

Testicular acid phosphatase induces odontoblast differentiation and mineralization

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Abstract Odontoblasts differentiate from dental mesenchyme during dentin formation and mineralization. However, the molecular mechanisms controlling odontoblast differentiation remain poorly understood. Here, we show that expression of testicular acid phosphatase (ACPT) is restricted in the early stage of odontoblast differentiation in proliferating dental mesenchymal cells and secretory odontoblasts. ACPT is expressed earlier than tissue-nonspecific alkaline phosphatase (TNAP) and partly overlaps with TNAP in differentiating odontoblasts. In MDPC-23 odontoblastic cells, expression of ACPT appears simultaneously with a decrease in β -catenin activity and is abolished with the expression of Phex and Dsp. Knockdown of ACPT in MDPC-23 cells stimulates cell proliferation together with an increase in active β -catenin and cyclin D1. In contrast, the overexpression of ACPT suppresses cell proliferation with a decrease in active β -catenin and cyclin D1. Expression

of TNAP, Osx, Phex and Dsp is reduced by knockdown of ACPT but is enhanced by ACPT overexpression. When ACPT is blocked with IgG, alkaline phosphatase activity is inhibited but cell proliferation is unchanged regardless of ACPT expression. These findings suggest that ACPT inhibits cell proliferation through β -catenin-mediated signaling in dental mesenchyme but elicits odontoblast differentiation and mineralization by supplying phosphate during dentin formation. Thus, ACPT might be a novel candidate for inducing odontoblast differentiation and mineralization for dentin regeneration.

Keywords ACPT · Localization · Odontoblasts · Proliferation · Differentiation

Introduction

Dentin is a calcified tissue produced by odontoblasts, which form a continuous single-cell layer at the periphery of the dental pulp. The functional odontoblast has a columnar shape with a long cellular process and its major function is to secrete matrix and regulate dentin mineralization (Ruch et al. 1995). As is well known, Wnt signaling promotes the proliferation of dental mesenchymal cells and dental pulp stem cells and inhibits their differentiation and mineralization (Scheller et al. 2008; Zhang et al. 2014; Bae et al. 2015). However, the regulatory mechanism involved in the transition from proliferation to differentiation during dentinogenesis is unclear.

Biom mineralization is important for the formation and function of mineralized tissues. Inorganic phosphate (Pi) is essential for extracellular matrix mineralization and plays an important role in the development and activity of osteogenic, odontoblastic and cementoblastic cells (Tada et al. 2011). The enzyme tissue-nonspecific alkaline phosphatase (TNAP), which is encoded by the *ALPL* gene, is one of the most intensively studied

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phosphatase molecules in mineralized tissues (Hessle et al. 2002; Millan 2013). TNAP hydrolyzes pyrophosphate to phosphate in mineralized tissue cells including osteoblasts, odontoblasts and cementoblasts (Rodrigues et al. 2012). The catalytic mechanism involves the formation of a serine phosphate at the active site that reacts with water at alkaline pH to release inorganic phosphate from the enzyme (Holtz and Kantrowitz 1999). Mutations in the *ALPL* gene result in hypophosphatasia, characterized by irregular dentin calcification, an enlarged pulp chamber and dysplasia or aplasia of the cementum (Olsson et al. 1996; Liu et al. 2010; Foster et al. 2015). Thus, the proper induction of TNAP is important for mineralizing tissue formation in development and regeneration processes (Liu et al. 2014; Zweifler et al. 2015; Foster et al. 2008).

Testicular acid phosphatase (ACPT), which is highly homologous to prostatic and lysosomal acid phosphatase, belongs to the acid phosphatase family (Yousef et al. 2001). One unique aspect of acid phosphatases is its optimum catalytic activity in acid media (Romas et al. 1979). The expression of ACPT is regulated by steroid hormones; ACPT has been shown to be up-regulated by androgens and down-regulated by estrogens in the prostate cancer cell line LNCaP. Its expression is significantly lower in testicular cancer tissues than in normal testicular tissues. In addition, ACPT has been reported to act as a tyrosine phosphatase to modulate signals that are mediated by ErbB4, which are important for neuronal development and synaptic plasticity (Fleisig et al. 2004). However, the expression and biological activity of ACPT are largely unknown in mineralized tissues. Here, we investigate the localization and functional characteristics of ACPT in odontoblast differentiation and dentin formation in order to understand its roles in odontoblast differentiation and mineralization.

