------------------------|
| Il1r1     | 17082     | NM_0010256602 | 3.91379                           |
| Maob      | 109731    | NM_172778  | 3.13405                           |
| Tmon47    | 192216    | NM_138751  | 2.99411                           |
| Cad200    | 17470     | NM_010818  | 2.36653                           |
| Gaitn13   | 271786    | NM_173030  | 2.09059                           |
| Tfrc      | 22042     | NM_011638  | 2.07298                           |
| Serpin3g  | 20715     | NM_009251  | 1.84646                           |
| Mpu21     | 100702    | NM_194336  | 1.77574                           |
| Zcchc5    | 213436    | NM_199468  | 1.74184                           |
| Illbrap   | 16174     | NM_010553  | 1.69067                           |
| Mmp13     | 17386     | NM_0096607 | 1.59059                           |
| Enpp1     | 18665     | NM_0081813 | 1.58652                           |
| Dkk1      | 13380     | NM_010051  | 1.36697                           |
| Dpep2     | 319446    | NM_176913  | 1.27646                           |
| Gabra3    | 14396     | NM_008767  | 1.26896                           |
| Ranbp3I   | 223332    | NM_190024  | 1.26017                           |
| Phex      | 18675     | NM_011077  | 1.23512                           |
| Expp6     | 320981    | NM_177304  | 1.23485                           |
| Serpin1   | 12258     | NM_009776  | 1.20661                           |
| Vdr       | 22337     | NM_009504  | 1.20173                           |

*FIGURE 3. Decreased expression of genes involved in phosphate homeostasis in Trps1-OE cells. A, qRT-PCR graphs depicting Phex, Vdr, and Fam20C mRNA expression during osteo-odontogenic differentiation in Trps1-OE cells in comparison with Cntr cells. Data are presented as the mean relative levels of Phex, Vdr, and Fam20C mRNA (normalized to β-actin) ± S.D. from three stable cell lines. Day 0 values for each cell line were arbitrarily set to 1 and used as a reference for remainder of days of differentiation. B, top panel, Western blots of Phex, Vdr, and Fam20C expression on day 6 and Phex expression on day 8 of differentiation on protein extracts isolated from Trps1-OE and Cntr cell lines. Bottom panel, results of densitometric quantification of the Western blot images. Protein levels were normalized to tubulin or Gapdh as depicted in the top panel. Asterisks denote statistically significant differences (*, p ≤ 0.05; **, p ≤ 0.005).
significant decrease of TNAP activity (Fig. 4C). Furthermore, undifferentiated Trps1-KD cells had dramatically reduced levels of TNAP mRNA (Alpl) in comparison with controls as demonstrated by qRT-PCR (Fig. 5A). This indicates that the loss of TNAP activity is due to decreased expression of the Alpl gene. Considering that Trps1-KD cells do not form mineralization nodules at all, we also analyzed expression of Phospho1, which codes for a phosphatase involved in the initiation of HA crystal formation. It has been demonstrated that expression of this gene is induced in odontoblasts prior to mineralization of the dentin (12, 13, 49). qRT-PCR and Western analyses show that Phospho1 is also significantly down-regulated in all Trps1-deficient odontoblastic cell lines in comparison with shScr control cells (Fig. 5, A and C).

Trps1 Is Required for Expression of Genes Initiating Mineralization—To investigate whether the effects of Trps1 deficiency are restricted to down-regulation of phosphatases that support HA formation or whether Trps1 is required for expression of mineralization-related genes in general, we compared global gene expression between preodontoblastic shScr and Trps1-KD cells (day 0 of differentiation). These analyses revealed a total of 204 genes that were significantly (over 2-fold) down-regulated in Trps1-deficient preodontoblastic cells in comparison with controls, including Alpl and Phospho1 genes that were among the most down-regulated genes in Trps1-KD cells (Table 2). The group of 10 most down-regulated genes in Trps1-KD cells also included other mineralization-related genes, such as Ibsp, Smpd3, and Galnt3, suggesting that Trps1 is required for expression of multiple genes that support mineralization.

