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Characterization of Pleiotrophin and Receptor Protein Tyrosine Phosphatase beta/zeta in Odontoblast and Osteoblast cell lines

Amena Tamkenath
University of Alabama at Birmingham

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By

AMENA TAMKENATH

HEIDI ERLANDSEN, COMMITTEE CHAIR
AMJAD JAVED
ANTON BOROVJAGIN
DOBRAWA NAPIERALA

A THESIS

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AMENA TAMKENATH, BDS

MASTERS in ORAL BIOLOGY

ABSTRACT

Pleiotrophin (PTN) is an extracellular matrix–associated growth factor and chemokine expressed in mesodermal and ectodermal cells. It plays an important role in osteoblast recruitment and differentiation. PTN has two well-known physiological receptors, protein tyrosine phosphatase β/ζ (RPTPRZ1), and N-syndecan. Protein tyrosine phosphatases are a group of enzymes that remove phosphate groups from phosphorylated tyrosine residues on proteins. Protein tyrosine phosphorylation is a common post-translation modification that can create novel recognition motifs for protein interactions and cellular localization, affect protein stability and regulate enzyme activity. Osteoblasts have recently been found to express the short transmembrane isoform of RPTPβ/ζ, but nothing is known regarding RPTPβ/ζ, expression during tooth development. There is also very limited information available about PTN and RPTP expression during odontoblast differentiation and tooth formation, and thus we aimed to establish the spatiotemporal expression pattern of PTN and RPTP during mouse odontogenesis. Immortalized mouse dental pulp (MD10-A11), odontoblast-like (M06-G3), mesenchymal/preosteoblasts (C3H10t1/2) and mature

mouse osteoblasts (ROS) cell lines were grown and samples prepared for immunocytochemistry, Western blot, and conventional PCR analysis. Finally, immunohistochemistry of sectioned mice mandibles and maxillaries at developmental stages E16, P3, P10 and P28 was performed. The experiments showed that PTN, at both the mRNA and protein level, was expressed in all tested cell lines. We observed initial expression of PTN in the inner enamel epithelium with prolonged expression in the ameloblasts and odontoblasts throughout their stages of maturation and strong expression in the terminally differentiated and enamel matrix–secreting ameloblasts and odontoblasts of the adult. RPTP was expressed at the mRNA level in all the cell lines investigated but at varying levels mainly as phosphacan (the extracellular domain of RPTPβ/ζ), and at the protein level RPTP was expressed in the mature MO6-G3 and ROS cells as Phosphacan short isoform (PSI), although the full-length form of Phosphacan cannot be ruled out at the present. These studies demonstrate that PTN and RPTPβ/ζ are expressed during tooth development. Localization of PTN and RPTP within mineralized tissue may indicate that both PTN and Phosphacan is involved in the production or homeostasis of mineralized tissue (bone and enamel).

Keywords: pleiotrophin, extracellular enamel matrix, immunohistochemistry

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I personally have many people that deserve thanks. First, I would like to thank Dr. Heidi Erlandsen, my mentor. She has guided me not only on my different projects and presentations, also has always been there at personal level whenever I needed her. I thank her from the bottom of my heart for everything she has done for me. Next, I want to thank Dr Amjad Javed for being there whenever I had questions. Also I would like to thank Dr Anton Borovjagin and Dr Dobrawa Napierala, both of whom have been generous with their time and participation with my project. I must also mention Jennifer Ames and Dr. Olga Mamaeva for helping me through my early days in the lab. Lastly I want to thank my mother Atiya Khatoon, without her support I would not have been able to be where I am today.

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INTRODUCTION

ODAM: Odontogenic Ameloblast associated Molecule

Human odontogenic ameloblast associated protein (hODAM or APin; UniProtKB/Swiss-Prot entry: A1E959) is a functionally unknown protein of 279 amino acids, rich in glutamine and proline. ODAM is expressed during the late bell stage of tooth development. A short version of ODAM is expressed in the amyloid deposits of CEOT (Calcifying Epithelial Odontogenic tumor)

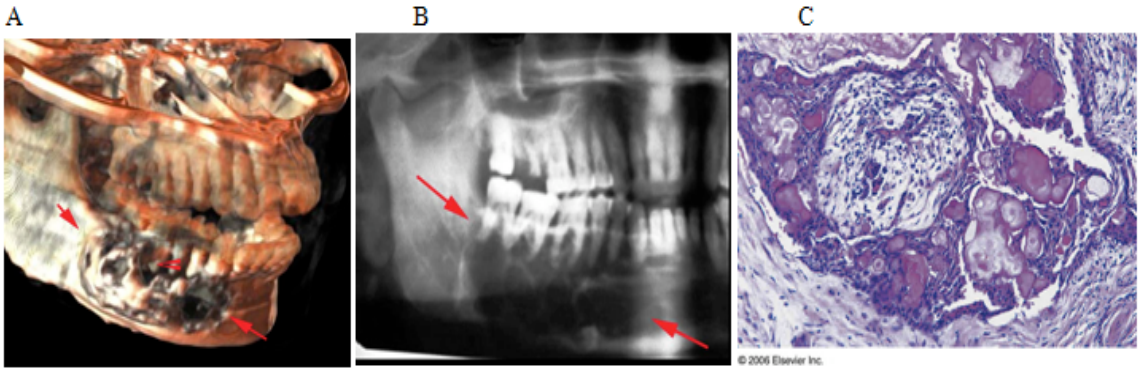


Figure 1: (A) 3D-CT image of CEOT lesion confirmed by biopsy. (B) X-ray image of same CEOT lesion. Images from: http://bhavin.typepad.com/radiology/2006/05/calcifying_epit.html. (C) Histological image of CEOT. Image from: http://www.pathconsultddx.com/pathCon/diagnosis?pii=S1559-8675%2806%2970617-9..

Two novel molecules, odontogenic ameloblast-associated protein (ODAM) and amelotin (AMTN) have recently been described as members of the secretory calcium-binding phosphoprotein (SCPP) gene cluster (Kawasaki and Weiss 2003; Sire et al., 2007; Kestler et al., 2008). The cDNA transcript of ODAM (FLJ20512) was originally cloned from the human KATO III cell-line (Sekiguchi et al., 1978) and has been detected in calcifying epithelial odontogenic tumor (CEOT)-associated amyloids designated as Apin (Solomon et al., 2003). The association of high ODAM expression with enamel maturation suggests a possible role for this protein in the final phases of enamel formation (Moffatt et al.,

2008). In addition to ameloblasts, ODAM is also expressed in odontoblasts, lactating mammary glands, nasal and salivary glands, tongue, and gingival tissue (Dey et al., 2001; Moffatt et al., 2008).Taken together, these data suggest a broad physiological role for ODAM; however, the precise function of ODAM is not clear. The goal of my initial research was to characterize the glycosylation and the proteolytic digestion pattern of human ODAM.

RESULTS:

To achieve the above stated goals first a series of deletion mutants of human ODAM were cloned(Fig 2). These plasmids carried epitop-tag either for bacterial(GST) or mammalian (His,V5) expression. Additional plasmid were constructed with GFP-ODAM fusion cDNA (table 1)

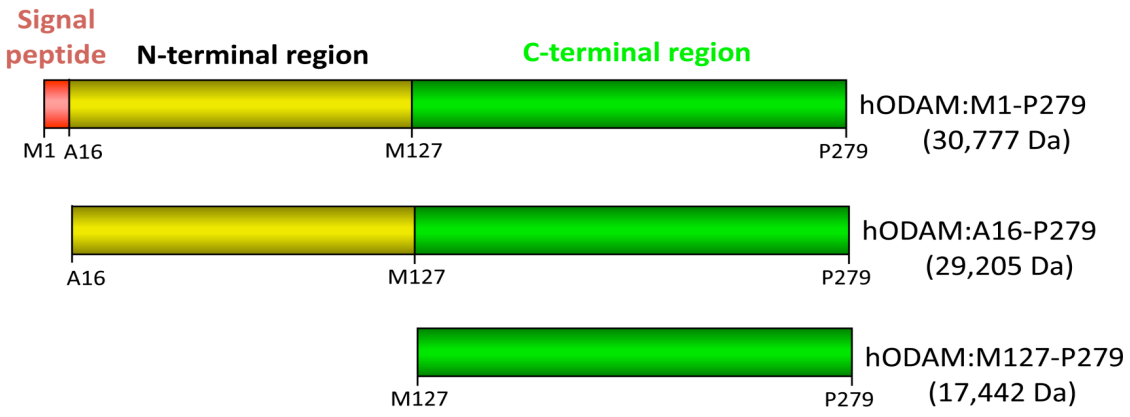


Figure 2: Three ODAM constructs were cloned into the Gateway system: full length; M1-P279, full length minus signal peptide; A16-P279, and C-terminal; M127-P279. All constructs were inserted into several expression vectors with either a fusion tag or purification tag (Table 1).