Materials and methods

Animals and tissue preparation

Mandibles were dissected from C57BL6J mice at postnatal day 1 and 7 (P7). The tissues were fixed in 4 % paraformaldehyde (PFA) and decalcified in 10 % EDTA solutions for 1 week at 4 °C. The decalcified tissues were dehydrated through a graded ethanol series, embedded in paraffin and sectioned at a thickness of 5 µm. All experimental procedures were approved by the Animal Welfare Committee of Chonbuk National University.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Kim et al. 2013). Briefly, sections were treated with 3 % hydrogen peroxide and incubated with rabbit polyclonal

antibodies against ACPT (1:200; Bioss, Woburn, Mich., USA), TNAP (1:50; Protein Tech, Chicago, Ill., USA), Dsp (1:200; Santa Cruz Biotechnology, Dallas, Tex., USA) and Ki-67 (1:200; Novus Biologicals, Littleton, Colo., USA). Histostain Plus rabbit primary (DAB) kit (Zymed Laboratories, San Francisco, Calif., USA) was used according to the manufacturer's instructions.

Cell cultures

MDPC-23 and OD11 cells, a dental papilla cell line and a mouse odontoblast cell line, respectively, were used for in vitro analyses (Hanks et al. 1998). MDPC-23 and OD11 cells were maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen, New York, N.Y., USA) with 10 % fetal bovine serum (FBS; Invitrogen) and 100 IU/ml penicillin-100 µg/ml streptomycin (Invitrogen). For primary dental pulp cells, the pulp of mandibular first molars from 5- to 7-day-old mice was isolated and digested in a solution of 3 mg/ml collagenase type I (Worthington Biochemical, Freehold, N.J., USA) and 4 mg/ml dispase (Boehringer, Mannheim, Germany) in the serum-free alpha modification of Eagle's medium (α -MEM; Invitrogen) for 1 h at 37 °C. Single cell suspensions were obtained by passing the cells through a 70-µm strainer (BD Labware, Franklin Lakes, N.J., USA) and cultured in a growth medium of α -MEM with 10 % FBS and 100 IU/ml penicillin-100 µg/ml streptomycin (Invitrogen) at 37 °C under 5 % CO₂. An osteoblast cell line, MC3T3-E1, was purchased from the American Type Culture Collection (Manassas, Va., USA) and was maintained in α -MEM with 10 % FBS and 100 IU/ml penicillin-100 µg/ml streptomycin at 37 °C under 5 % CO₂. To induce cell differentiation and mineralized nodule formation of MDPC-23 and OD11, 80–90 % confluent cells were cultured in osteogenic medium (OM) consisting of growth media supplemented with 50 µg/ml ascorbic acid (Sigma Aldrich, St. Louis, Mo., USA) and 10 mM β -glycerophosphates (Sigma Aldrich) for up to 6 days. The cells were treated with polyclonal rabbit anti-ACPT IgG or normal rabbit IgG (2 µg/ml) in the medium for the indicated duration.

DNA constructs and transfection

A full-length open reading frame of mouse *Acpt* (accession no. NM_001195034), cloned into the pCMV6 vector, was purchased from OriGene Technologies (Rockville, Md., USA). The clone was engineered to express the complete ACPT protein with a C-terminal FLAG. A green fluorescent protein (GFP) construct was also transfected for a control. Transfection experiments were performed with Lipofectamine LTX and PLUS reagent (Invitrogen) according to the manufacturer's instructions. After 24 h, transfected cells

were harvested for whole-cell lysate preparation or cultured with OM for further differentiation.

RNA interference

Cells were seeded onto a 24-well plate and grown to 60–70 % confluence. They were transiently transfected with 20 nM ACPT short interfering RNA (siRNA; Ambion, Carlsbad, Calif., USA) targeting exon 6 of *Acpt* and control siRNA (Ambion) for 6 h by using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. After 48 h, transfected cells were harvested for whole-cell lysate preparation or cultured with OM for mineralization.

Proliferation assay

Proliferation rates of MDPC-23 were measured by using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. In brief, cells were cultured in 24-multiwell plates and transfected with siRNA and *Acpt* constructs for 6 h; they were then treated with the kit solution at 10 μ l/well after a 48-h incubation. Absorbance was measured spectrophotometrically at 450 nm.

