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The protein tyrosine phosphatase Rptpζ is expressed in differentiated osteoblasts and affects bone formation in mice

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Abstract

Tyrosine phosphorylation of intracellular substrates is one mechanism to regulate cellular proliferation and differentiation. Protein tyrosine phosphatases (PTPs) act by dephosphorylation of substrates and thereby counteract the activity of tyrosine kinases. Few PTPs have been suggested to play a role in bone remodeling, one of them being Rptpζ, since it has been shown to be suppressed by pleiotrophin, a heparin-binding molecule affecting bone formation, when over-expressed in transgenic mice. In a genome-wide expression analysis approach we found that *Ptprz1*, the gene encoding Rptpζ, is strongly induced upon terminal differentiation of murine primary calvarial osteoblasts. Using RT-PCR and Western Blotting we further demonstrated that differentiated osteoblasts, in contrast to neuronal cells, specifically express the short transmembrane isoform of Rptpζ. To uncover a potential role of Rptpζ in bone remodeling we next analyzed the skeletal phenotype of a Rptpζ-deficient mouse model using non-decalcified histology and histomorphometry. Compared to wildtype littermates, the Rptpζ-deficient mice display a decreased trabecular bone volume at the age of 50 weeks, caused by a reduced bone formation rate. Likewise, Rptpζ-deficient calvarial osteoblasts analyzed *ex vivo* display decreased expression of osteoblast markers, indicating a cell-autonomous defect. This was confirmed by the finding that Rptpζ-deficient osteoblasts had a diminished potential to form osteocyte-like cellular extensions on Matrigel-coated surfaces. Taken together, these data provide the first evidence for a physiological role of Rptpζ in bone remodeling, and thus identify Rptpζ as the first PTP regulating bone formation *in vivo*.

Keywords: Protein tyrosine phosphatases; *Ptprz1*; Osteoblast; Bone formation; Mineralization

Introduction

The recent advances in molecular biology, including human and mouse genetics, have helped to identify several genes involved in osteoblast differentiation and bone formation. At least two transcription factors, Runx2 and Osterix, have been demonstrated to be required for the formation of functional osteoblasts *in vivo* by regulating the expression of target genes [1,2]. The discovery of the *Lrp5* gene as a major determinant of bone formation in humans has further led to the identification of a Runx2-independent regulation of osteoblast differentiation involving the canonical Wnt-signaling pathway [3–6]. Despite this increasing knowledge about the early stages of osteoblast differentiation, there are however still some open questions concerning the terminal steps associated with extracellular matrix mineralization and differentiation into osteocytes.

In an attempt to identify genes induced upon terminal osteoblast differentiation, we have applied a genome-wide expression analysis using Affymetrix Gene Chip hybridization. One of the genes, whose expression was strongly induced between day 5 and

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day 25 of primary osteoblast differentiation, encodes the receptor protein tyrosine phosphatase Rptpζ. Rptpζ, that was initially found specifically expressed by neuronal cells, appeared to be an interesting candidate to be involved in terminal osteoblast maturation based on its domain structure [7–10]. First, an N-terminally located carbonic anhydrase domain could potentially be involved in the local control of acidification, which could in theory influence mineral formation in newly formed bone. Second, the two intracellularly located protein tyrosine phosphatase domains could be required for the arrest of osteoblast proliferation during terminal differentiation, thereby allowing the cells to reach a post-mitotic state. Third, the extracellular domain of Rptpζ could be involved in the development of the characteristic stellate osteocyte morphology, since the addition of a non-transmembrane splice variant of Rptpζ to laminin substrates was found to cause a striking reduction of neurite outgrowth in explants of the dorsal root ganglion [7,11]. Moreover, an involvement of Rptpζ in bone formation has been postulated previously, based on a possible connection with the actions of the growth-factor-induced extracellular matrix protein pleiotrophin (Ptn) [12]. Ptn, when overexpressed in transgenic mice, has been reported to cause increased bone density and protection against ovariectomy-induced bone loss [13–15]. Since Ptn has been demonstrated to bind to Rptpζ in glioblastoma cells, thereby inhibiting its tyrosine phosphatase activity, it appeared likely that Rptpζ may also be involved in the regulation of bone formation [16].

Here we report for the first time a full histomorphometric analysis of the skeletal phenotype of Rptpζ-deficient mice. At the age of 50 weeks we observed a reduction of the trabecular bone volume, explained by decreased bone formation in the face of normal bone resorption. Rptpζ-deficient primary calvarial cells display decreased expression of osteoblast differentiation markers and a diminished potential to form osteocyte-like cellular extensions on Matrigel-coated surfaces. Taken together, our data provide the first evidence for a previously unrecognized role of Rptpζ in bone formation and expand our knowledge about the genetic mechanisms underlying osteoblast maturation.