Table 1: *Human ODAM protein expression constructs made for mammalian expression.*

Construct	Expression vector	Fusion protein or purification tag
M1-P279	pDEST53	N-terminal GFP
A16-P279	pDEST53	N-terminal GFP
M127-P279	pDEST53	N-terminal GFP
M1-P279	pDEST27	N-terminal GST
A16-P279	pDEST27	N-terminal GST
M127-P279	pDEST27	N-terminal GST
M1-P279	pcDNA3.1/nV5-DEST	C-terminal V5 epitope
A16-P279	pcDNA3.1/nV5-DEST	C-terminal V5 epitope
M127-P279	pcDNA3.1/nV5-DEST	C-terminal V5 epitope
M1-P279	pcDNA-DEST40	C-terminal V5 epitope and 6X-His tag
A16-P279	pcDNA-DEST40	C-terminal V5 epitope and 6X-His tag
M127-P279	pcDNA-DEST40	C-terminal V5 epitope and 6X-His tag
M1-P279	pDEST26	N-terminal 6X-His tag
A16-P279	pDEST26	N-terminal 6X-His tag
M127-P279	pDEST26	N-terminal 6X-His tag

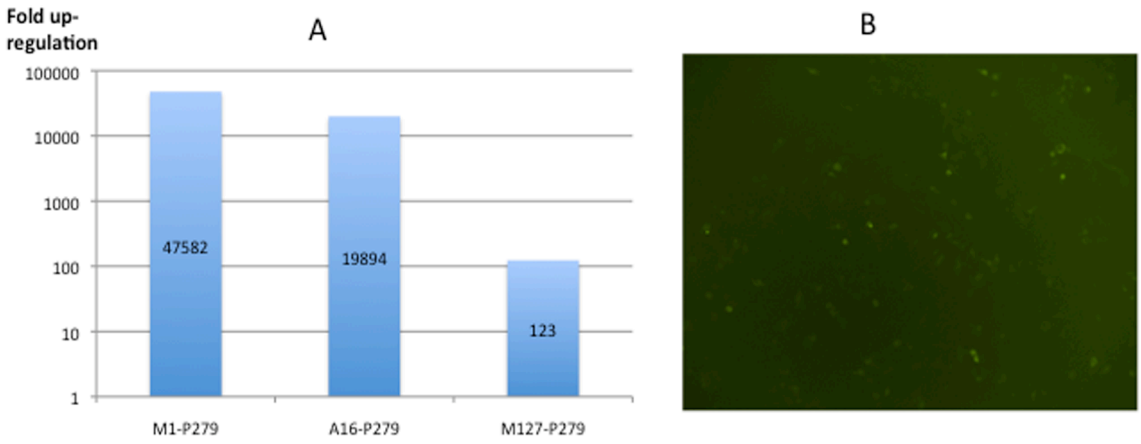


Figure 3: (A) Quantitative RT-PCR with ODAM GFP (pcDNA-DEST53) constructs transfected into HEK293 cells. Commercially available ODAM primers from SA Biosciences were used.

(B) Visualization of HEK293 cells transfected with M127- P279 pcDNA-pDEST 53. We over-expressed human ODAM in HEK293 cells with a maximum transfection efficiency of 20-30% using the green fluorescent construct (Fig A, B).

Unfortunately, after many unsuccessful trials of protein expression in *Escherichia coli*, we attempted to transfect the ODAM construct into mammalian cells for protein overexpression. We tested multiple constructs (Table 1) in many different cell lines but could not get the transfection efficiency to work very well (maximum efficiency obtained was around 20-30%, thus we were unable to obtain any protein for further study. After attempting transfections for more than a year, we switched to studying Pleotrophin and Receptor protein tyrosine phosphatase proteins.

PLEIOTROPHIN:

Pleiotrophin (PTN the protein, *ptn* the gene), is a protein of many names, it is also known as heparin-binding brain mitogen (HBBM), heparin-binding growth factor 8 (HBGF-8), neurite growth-promoting factor 1 (NEGF1), heparin affinity regulatory peptide (HARP), heparin binding growth associated molecule (HB-GAM) or Osteoblast-Specific Factor-1 (OSF-1). Pleiotrophin is an 18-kDa growth factor that has high affinity for heparin. It is structurally related to midkine and retinoic acid-induced heparin-binding protein. Pleiotrophin is 136 amino acids and is a secreted extracellular cytokine that signals diverse functions, including lineage-specific differentiation of glial progenitor cells, neurite outgrowth, and angiogenesis. Pleiotrophin gene expression is found in cells in early differentiation during different development periods and upregulated in cells with an early differentiation phenotype in wound repair. The PTN gene is a protooncogene. It is strongly expressed in different human tumor cells and expression of the PTN gene in tumor cells *in vivo* accelerates growth and stimulates tumor angiogenesis. Separate independent domains have been identified in the PTN protein to signal transformation and tumor angiogenesis.

RECEPTOR PROTEIN TYROSINE PHOSPHATASE:

Pleiotrophin is the first ligand of any of the known transmembrane tyrosine phosphatases RPTPβ/ζ. A hypothesis for PTN–induced signaling through the RPTPβ/ζ receptor has been published (Meng *et al*., 2000). Extracellular PTN binding inactivates the intracellular phosphatase domain of the RPTPβ/ζ by imposing a dimerization of the receptor, thereby inactivating the intracellular D1 phosphatase domain (Figure 4). Experimentally, the interaction of PTN and RPTPβ/ζ increases the steady-state tyrosine

phosphorylation of intracellular RPTPβ/ζ-D1 substrates, such as beta-catenin (Meng *et al*. 2000). Pleiotrophin regulates both normal cell functions and different pathological conditions at many levels. It signals these functions through RPTPβ/ζ as well as SDC3 (N-Syndecan) and potentially also Anaplastic Lymphoma Kinase (ALK). N-Syndecan and RPTPβ/ζ have been implicated in neurite outgrowth while RPTPβ/ζ and ALK have been shown to mediate cellular migration induced by pleiotrophin as well as the mitogenic, angiogenic, and transforming activities of this growth factor. Protein tyrosine phosphatase β/ζ (RPTPβ/ζ) is mainly expressed in the brain, and has been extensive studied during brain development, and there is thus a shortage of information about its expression in other tissues. In the early cortical development, RPTPβ/ζ has been localized along radial glial fibers and on migrating neurons, suggesting that this receptor-type phosphatase is involved in neuronal migration (Canoll *et al.,* 1993). RPTPβ/ζ consists of an NH_2-terminal carbonic anhydrase-like (CAH) domain, a fibronectin type III (Fn-III) domain, a large cysteine-free region, a transmembrane segment, and two tyrosine phosphatase domains, D1 (active) and D2 (inactive).

ISOFORMS OF RPTP B/Z:

There exist four splice variants of this molecule (Figure 5):

(1) the full-length RPTPβ/ζ (RPTPβ/ζ-A),

(*2*) the short form of RPTPβ/ζ (RPTPβ/ζ-B), in which most of the cysteine-free region is deleted,

(3) the secreted form of RPTPβ/ζ (RPTPβ/ζ-C), which corresponds to the extracellular region of PTPζ-A, and is also known as 6B4-proteoglycan/Phosphacan, and the last form called

(4) Phosphacan short isoform (PSI), which was recently discovered and is similar to RPTPβ/ζ-C/Phosphacan, only shorter. All these splice variants are synthesized as chondroitin sulfate proteoglycans in the brain, suggesting that the chondroitin sulfate portion is essential for the receptor function. RPTPβ/ζ has been shown to bind various cell adhesion and extracellular matrix molecules such as F3/contactin, N-CAM, L1, TAG1, and tenascin (Grumet et al., 1994; Milev et al., 1994, 1996; Peles *et al.,* 1995). Furthermore, RPTPβ/ζ binds pleiotrophin/heparin-binding growth-associated molecule (HB-GAM) (Maeda *et al.,* 1996). Chondroitin sulfate and the protein portion of RPTPβ/ζ together constitute the binding site for pleiotrophin, and various glycosaminoglycans inhibit RPTPβ/ζ-pleiotrophin binding (Maeda *et al., 1996*).