It has already been demonstrated that undifferentiated 17A cells are positive for osteogenic transcription factors (42, 43). Runx2 and its downstream target Osx (coded by the Sp7 gene) are the key transcription factors that control development of cells producing mineralizing matrix and directly regulate expression of osteogenic genes. From the group of osteogenic transcription factors, only expression of the Sp7 gene was significantly decreased in Trps1-KD cells in the gene array experiment (data not shown). This was confirmed by qRT-PCR on mRNA isolated from three stable Trps1-KD cell lines (Fig. 5B). qRT-PCR analyses also demonstrated significant down-regulation of Runx2 mRNA in Trps1-KD cell lines (Fig. 5B). Western blot analyses demonstrated a more dramatic decrease of both Sp7 and Runx2 at the protein level (Fig. 5C). There was no significant change in expression of the Atf4 gene, which codes for a transcription factor that regulates function of
more mature osteogenic cells (Fig. 5B). These results suggest that in preodontoblastic cells Trps1 supports expression of genes involved in the early stages of osteo-odontogenic differentiation.

To follow up on this hypothesis, we analyzed expression of matrix proteins osteopontin (Opn, coded by the Spp1 gene) and matrix Gla protein (MGP) that are activated along with TNAP during early osteo-odontogenic differentiation. However,
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Unlike TNAP, Opn and MGP are inhibitors of mineralization. We found that expression of Opn and Mgp in undifferentiated Trps1-KD cells is higher than in shScr (Fig. 5D). It has been reported that Opn is up-regulated upon TNAP deficiency and by increased PPi levels (14, 50); therefore, we analyzed expression of a PPi transporter Ank but detected no significant difference in Ank expression between Trps1-KD cells and controls (Fig. 5E). Similarly, there were no changes in expression of P1 transporters PiT1 and PiT2 (Fig. 5E). In summary, depletion of Trps1 in preodontoblastic cells results in the loss of the mineralization potential associated with significant down-regulation of phosphatases specifically involved in HA formation, as well as decreased expression of transcription factors essential for the osteogenic program.

Trps1 Deficiency Affects Matrix Vesicles—The inability of Trps1-deficient cells to initiate the mineralization process and the significant down-regulation of phosphatases associated with MV function suggest defects in MVs. AFM imaging was employed to investigate the size and number of MVs isolated from Trps1-KD cell lines with respect to control cell lines (Fig. 6). MVs from both Trps1-KD and shScr cells had an average diameter of ~70 nm. However, from the same number of cells, ~10-fold fewer MVs were isolated from Trps1-KD cell lines in comparison with shScr cells, suggesting that fewer MVs are made by Trps1-KD cells. This implicates Trps1 in MV biogenesis and suggests that Trps1 is required for MV-dependent initiation of the mineralization process.

DISCUSSION

Trps1 demonstrates a dynamic and specific expression pattern during odontoblast development. It is highly expressed in preodontoblasts and then is down-regulated upon odontoblast maturation suggesting a Trps1 role in odontoblast differentiation (39). Trps1 expression in secretory odontoblasts is maintained at much lower levels than in progenitors (41). This, in turn, suggests that formation of dentin by mature odontoblasts does not require Trps1 or that Trps1 may have a negative effect on their function. We have recently demonstrated that sustained high Trps1 expression in secretory odontoblasts has deleterious effects on dentin formation (41); however, the role of Trps1 in odontoblast differentiation and function is unknown.

In these studies, using the 17A odontoblastic cell line, we delineated the consequences of Trps1 deficiency and up-regulation on the odontoblast-regulated mineralization process. Based on our findings, we propose the following model of the Trps1 role in odontoblasts (Fig. 7). The function of Trps1 in odontoblasts was determined by AFM imaging and microarray analysis. The table lists the ten most up-regulated and down-regulated genes in undifferentiated 17A preodontoblastic cells deficient for Trps1. The role of Trps1 in odontoblasts is further supported by the observation that Trps1 deficiency affects matrix vesicles.

Table 2: Ten most up-regulated and down-regulated genes in undifferentiated 17A preodontoblastic cells deficient for Trps1