Materials and methods

Gene expression analysis

Primary osteoblasts were isolated from the calvariae of 3 days old wildtype mice by sequential digestion steps with collagenase and dispase as described previously [17]. Cells were seeded at an initial density of 300,000 cells per 100 mm-dish and grown until 80% confluency for 3 days (d0). After the addition of ascorbic acid and β-glycerophosphate the cultures were allowed to differentiate. Since extracellular matrix mineralization, determined by von Kossa staining, was absent at d5 and fully pronounced at d25, these two stages were chosen for comparison by Affymetrix Gene Chip hybridization. Total RNA was extracted using the TRIzol® reagent (Invitrogen) and further purified using the RNeasy Midi Kit (Qiagen). The targets for Affymetrix DNA microarray analysis were prepared according to the manufacturer's instructions. The amount of total RNA used for the cDNA synthesis was 10 μg for each reaction. Gene Chip microarrays (Affymetrix Murine U74v2 Gene Chips A and B) were hybridized with the targets for 16 h at 45 °C, washed and stained using the Affymetrix Fluidics Station according to the Gene Chip Expression Analysis Technical Manual. Microarrays were scanned with the Hewlett-Packard-Agilent Gene Chip scanner, and the signals were processed using the Gene Chip expression analysis algorithm (Affymetrix). To compare samples and experiments, the trimmed mean signal of each array was scaled to a target intensity of 100. Absolute and comparison analyses were performed with Affymetrix MAS 5.0 and DMT software using default parameters. Annotations were analyzed with interactive query analysis at www.affymetrix.com.

Bone marrow cells were isolated and induced to differentiate into osteoclasts and osteoblasts as described [18]. For RT-PCR expression analysis RNA was reverse transcribed using the Cloned AMV First-Strand cDNA synthesis Kit (Invitrogen) according to the manufacturer's instructions. PCR reactions were performed under standard conditions using the following primer pairs: *Ptprz1 (A)*, 5′-AGACTGGAACTAACCTGCCACCAC-3′ and 5′-AGCATCTGGAGAAAACGACTCG-3′; *Ptprz1 (B)*, 5′-AGCAAAAGTCCCCAGCAAGTG-3′ and 5′-TCCCTAATGGAACAGAATGGTCAG-3′; *Ptprz1 (C)*, 5′-TGGTGGTTATGATTCCAGATGGTC-3′ and 5′-AAGTTCCTGCTGTTACTCCCCCG-3′; *Gapdh*, 5′-GACATCAAGAAGGTGGTGAAGCAG-3′ and 5′-CTCCTGTTATTATGGGGGTCTGG-3′; *Calcr*, 5′-ATGGTGGCTCTGGTGGTCAACTTC-3′ and 5′-TCACATTCAAGCGGATGCG-3′; *Bglap*, 5′-TCCAAGCAGGAGGGCAATAAG-3′ and 5′-GCGTTTGTAGGCGGTCTTCAAG-3′; *Ibsp*, 5′-ACGCCACACTTTCCACACTCTC-3′ and 5′-TCATCCACTTCTGCTTCTTCGTTC-3′. The PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. Bands derived from the differentiated osteoblasts were isolated using the QuiaexII gel extraction kit (Quiagen), subcloned into the vector pCR2.1TOPO (Invitrogen) and verified by automated DNA sequencing.

Immunohistochemistry and Western Blot analysis

For immunohistochemistry the calvariae were decalcified and sectioned as described [19]. Sections were incubated with a 1:100 dilution of a polyclonal antiserum raised against the C-terminal tail of Rptpζ. The specificity of this antiserum has previously been verified on wildtype and Rptpζ-deficient brain sections (Lamprianou et al., unpublished data). For controls we used a commercially available normal rabbit serum (Dako). A biotinylated secondary antibody against rabbit-IgG (Dako) was detected by a conjugate of streptavidin and horseradish peroxidase using diaminobenzidine as a substrate.

For protein extraction cells were washed two times with cold PBS and lysed in HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X100, 10% glycerol) including 5 mM sodium fluoride, 2 mM sodium vanadate, 1% octylglycopyranoside and protease inhibitors. After 10 min on ice the cellular debris was removed by centrifugation, and the protein concentration was determined using the Bio-Rad protein assay according to the manufacturer's instructions. The cells used for phosphotyrosine analysis were serum-starved overnight, stimulated for 10 min with 50% FBS and then serum-starved for 30 min, before protein extracts were taken as described above. Isolated proteins (20 μg of each sample) were separated by SDS-PAGE (7.5% or 7.5%–18% acrylamide gels, respectively) and transferred to a HybondECL™ membrane (Amersham) thereafter. Blots were incubated with the corresponding antibodies in Tris-buffered saline including 0.1% Tween 20, and bound secondary antibodies were visualized using the ECL plus Western Blotting Detection System (Amersham) according to the manufacturer's instructions. Antibodies against MAGI-1, β-catenin and phosphotyrosine residues (PY99) were purchased from Santa Cruz Biotechnology Inc., USA.

Skeletal analysis

Skeletons from 3 days old wildtype and Rptpζ-deficient mice (129SV/Ev genetic background) were fixed in 95% EtOH after removal of the internal organs. Staining with alcian blue and alizarin red was performed as described [20]. Skeletons from 30 and 50 weeks old wildtype and Rptpζ-deficient littermates were fixed in 3.7% PBS-buffered formaldehyde for 18 h at 4 °C and stored in 80% EtOH thereafter. Whole skeletons were analyzed by contact radiography using a Faxitron Xray cabinet (Faxicon Xray Corp., USA). The lumbar vertebral bodies and one tibia of each mouse were dehydrated in ascending alcohol concentrations and then embedded in methylmetacrylate as described previously [21]. Sections of 5 μm thickness were cut in the sagittal plane on a Microtec rotation microtome (Techno-Med GmbH, Germany). Sections were stained by toluidine blue, van Gieson/von Kossa and Giemsa staining procedures as described [21]. Non-stained sections of 12 μm thickness were used to determine the bone formation rate. Cortical thickness was determined by cross-sectional μCT scanning of the femora as described previously [22].