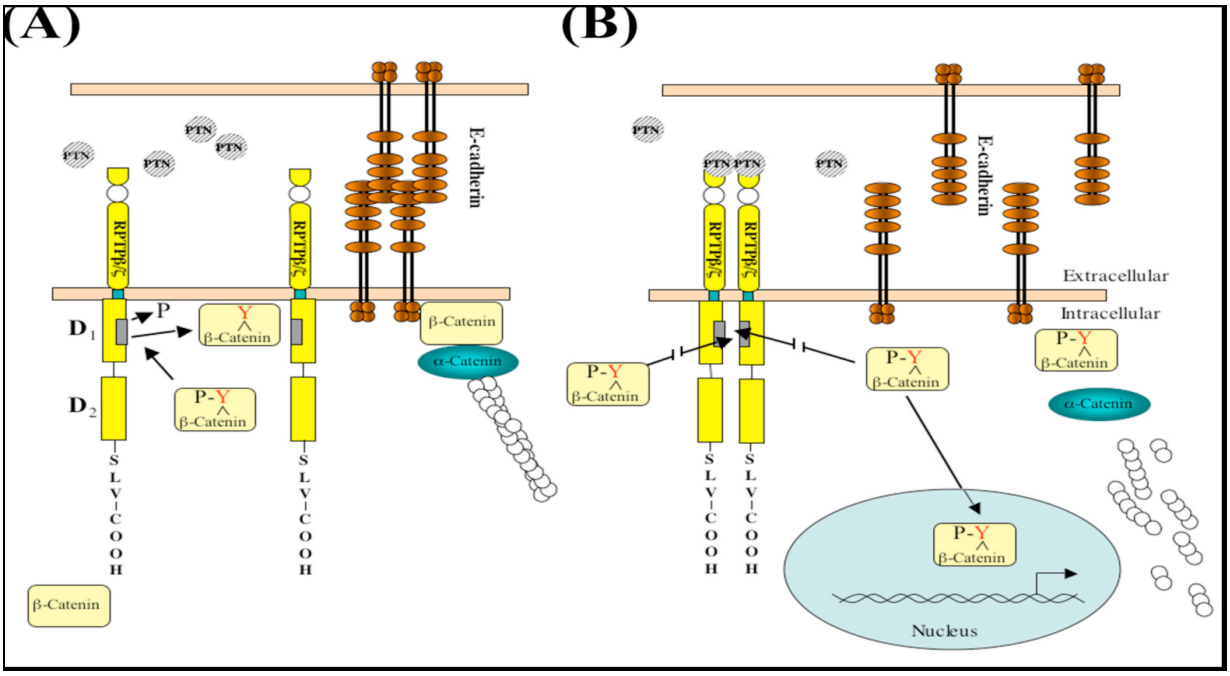


Figure 4: Diagram showing the hypothetical action/signaling of PTN through RPTPβ/ζ.

(Figure is from Meng *et al*., 2000 (A) shows the result when Pleiotrophin does not bind to the receptor. The intracellular domain of RPTPβ/ζ dephopharylates intracellular substrates such as β-catenin and is available to be attached to α-catenin and E-cadherin that is essential for cell adhesion. (B): Extracellular PTN binding inactivates the intracellular phosphatase domain of the RPTP by imposing a dimerization of the active D1 phosphatase domain. The interaction of PTN and RPTPβ/ζ increases steady-state tyrosine phosphorylation of intracellular substrates, such as beta-catenin, and beta-catenin can not longer interact with α-catenin and E-cadherin, and a loss of cell-cell adhesion is observed.

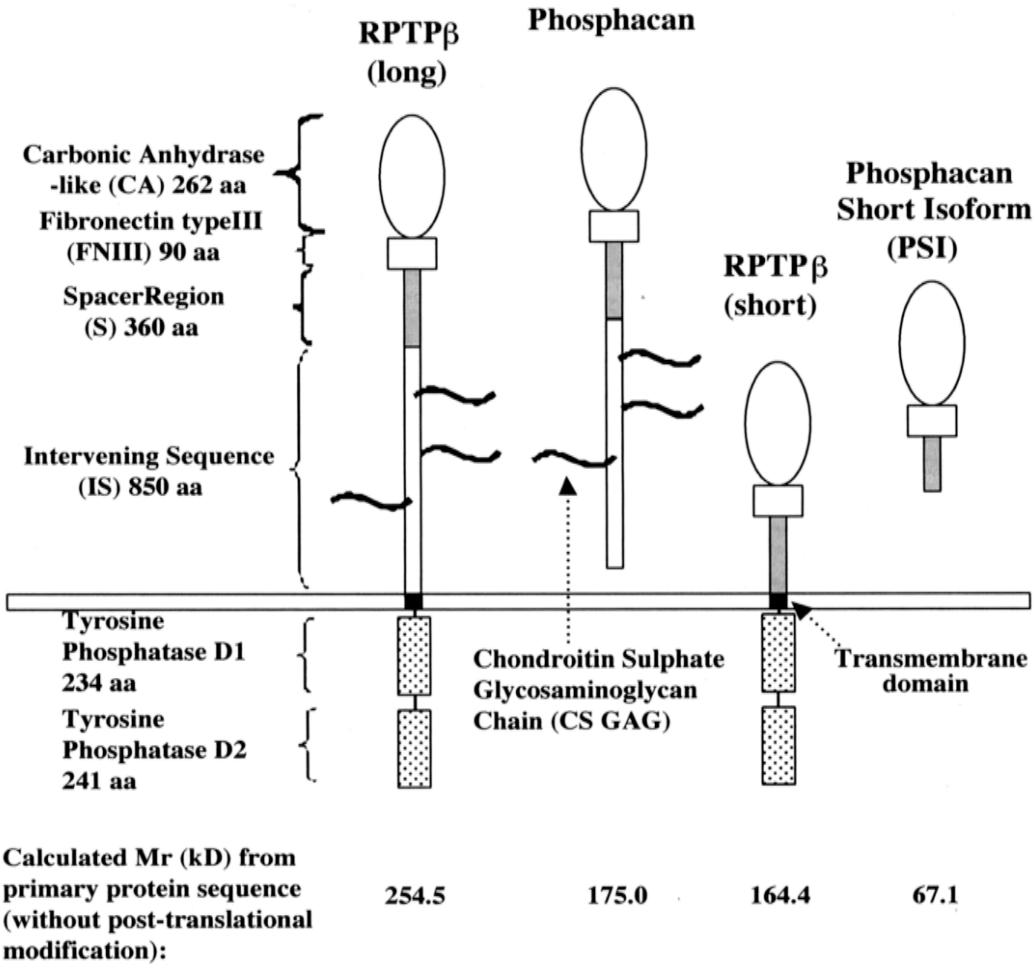


Figure 5: Diagram showing the four different isoforms of the Receptor Protein Tyrosine Phosphatase β/ζ (Figure is from Garwood *et al.* 2003).

RESEARCH PROBLEM

The development of teeth or odontogenesis in mammals is a highly ordered process that is mediated by multiple transcriptional regulators, signaling molecules and involves the association of epithelial cells, mesenchymal cells and the extracellular matrix. Pleiotrophin and its receptor protein tyrosine phosphatase was initially recognized as a neurite outgrowth-promoting factor present in rat brain around birth and as a mitogen toward fibroblasts isolated from bovine uterus tissue. Together with midkine these growth-factors constitute a family of (developmentally regulated) secreted heparin-binding proteins now known as the neurite growth-promoting factor (NEGF) family. During embryonic and early postnatal development, pleiotrophin is expressed in the central and peripheral nervous system and also in several non-neural tissues, notably lung, kidney, gut and bone. Pleiotrophin is also expressed by several tumor cells and is thought to be involved in tumor angiogenesis. PTN was synthesized by osteoblasts at an early stage of osteogenic differentiation and was present at sites of new bone formation, where PTN was stored in the new bone matrix (Tare, *et al*). RPTPβ/ζ was recently found in osteoblasts (Schinke, *et al,* 2008). Little is known regarding PTN and RPTPβ/ζ expression during tooth development. The research plan will result in determination of spatio-temporal expression pattern of PTN and RPTPβ/ζ during mouse odontogenesis.

SPECIFIC AIMS:

The specific aims of my project were:

1) Investigation of the mRNA and protein expression of Pleiotrophin and its Receptor Protein Tyrosine Phosphatase β/ζ in odontoblast and osteoblasts cell lines by immunocytochemistry, RT-PCR and western blot.

2) Investigation of the spatio-temporal expression pattern of PTN and RPTPβ/ζ during odontogenesis.

MATERIALS AND METHODS:

Antibodies:

A primary rabbit polyclonal antibody raised against recombinant human PTN (ab14025) was purchased from Abcam (Cambridge, MA), together with another goat polyclonal PTN antibody raised against human PTN produced recombinantly in Sf21 insect cells (Abcam; ab10849). A primary mouse monoclonal antibody against amino acids 94 to 168 of human PTN was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), PTN (H-6). Two RPTPβ/ζ primary mouse antibodies were purchased from R&D Systems (Immunogen: rhRPTPβ/ζ amino acids 302-754) and BD Transduction laboratories (Immunogen: human RPTPβ/ζ amino acid 2098-2307), and one rabbit primary RPTPβ/ζ antibody was purchased from Abcam (ab12554) (Immunogen: amino acids 1850-1950 of human RPTPβ/ζ/Phosphacan).

Methods

Localization of PTN and RPTP Protein Using Immunohistochemistry

Mice (C57BL/6) embryonic day 16 (E16) to day 18 (E18), newborn, and postnatal days 1, 3, 5, 7, 10, 14, and 28 were sacrificed and fixed in 4% paraformaldehyde for shipment from The Scripps Research Institute (Dr. Deuel's laboratory) to The Institute of Oral Health at the University of Alabama at Birmingham. The mouse heads were sectioned into mandibles and maxillaries and demineralized in 10% EDTA if older than 5 days, processed, embedded in paraffin, sectioned and mounted on silane-treated slides. Immunohistochemistry (IHC) was performed with the goat polyclonal PTN antibody (Abcam, ab14025) and RPTPβ/ζ rabbit primary antibody (Abcam, ab12554) at a dilution

of 1:50 or 1:100, followed by the corresponding secondary antibody horseradish peroxidase (HRP) conjugate system, visualized by 3–3′tetrachloride diaminobenzidine (DAB) oxidization, counterstained with hematoxylin and photographed using a Nikon. 10% BSA was used instead of primary antibody for negative controls.