| Gene name   | Entrez ID | Accession      | Log fold change (Trps1-KD vs. shScr) |
|-------------|-----------|----------------|-----------------------------------|
| Prl2c3      | 18812     | NM_011118      | 5.05802                           |
| Epha7       | 13841     | NM_010141      | 4.44097                           |
| Npr3        | 18162     | NM_001039181   | 4.09527                           |
| Vcam1       | 22329     | NM_011693      | 3.91533                           |
| Igf1        | 16000     | NM_00111275    | 3.6016                            |
| Met         | 17295     | NM_008591      | 3.55918                           |
| Fha1        | 14118     | NM_007993      | 3.40414                           |
| Atp1b1      | 11931     | NM_009721      | 3.32497                           |
| Tmem66a     | 20349     | NM_011348      | 2.96628                           |
| Arhgap29    | 214137    | NM_172325      | 2.94997                           |
| Gpr141      | 353346    | NM_181754      | 2.94997                           |
| Sema3e      | 20349     | NM_011348      | 2.96628                           |
| Arhgap29    | 214137    | NM_172325      | 2.94997                           |
| Gpr141      | 353346    | NM_181754      | 2.94997                           |
| Ibsp        | 15891     | NM_008318      | 3.48697                           |
| Smpd3       | 51801     | NM_178401      | 3.03233                           |
| Ibsp        | 15891     | NM_008318      | 3.48697                           |
| Smpd3       | 51801     | NM_178401      | 3.03233                           |
| Phospho1    | 237928    | NM_153104      | 2.89978                           |
| Senataa     | 20358     | NM_011693      | 2.89978                           |
| Ramp1       | 51801     | NM_178401      | 2.88211                           |

FIGURE 6. Trps1 deficiency affects matrix vesicles. A, representative AFM images (scan size 10 × 10 μm) of MVs isolated from Trps1-KD and shScr cell lines. Topography (top panel) and three-dimensional surface rendering (bottom panel) are shown. B, number of MVs (mean ± S.D.) isolated from Trps1-KD and shScr cell lines. Asterisks denote statistically significant differences (**, p < 0.005). C, diameter distributions for MVs (n = 100 for each cell line) isolated from Trps1-KD and shScr cell lines.
is context-dependent and is specified by the odontoblast differentiation stage. In preodontoblasts, \( \text{Trps1} \) is required for the MV-dependent initiation of mineralization by supporting expression of major phosphatases and transcription factors required for this process. During the later stages of the mineralization process, \( \text{Trps1} \) acts as a mineralization inhibitor by repressing expression of genes involved in the mineral propagation within the ECM.

In summary, our studies of an in vitro model of odontoblast differentiation and mineralization provide an understanding of the biological significance of the dynamic changes of \( \text{Trps1} \) in mineralization is context-dependent and is specified by the odontoblast differentiation stage. In preodontoblasts, \( \text{Trps1} \) is required for MV-dependent initiation of the mineralization process by supporting expression of major MV-associated phosphatases involved in HA formation and for expression of key osteogenic transcription factors. However, in the mature cells, which actively produce mineralizing matrix, \( \text{Trps1} \) acts as a repressor by suppressing expression of genes involved in propagating HA within the ECM. We propose that down-regulated expression of the genes involved in the initiation of mineralization is an indirect effect of \( \text{Trps1} \) deficiency and may be due to up-regulation of another factor that directly represses these genes. This mechanism incorporates the findings from our in vitro studies into the well established molecular function of \( \text{Trps1} \) as a transcriptional repressor (31–35). We cannot definitively rule out the possibility that in preodontoblasts \( \text{Trps1} \) directly activates a subset of mineralization genes, as it has been demonstrated for Wnt pathway genes in hair follicle progenitor cells (36). However, thus far, there is no evidence that \( \text{Trps1} \) can act as a transcriptional activator in cells other than hair follicle progenitors.