Cell Cultures

Mouse immortalized dental pulp mesenchymal cells, pre-odontoblasts (MD10-A11), and odontoblast-like (M06-G3) were plated and cultured approximately 90% confluency under culture conditions as previously described (MacDougall et al. 1998) and further grown on four-chamber glass slides (Lab Tek II; Nalge Nunc International, Rochester, NY) until confluent for further use with immunocytochemistry (ICC). The mature osteoblasts (ROS) and mesenchymal or preosteoblasts were grown similarly, except that the media used was Alpha MEM with 5% FBS.

Immunofluorescence

Immunofluorescence was performed as for IHC with the goat polyclonal PTN primary antibody (Abcam; ab10849) and Rabbit RPTP/Phosphacan primary antibody (Abcam: ab12554) at 1:50 with overnight incubation and using an Alexa Fluor 388–conjugated, anti-goat IgG secondary antibody (PTN) and anti-rabbit IgG secondary antibody (RPTP). The cells were then counterstained with 4,′6-diamidino-2-phenylindole (DAPI) to visualize the nuclei, before being photographed using a Roper Scientific (Trenton, NJ) digital camera at the same exposure time for the different cell lines. The FITC and DAPI images were merged using the NIS-Elements software (Nikon).

Isolation of RNA

Cells were harvested at 100% confluency for isolation of total RNA using the Qiagen RNeasy kit. Total RNA/mRNA isolation reagent kit according to manufacturer's protocol (Qiagen sciences, Maryland). The isolated RNA was resuspended in 10µl sterile water and the concentration determined.

Reverse Transcription/PCR Amplification

In total, 1 µg RNA was converted to cDNA using iScript (Applied Biosystems, Foster City, CA) with random hexamers. An aliquot of the resulting cDNA was amplified with REDTaq DNA Polymerase and specific PTN, RPTPβ/ζ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer sets (10 pM each primer, Table 2) during 35 cycles of RT-PCR (Rappolee et al. 1989). The RT-PCR primers were designed using the accession codes NM_008973 (*Mus musculus* PTN) and NC_000072 (*M. musculus* GAPDH). The RT-PCR PTN primers were designed to cover several exon/intron boundaries to distinguish mRNA amplicons and alternative splicing forms and rule out genomic DNA contamination. RPTP primers were from Sckinke et al. The PCR products were separated on a 2% agarose gel (PTN) and 1% agarose gel for RPTP. Gels were stained with ethidium bromide and images captured with an Alpha Imager 2000 gel documentation and analysis system (Alpha Innotech, Santa Clara, CA).

Table 2: Designed primers used for conventional RT-PCR

Primer name	Primer Sequence
mRPTP-A (+)	5'-TGTGAGGGCTTAAAACACCC-3'
mRPTP-A (-)	5'-CAGCTCACCTCCAGTCACAA-3'
mRPTP-B (+)	5'-AGCAAAAGTCCCCAGCAAGTG-3'
mRPTP-B (-)	5'-TCCCTAATGGAACAGAATGGTCAG-3'
mRPTP-C (+)	5'-TGGTGGTTATGATTCCAGATGGTC-3'
mRPTP-C (-)	5'-AAGTTCCTTGCTGTTACTCCCCCG-3'
mPTN(+)	5'-AAAAATGTCGTCCCAGCAGCAAT-3'
mPTN(-)	5'-GCTCCAAACTGCTTCTTCCA-3'
mGAPDH(+)	5'-GACATCAAGAAGGTGGTGAAGCAG-3'
mGAPDH(-)	5'-CTCCTGTTATTATGGGGGTCTGG-3'

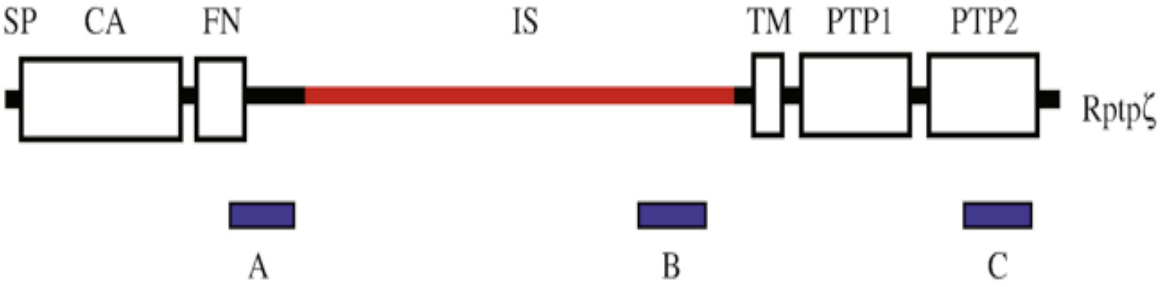


Figure 6: Schematic of the recognition sites of the designed RPTPβ/ζ primers on the domain structure of RPTPζ. The N-terminal signal peptide (SP) is followed by a carbonic anhydrase (CA) and fibronectin (FN) domain. From Schinke *et al.* (2008). **Primer pair A** amplifies the C-terminal portion of the Fibronectin-type III region, which is present in all the four isoforms of RPTPβ/ζ. **Primer pair B** amplifies the Intervening Sequence (IS) of RPTPβ/ζ. The IS is only present in phosphacan and the long transmembrane isoform of RPTPβ/ζ. **Primer pair C** amplifies a region in the Intracellular D1 phosphatase domain, which is present in long and short isoform of RPTPβ/ζ. Using all three primer pairs we can distinguish which isoform of RPTPβ/ζ is present.

PTN Protein Expression and Western Blot Analysis:

Dental cell lines and osteoblasts /preosteoblasts were lysed in RIPA lysis buffer, then sonicated using the Bioruptor (Diagenode Inc, Denville, NJ), and the media containing protein was collected and frozen before further use.

Total amount of soluble proteins from cell cultures was quantified using the BCA Protein Assay kit (Pierce, Rockford, IL), SDS-PAGE resolved using 4% to 12% precast gels (Invitrogen, Carlsbad, CA), and electroblotted to poly(vinylidene difluoride) (PVDF) membranes using the NuPAGE wet transfer apparatus for 1.5 hrs-3hrs at 30V. Bound proteins were detected with the PTN primary antibody (Abcam; ab14025, 1:1000 dilution) Rabbit RPTP primary antibody (Abcam; ab12554), Mouse RPTP Primary (BD Transduction laboratories) or Mouse RPTP primary (R&D Biosciences) and species-specific secondary antibody (Odyssey infrared imaging systems) at a dilution of 1:1000. PTN and RPTP on the membrane imaged by Odyssey infrared imaging systems.

RESULTS

Expression of PTN and RPTP at mRNA level in odontoblasts and osteoblast cells:

PTN expression was first tested in dentally derived mouse cell lines at the mRNA level by conventional RT-PCR. PTN mRNA transcripts were detected in all ectomesenchymally derived cell lines tested at similar relative levels, after normalization to the GAPDH gene. RPTPβ/ζ mRNA was detected in all the odontoblast cell lines(MD10A11,MD10D3) and the C3HT10t1/2 pre-osteoblasts(Figure 7).

Expression of PTN and RPTP protein in odontoblast and osteoblast cell:

The size and the complexity of the PTN protein were analyzed by Western blot (Figure 8). The presence of PTN protein in the mouse odontoblast-like cell lines M06-G3, and MD10-A11 was confirmed. The protein migrated as a major monomer of ~23 kDa in the mesenchymal-derived cells as well as the mouse brain extract positive control. Furthermore, PTN dimers and putative trimers can be seen at ~43 kDa and ~51 kDa in the dental mesenchymal cells only (M06-G3, MD10-A11, and MD10-D3. Interestingly, the PTN dimers and trimers were not detected within the control mouse brain extract sample. RPTP protein was detected in MO6-G3 and ROS cells. The protein which appears to migrate at 67kDa on the Western Blot probed with the antibody towards Phosphacan, might be Phosphacan short isform (PSI). The two bands seen close together might be glycosylated and unglycosylated PSI and was only detected with MO6-G3 cells.

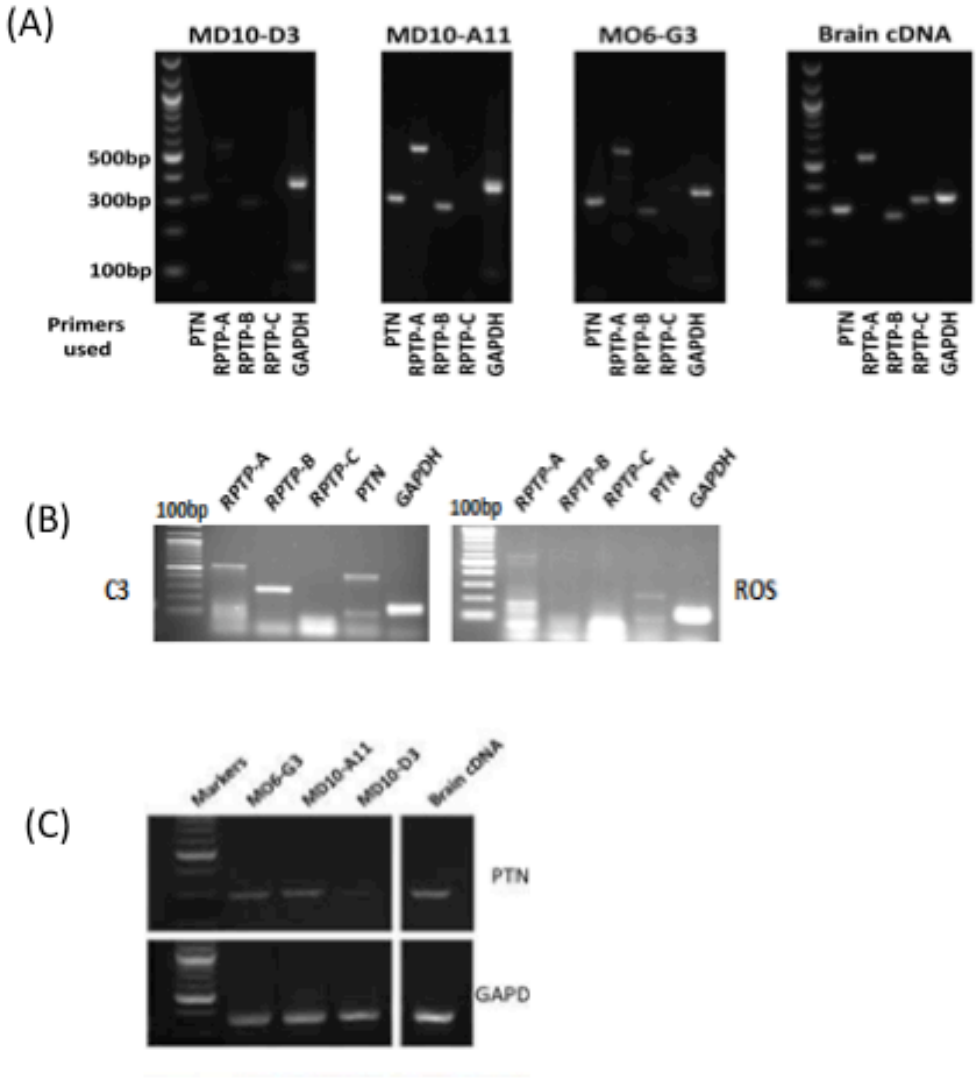


Figure 7 (A)(B): Conventional RT-PCR using RPTP mouse primers and cDNA from MD10-D3, MD10-A11, MO6-G3, C3H10t1/2 and ROS cells. Positive control with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers. RT-PCR expression analysis with primers amplifying the three different regions of the RPTPβ/ƒ cDNA. Only the brain cDNA contains the region encoding the full-length form of RPTPβ/ƒ. The expression of PTN and GAPDH is shown for reference. (C) Conventional RT-PCR using pleiotrophin (PTN) mouse primers and cDNA from M06-G3 (lane 2), MD10-A11 (lane 3), MD10-D3 (lane 4), and mouse brain cDNA (PTN positive control, lane 5.

(A) (B)

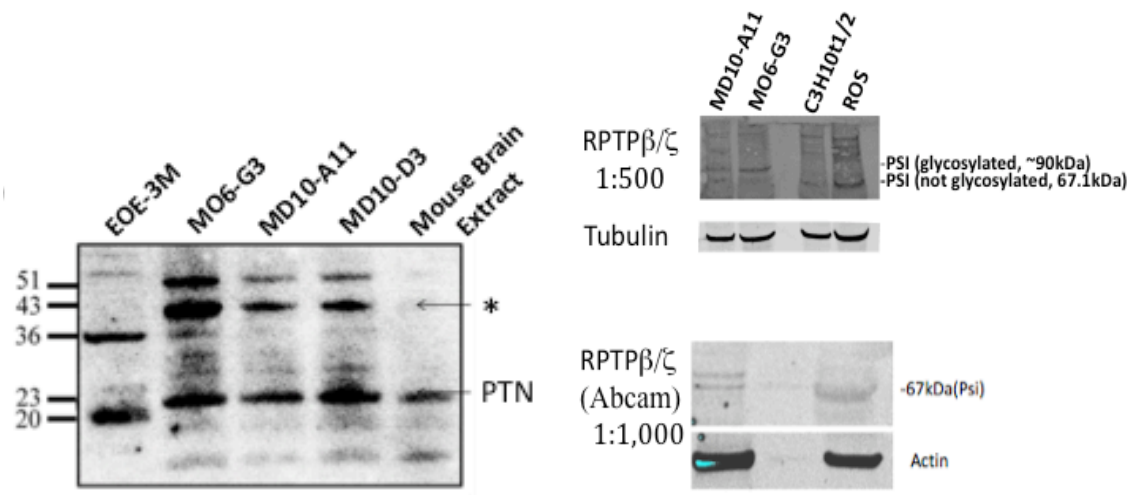


Figure 8 (A): Western blot for PTN of cell lysates from mouse ameloblast-like (EOE-3M), M06-G3, MD10-D3, MD10-A11 cells. Positive control is shown in the right lane; mouse brain extract (Santa Cruz Biotechnology). (B) Western Blots for RPTPβ/ζ of cell lysates from MD10-A11, MO6-G3, C3, and ROS cells. Top western blot is probed with the RPTPβ/ζ antibody from BD Transduction laboratories, and the bottom against the RPTPβ/ζ Abcam antibody.

Expression of PTN and RPTP at different time points during odontogenesis:

Again the PTN and RPTP proteins were investigated by immunohistochemistry (Figures 9-11). We saw an initial expression of PTN protein in the inner enamel epithelium (E-16) and along the dental follicle with prolonged expression in the ameloblasts throughout their stages of maturation (days 1-28 postnatal) (Figure 9). PTN was also expressed at lower levels in the dental pulp mesenchyme (E-16) with increased

staining associated with odontoblast cytodifferentation (Figure 9). Expression was also detected within sub-populations of the stellate reticulum, the dental follicle and the periodontal ligament. Strong staining was seen in the adult mouse incisor at P28 (Figure 10), specifically in the odontoblasts, the enamel (including Tomes' processes), at the dentino-enamel junction (DEJ), the secreting end of the ameloblast layer as well as the stratum intermedium (SI). Immunohistochemistry on adult (P28) mouse molars also detected strong staining especially in the odontoblasts, dentin tubules/odontoblast processes, PDL as well as at the root apex (Figure 10). Positive staining was detected as expected within the forming alveolar bone within the osteoblasts and embedded osteocytes (Figures 9 and 10).

RPTP was also expressed in bone at the time-points tested, from PN1 to PN14, but at E16 the protein was expressed only in the dental sac (future alveolar bone region)(Figure 11). Upon onset of mineralization (P1), RPTPβ/ζwas expressed in the forming alveolar bone, and some staining could be seen in at the dentino-enamel junction (DEJ). At P10, the enamel shows strong staining for RPTPβ/ζ, as well as the secreting ends of both the odontoblasts and the ameloblasts (Figure 11), as well as in the alveolar bone.