In these studies, we determined that in preodontoblastic cells expression of major osteogenic transcription factors \( \text{Runx2} \) and \( \text{Sp7} \) is decreased upon \( \text{Trps1} \) depletion. This contradicts our earlier data from analyses of \( \text{Trps1}^{\Delta GT/\Delta GT} \) endochondral bones where we demonstrated up-regulation of \( \text{Runx2} \) in perichondrium and cartilage of \( \text{Trps1} \) mutant mice (32). This may reflect a combination of cell autonomous and nonautonomous mechanisms regulating mineralization of the perichondrium versus effects of only cell autonomous mechanisms in the in vitro system. For example, Ihh signaling, which is known to up-regulate \( \text{Runx2} \) in endochondral bones, is increased in the perichondrium of \( \text{Trps1}^{\Delta GT/\Delta GT} \) mice, implicating cell nonautonomous mechanisms in the accelerated perichondrial mineralization in \( \text{Trps1} \)-deficient mice (32). Alternatively, this may indicate that different molecular networks regulate mineralization of dentin and perichondrium. This possibility is supported by studies, which discovered distinct pools of \( \text{Runx2} \)-dependent genes in bone and dental mesenchyme (51). Similarly, the upstream regulation of \( \text{Runx2} \) may be different in bones and teeth. In developing endochondral bones, the loss of \( \text{Trps1} \) is sufficient to up-regulate \( \text{Runx2} \) (32), although in preodontoblastic cells this effect may be counteracted by simultaneous up-regulation of another repressor of \( \text{Runx2} \). The same mechanism involving another repressor may underlie the observed down-regulation of \( \text{Alpl} \) and \( \text{Phospho1} \) in \( \text{Trps1-KD} \) cells. Therefore, we propose that the observed down-regulation of \( \text{Alpl} \), \( \text{Phospho1} \), \( \text{Runx2} \), and \( \text{Sp7} \) in \( \text{Trps1-KD} \) cells is an indirect consequence of \( \text{Trps1} \) deficiency and results from the released inhibition of a yet unidentified repressor.

Results of our in vitro studies, considered together with the \( \text{Trps1} \) expression pattern in odontoblasts, provide first insights into the \( \text{Trps1} \) function in dentinogenesis in vivo. We have shown here that \( \text{Trps1} \) depletion from preodontoblastic cells results in the loss of the mineralization potential and down-regulation of multiple mineralization-related genes. This supports the hypothesis that \( \text{Trps1} \) is required for odontoblast maturation. Interestingly, the genes most affected by \( \text{Trps1} \) deficiency are those associated with MVs, cellular structures that initiate the mineralization process (Table 2). In vivo, a combined deficiency of TNAP and \( \text{PHOSPHO1} \) (\( \text{PHOSPHO1}^{-/-} \); \( \text{Alpl}^{-/-} \) mice), two phosphatases that are significantly down-regulated in \( \text{Trps1-KD} \) cells, resulted in a significant decrease of dentin mineralization and was sufficient to almost completely deplete MVs from the mantle dentin (49). Consistent with these data from animal models, loss of TNAP and \( \text{PHOSPHO1} \) in \( \text{Trps1} \)-deficient odontoblastic cells is associated with a decreased number of MVs. Considering that MVs are abundant in mantle dentin, the first dentin layer formed by newly differentiated odontoblasts, but not in the remaining (circumpulpal) dentin formed by mature odontoblasts (2, 52–56), this suggests that \( \text{Trps1} \) is involved in the formation of mantle dentin. The role of \( \text{Trps1} \) in the biogenesis of mineralization-competent MVs remains to be determined.

Mineralization of circumpulpal dentin relies on molecular mechanisms different from those that drive mantle dentin formation (57), as underscored by analyses of human conditions associated with dentin defects. In hypophosphatemia caused by either \( \text{PHEX} \) or \( \text{VDR} \) mutations, formation of circumpulpal dentin is severely disrupted, although mantle dentin is unaffected, demonstrating the crucial role of phosphate homeostasis genes in the later stages of dentin formation (28, 58–62). Consistent with their role in the function of mature odontoblasts, expression of \( \text{Phex} \) and \( \text{Vdr} \) is low in 17A cells prior to mineralization nodule formation and then increases during the propagation of mineralization. This up-regulation of \( \text{Phex} \) and \( \text{Vdr} \) in the later phase of mineralization is hindered by \( \text{Trps1} \) overexpression, whereas \( \text{Trps1} \) has no effect on expression of these genes during the mineralization initiation phase. Our studies demonstrate that \( \text{Trps1} \) specifically represses genes that are required for circumpulpal dentin mineralization, and this function is restricted to mature cells that actively support propagation of mineralization in the ECM. This agrees with our recent in vivo studies demonstrating that sustained high \( \text{Trps1} \) expression in secretory odontoblasts results in severely abnormal dentin and direct repression of the \( \text{Dspp} \) gene. However, TNAP levels remain unchanged.

In summary, our studies of an in vitro model of odontoblast differentiation and mineralization provide an understanding of the biological significance of the dynamic changes of \( \text{Trps1} \) in mineralization.
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expression during dentinogenesis. We uncovered that although Trps1 is required for the initiation of odontoblast-regulated mineralization, down-regulation of Trps1 in secretory odontoblasts is necessary to allow for the formation of circumpulpal dentin.

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