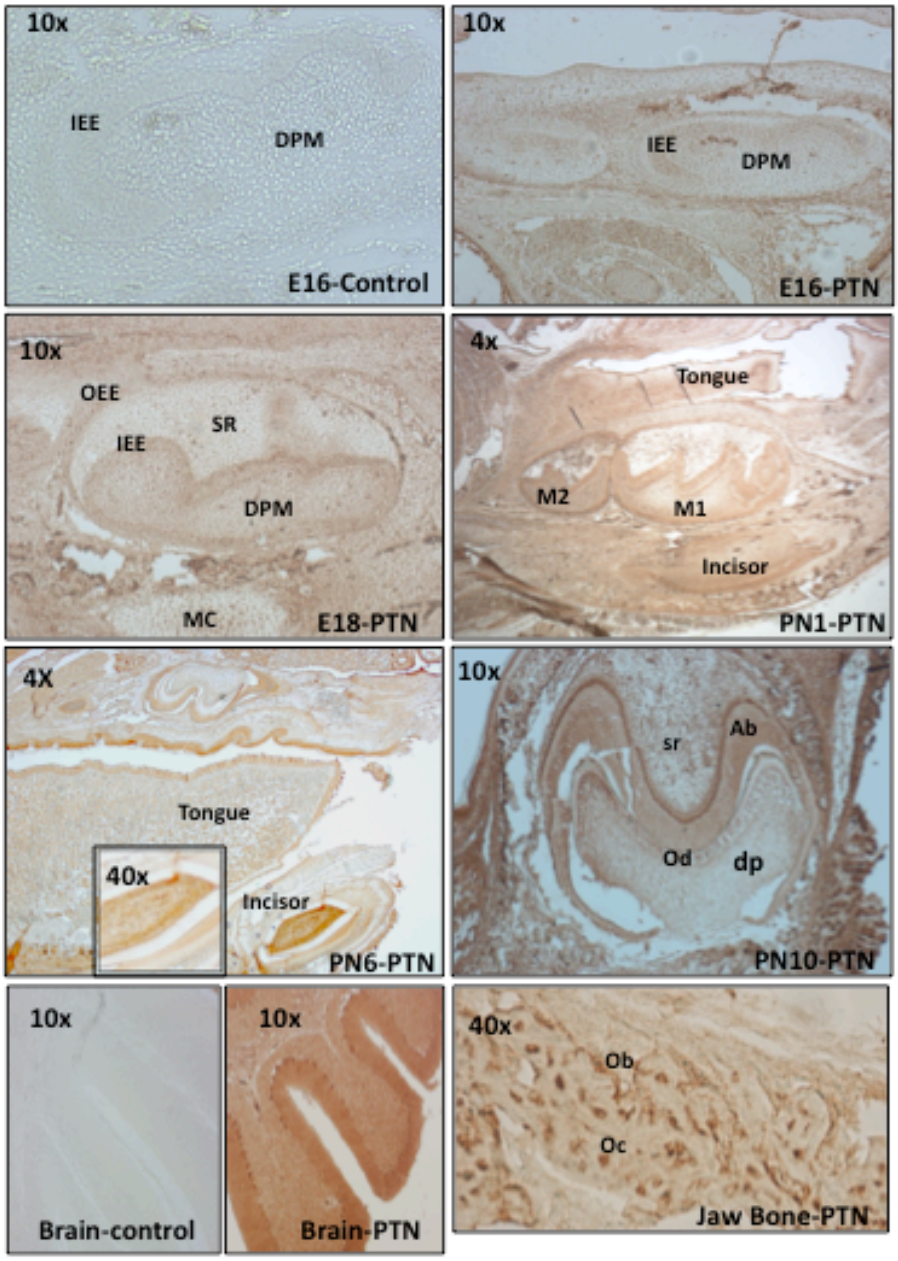
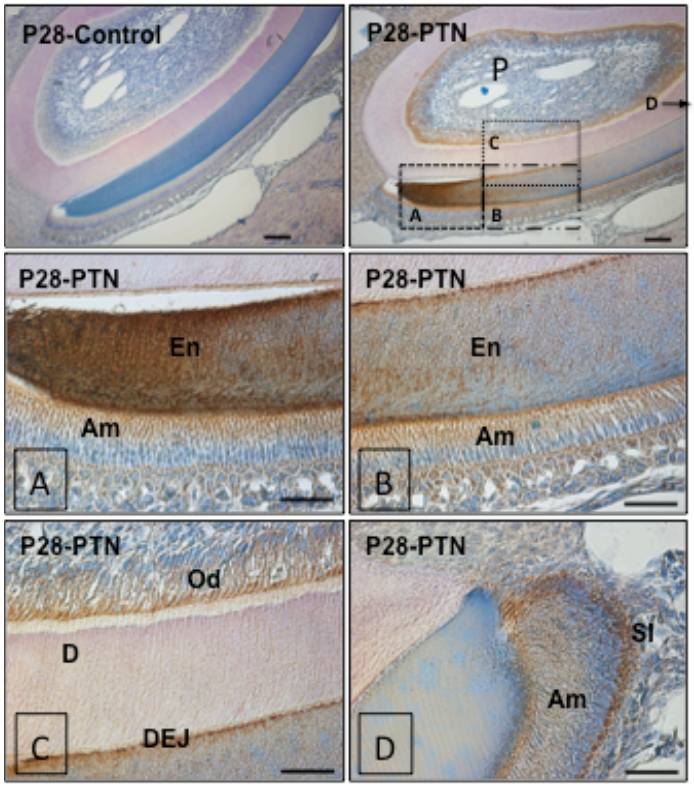


Figure 9: Immunohistochemistry of mouse tissue sections at developing stages E16, E18, P1, P6 and P10). PTN antibody (Abcam, ab14025) dilutions were 1:50. Positive PTN controls for brain and bone as well as representative negative controls (using BSA instead of primary antibody) are shown. IEE; Inner Enamel Epithelium, OEE; Outer Enamel Epithelium, DPM; Dental pulp mesenchyme, SR; Stellate Reticulum, MC; Meckels Cartilage, M1 and M2; Molar 1 and 2, dp; Dental pulp, Od; Odontoblasts, AB; Ameloblasts, Ob, Osteoblasts, OC; Osteocytes.

(A)



(B)

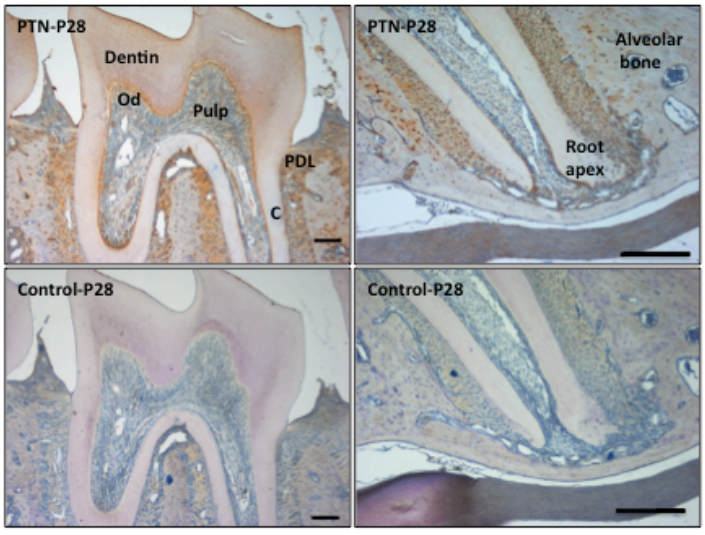


Figure 10 (A): Immunohistochemistry of mouse incisor and (B) molar and root tissue sections in the adult mouse (P28). Pleiotrophin (PTN) antibody (Abcam; ab14025) dilutions were 1:50. Negative controls (using BSA instead of primary antibody) are shown. Negative controls (using BSA instead of primary antibody) are shown.

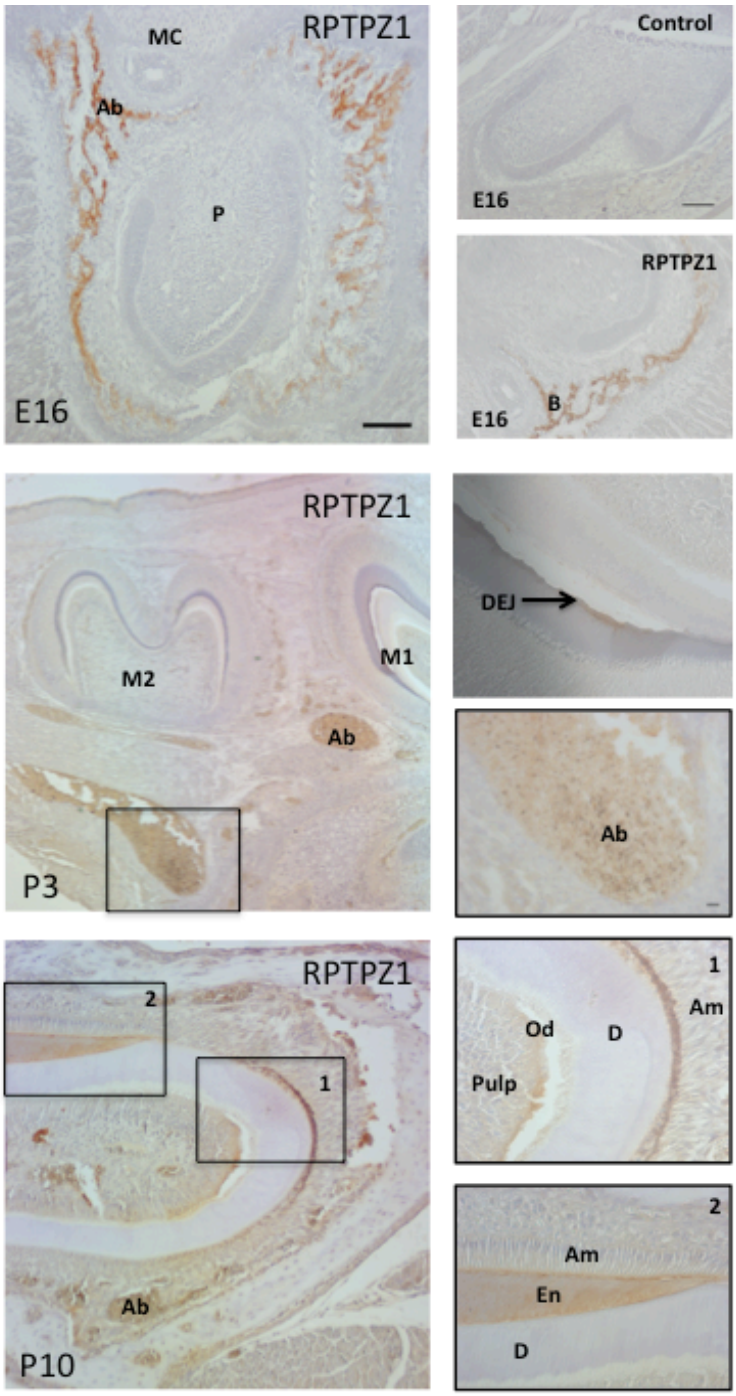


Figure 11: Immunohistochemistry of mouse molar and incisor at E16, PN3 and PN10. RPTP antibody (Abcam) dilutions were 1:50. Negative controls (using 10% BSA instead of primary antibody) are shown.

Perinuclear Expression of PTN and RPTPβ/ζ in odontoblasts and osteoblast cells:

Finally the presence of PTN and RPTPβ/ζ was confirmed at the protein level by immunocytochemistry in the same odontoblasts and osteoblasts cell lines (Figures 12 and 13). RPTPβ/ζ and PTN were expressed in MO6-G3, MD10-A11 and C3H10T1/2 cells when compared to the control. Intense staining was seen in the peri-nuclear and cytoplasmic region.

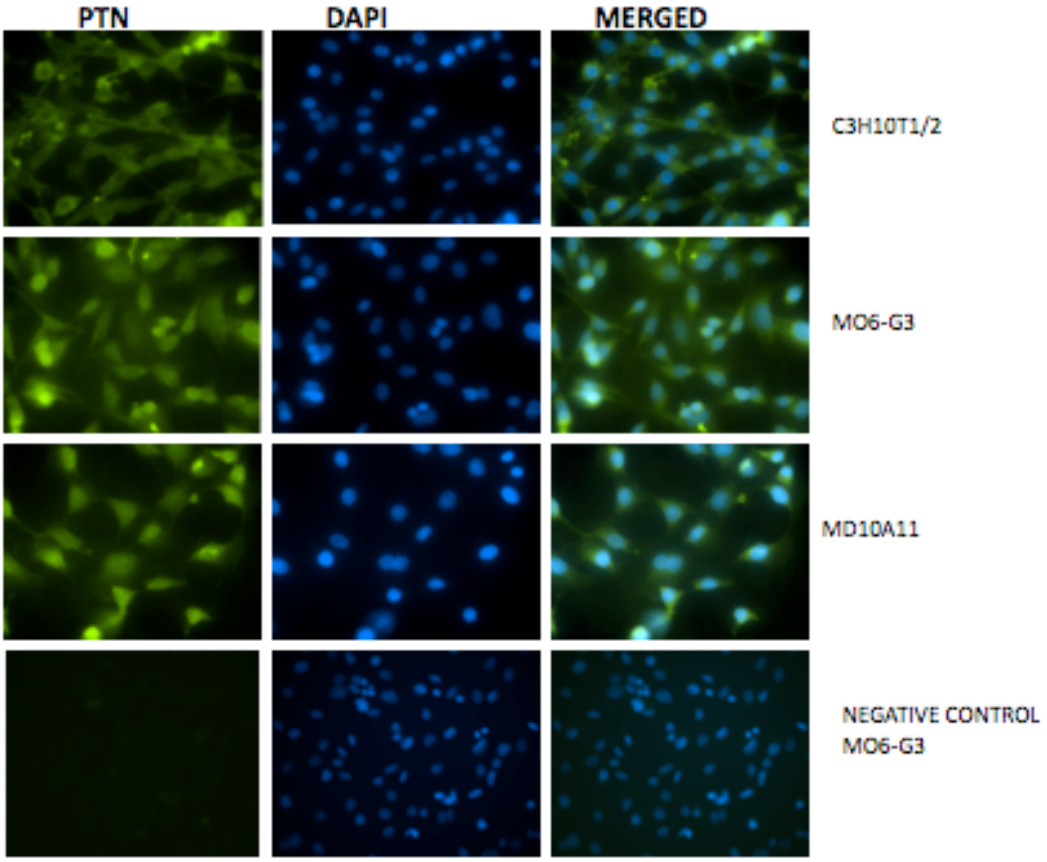


Figure 12: Insitu Immunofluorescence of mouse M06-G3, MD10-A11, and C3H10t1/2 cells stained with the PTN antibody (Abcam; ab10849) at a1:50 dilution. Magnification is 10x.The negative control is 10% BSA instead of primary antibody.

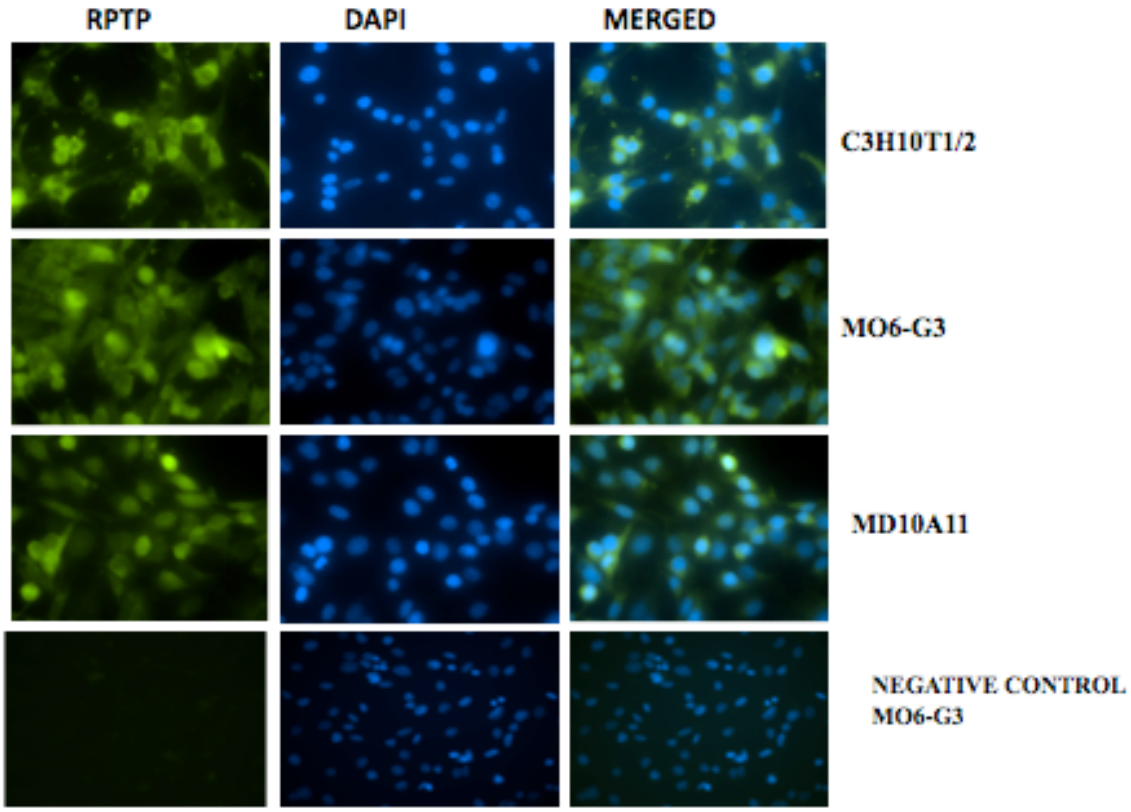


Figure 13: Insitu Immunofluorescene of MD10A11,MO6-G3 and C3H10T1/2 cells with RPTPb/z antibody.RPTPβ/ζ primary antibody (Abcam: ab12554) at a 1:50 dilution. The secondary antibody was tagged with Alexa Fluor 388, followed by DAPI counterstaining. The same exposure time was used for all images. The negative control is with 10% BSA instead of primary antibody. Magnification is 10x.

SUMMARY

These studies demonstrate that PTN and Receptor PTPβ/ζ are expressed during tooth development. Localization of PTN within the odontoblasts and ameloblasts may indicate that PTN is involved in the production or homeostasis of mineralized tissue. The presence of Phosphacan/RPTPβ/ζ in odontogenesis especially in the odontoblast and ameloblast layer indicates that it is involved in dentin and/or enamel formation. Together these results indicate that PTN and RPTPβ/ζ are involved not only in bone formation but also tooth formation, and thus are involved in the formation of mineralized tissues.

DISCUSSION

This study demonstrates that PTN and RPTPβ/ζ are both expressed during development in bone and also in the tooth, and that, rather unexpectedly, PTN is also expressed in the adult mouse molar and incisor. Using an assortment of cell biology techniques, such as polymerase chain reaction, immunohistochemistry, immunocytochemistry, and Western blots, we detected PTN and RPTPβ/ζ within a number of mineralizing cell-types. There was an increase in PTN staining associated with the terminal cytodifferentiation of ameloblasts and also at the secreting ends of the ameloblasts, which suggests that PTN is an extracellular enamel matrix molecule and could be involved in enamel matrix maturation. PTN and RPTPβ/ζ expression in odontoblasts was confirmed by both RT-PCR and Western blot analysis on immortalized mouse cells. PTN has the ability to promote migration, expansion, adhesion, and differentiation of human osteoprogenitor cells (Yang et al. 2003), and it is possible that PTN (and RPTPβ/ζ) also has this function during ameloblast cytodifferentiation and maturation. PTN has been shown to have multiple effects on bone formation, and the effects are dependent on the concentration of PTN and the timing of its presence. To explain the multiple effects, it has been proposed that PTN is an accessory-signaling molecule, which is involved in a variety of processes in bone formation (Tare *et al.* 2002). PTN can thus enhance or inhibit primary responses depending on the prevailing concentrations, the primary stimulus, and the availability of appropriate receptors (syndecans and/or RPTP β/ζ) (Tare *et al.* 2002). One of the most prevalent integrins αvβ3, is expressed on almost all the cells originating from the mesenchyme, and the presence of integrin αvβ3 seems to be a key factor that determines

whether PTN will have a stimulatory or inhibitory effect on endothelial cell migration (Mikelis *et al*).

RPTPβ/ζ and phosphacan have punctual spatiotemporal expression patterns that suggests a potential role for these proteins in various developmental processes, including cell migration, differentiation, (Garwood *et al*. 2003) Both the PTN receptors syndecan-1 and -3 and RPTPβ/ζ are known to have multiple CS chains the size and extent of CS oversulfation vary during vertebrate developmental stages. Syndecan-1 and -3 are known to be present in tooth epithelium during development and are both cell-surface heparan sulfate proteoglycans that act as receptors for not only PTN but also several matrix molecules and growth factors, such as FGF (Thesleff et al. 1995).

From our studies, we can conclude that PTN and RPTPβ/ζ are expressed throughout odontogenesis, especially during the mineralization process in bone and teeth, but the exact role and function that this receptor-ligand pair play in odontogenesis remains unknown and a topic for further studies.

FUTURE DIRECTIONS

For this project there are several things that can be done in the future. One direction is to look at the expression of PTN and RPTPβ/ζ at different time points during the differentiation of pre-odontoblast and osteoblast cells. Furthermore, one can also determine the up and down regulation of other mineralized genes, such as Amelogenin, Ameloblastin, Enamelin compared to PTN and RPTPβ/ζ in the mineralized cells.

REFERENCES

1. Arai H, Higuchi S, Muramatsu T, Iwatsubo T, Sasaki H, Trojanowski JQ. 1994. Apolipoprotein E gene in diffuse Lewy body disease with or without co-existing Alzheimer's disease. *Lancet*. 344(8932):1307.

2. Bernard-Pierrot I, Heroult M, Lemaitre G, Barritault D, Courty J, Milhiet PE. 1999. Glycosaminoglycans promote HARP/PTN dimerization. *Biochem Biophys Res Commun*. 266(2):437–442.

3. Canoll PD, Barnea G, Levy JB, Sap J, Ehrlich M, Silvennoinen O, Schlessinger J, Musacchio JM. 1993, *Brain Res Dev Brain Res.*75(2):293-8.

4. Deuel TF, Zhang N, Yeh HJ, Silos-Santiago I, Wang ZY. 2002. Pleiotrophin: a cytokine with diverse functions and a novel signaling pathway. *Arch Biochem Biophys*. 397(2):162–171.

5. Garwood J, Heck N, Reichardt F, Faissner A. 2003, *J Biol Chem.* 278(26):24164-73.

6. Grumet M, Milev P, Sakurai T, Karthikeyan L, Bourdon M, Margolis RK, Margolis RU. 1994, *J Biol Chem.* 269(16):12142-6.

7.Hamma-Kourbali Y, Bernard-Pierrot I, Heroult M, Dalle S, Caruelle D, Milhiet PE, Fernig DG, Delbe J, Courty J. 2008. Inhibition of the mitogenic, angiogenic and tumorigenic activities of pleiotrophin by a synthetic peptide corresponding to its thrombospondin repeat-I domain. *J Cell Physiol*. 214(1):250–259.

8. Comparison of expression patterns between CREB family transcription factor OASIS and proteoglycan core protein genes during murine tooth development. *Anat Embryol (Berl)*. 206(5):373–380

9. Imai S, Kaksonen M, Raulo E, Kinnunen T, Fages C, Meng X, Lakso M, Rauvala H. 1998. Osteoblast recruitment and bone formation enhanced by cell matrix-associated heparin-binding growth-associated molecule (HB-GAM). *J Cell Biol*. 143(4):1113–1128

10. Kinnunen A, Kinnunen T, Kaksonen M, Nolo R, Panula P, Rauvala H. 1998. Nsyndecan and HB-GAM (heparin-binding growth-associated molecule) associate with early axonal tracts in the rat brain. *Eur J Neurosci*. 10(2):635–648.

11. MacDougall M, Selden JK, Nydegger JR, Carnes DL. 1998. Immortalized mouse odontoblast cell line M06-G3 application for in vitro biocompatibility testing. *Am J Dent*. 11(Spec No):S11–S16.

12. Maeda N, Nishiwaki T, Shintani T, Hamanaka H, Noda M. 1996. 6B4 proteoglycan/phosphacan, an extracellular variant of receptor-like protein-tyrosine phosphatase zeta/RPTPbeta, binds pleiotrophin/heparin-binding growth-associated molecule (HB-GAM). *J Biol Chem*. 271(35):21446–21452.

13. Maeda N, Fukazawa N, Hata T. 2006. The binding of chondroitin sulfate to pleiotrophin/heparin-binding growth-associated molecule is regulated by chain length and oversulfated structures. *J Biol Chem*. 281(8):4894–4902.

14. Meng K, Rodriguez-Pena A, Dimitrov T, Chen W, Yamin M, Noda M, Deuel TF. 2000. Pleiotrophin signals increased tyrosine phosphorylation of beta-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase beta/zeta. *Proc Natl Acad Sci U S A*. 97(6):2603–2608.

15. Mikelis C Sfaelou E Koutsioumpa M Kieffer N Papadimitriou E 2009 Integrin alpha(v)beta(3) is a pleiotrophin receptor required for pleiotrophin-induced endothelial cell migration through receptor protein tyrosine phosphatase beta/zeta. *FASEB J*.

23(5):1459–1469

16. Milev P, Friedlander DR, Sakurai T, Karthikeyan L, Flad M, Margolis RK, Grumet M, Margolis RU. 1994, *J Cell Biol.* 127(6 Pt 1):1703-15.

17. Milev P, Maurel P, Häring M, Margolis RK, Margolis RU. 1996, *J Biol Chem.* 271(26):15716-23.

18. Mitsiadis TA, Salmivirta M, Muramatsu T, Muramatsu H, Rauvala H, Lehtonen E, Jalkanen M, Thesleff I. 1995. Expression of the heparin-binding cytokines, midkine (MK) and HB-GAM (pleiotrophin) is associated with epithelial-mesenchymal interactions during fetal development and organogenesis. *Development*. 121(1):37–51.

19. Muramatsu H, Zou P, Kurosawa N, Ichihara-Tanaka K, Maruyama K, Inoh K, Sakai T, Chen L, Sato M, Muramatsu T. 2006. Female infertility in mice deficient in midkine and pleiotrophin, which form a distinct family of growth factors. *Genes Cells*. 11(12):1405–1417.

20. Peles E, Schlessinger J, Grumet M. 1998, *Trends Biochem Sci.* 23(4):121-4.

21. Schinke T, Gebauer M, Schilling AF, Lamprianou S, Priemel M, Mueldner C, Neunaber C, Streichert T, Ignatius A, Harroch S, et al. 2008. The protein tyrosine phosphatase Rptpzeta is expressed in differentiated osteoblasts and affects bone formation in mice. *Bone*. 42(3):524–534.

22. Taravini IR, Ferrario JE, Delbe J, Ginestet L, Debeir T, Courty J, Murer MG, Gershanik OS, Raisman-Vozari R. 2005. Immunodetection of heparin-binding growth associated molecule (pleiotrophin) in striatal interneurons. *Brain Res*. 1066(1–2):196–200

23. Tare RS, Oreffo RO, Clarke NM, Roach HI. 2002. Pleiotrophin/osteoblaststimulating factor 1: dissecting its diverse functions in bone formation. *J Bone Miner Res*. 17(11):2009–2020.

24. Tezuka K, Takeshita S, Hakeda Y, Kumegawa M, Kikuno R, Hashimoto-Gotoh T. 1990. Isolation of mouse and human cDNA clones encoding a protein expressed specifically in osteoblasts and brain tissues. *Biochem Biophys Res Commun*. 173(1):246–251

25. Yang X, Tare RS, Partridge KA, Roach HI, Clarke NM, Howdle SM, Shakesheff KM, Oreffo RO. 2003, *J Bone Miner Res*. 18(1):47-57.