Activity of alkaline phosphatase and acid phosphatase

Quantitative alkaline phosphatase activity was determined by an assay based on the hydrolysis of p-nitrophenylphosphate (p-NPP) to p-nitrophenol (p-NP). Cell layers were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 50 mM TRIS–HCl buffer (pH 7.0) containing 1 % (v/v) Triton X-100 (Sigma Aldrich) and 1 mM phenylmethane sulfonyl-fluoride (Sigma Aldrich). Whole-cell lysates were assayed by adding 1 mg/ml p-NPP as a substrate in 0.1 M glycine buffer (pH 10.4) containing 1 mM ZnCl₂ (Sigma Aldrich) and 1 mM MgCl₂ (Sigma Aldrich) to each tube for 15 min at 37 °C. Reactions were stopped by adding NaOH (final concentration of 0.6 N) and absorbance was read spectrophotometrically at 405 nm. Enzyme activity was expressed as OD 405/min per milligram of protein. Quantitative acid phosphatase activity was determined by means of the Acid Phosphatase Activity Colorimetric Assay Kit (BioVision, Milpitas, Calif., USA) according to the manufacturer's instructions. The concentration of protein in each cell lysate was measured by using a DC Protein Assay (Bio-Rad Laboratories, Hercules, Calif., USA).

Mineralization induction and alizarin red S staining

To induce cell differentiation and mineralized nodule formation of MC3T3-E1 and dental pulp cells, 95 % confluent cells were cultured in OM consisting of α -MEM with 10 % FBS, 50 μ g/ml ascorbic acid, 10 mM β -glycerophosphates and 10 nM dexamethasone (Sigma Aldrich) for up to 3 weeks. Mineral nodule

formation was observed by staining the cells with 40 mM alizarin red S (pH 4.2) after fixation with 4 % PFA for 10 min. The amount of alizarin red S that bound to the minerals was quantified by destaining the samples in 10 mM sodium phosphate containing 10 % cetylpyridinium chloride (pH 7.0) for 15 min at room temperature. The amount of alizarin red S in the destaining solution was measured at OD 562 nm.

Western blot analysis

Proteins (30 μ g) were dissolved in sample buffer and electrophoresis was carried out at a current of 25 mA for 2 h. The protein bands were transferred from the gels onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Membranes were blocked for 1 h with 5 % nonfat dry milk in PBS containing 0.1 % Tween-20 (PBS-T) and incubated overnight with anti-rabbit ACPT (1:500; Bioss), active β -catenin (1:1000, Cell signaling, Beverly, Mass., USA), Dsp (1:200, Santa Cruz Biotechnology), Phex (phosphate regulating endopeptidase homolog, X-linked, 1:500; Bioss), cyclin D1 (1:500, Abcam), β -actin (1:10000; Santa Cruz Biotechnology), Osx (1:500; Abcam, Cambridge, Mass., USA) and anti-mouse FLAG IgG diluted (1:2000; Sigma Aldrich) in PBS-T buffer at 4 °C. After being washed, the membranes were incubated with anti-rabbit or mouse-IgG (1:5000 dilution) conjugated to horseradish peroxidase (Santa Cruz Biotechnology) for 1 h. Labeled protein bands were detected by using an enhanced chemiluminescence (ECL) system (Amersham Biosciences, Buckinghamshire, UK). Protein expression levels were analyzed with the ImageQuant TL 1D gel analysis program (Amersham Biosciences).

Statistical analysis

Data are presented as means \pm standard errors from three or more separate experiments, as indicated. Normal data with equal variance were analyzed by using Student's *t*-test (for single comparison) or one-way analysis of variance with Tukey's procedure (for multiple comparisons). Significance was assigned at $P \leq 0.05$ as indicated.

Results

Temporo-spatial localization of ACPT during tooth development

In the mandibular incisor of neonatal mice, ACPT was restricted to early differentiating odontoblasts (Fig. 1a); it was not expressed in mature secretory odontoblasts or in proliferating cells in the dental mesenchyme near the cervical loop. Interestingly, TNAP, the other phosphatase, was localized to late differentiating odontoblasts and mature secretory odontoblasts but was not expressed in either proliferating cells in the dental

mesenchyme or in early differentiating odontoblasts in which ACPT exhibited limited localization (Fig. 1b). For comparison, we performed immunohistochemistry with the cell proliferation

marker, Ki67 (van Oijen et al. 1998) and the odontoblast differentiation marker, Dsp (Suzuki et al. 2009). Ki67-positive cells were mainly found in the inner enamel epithelium of the cervical

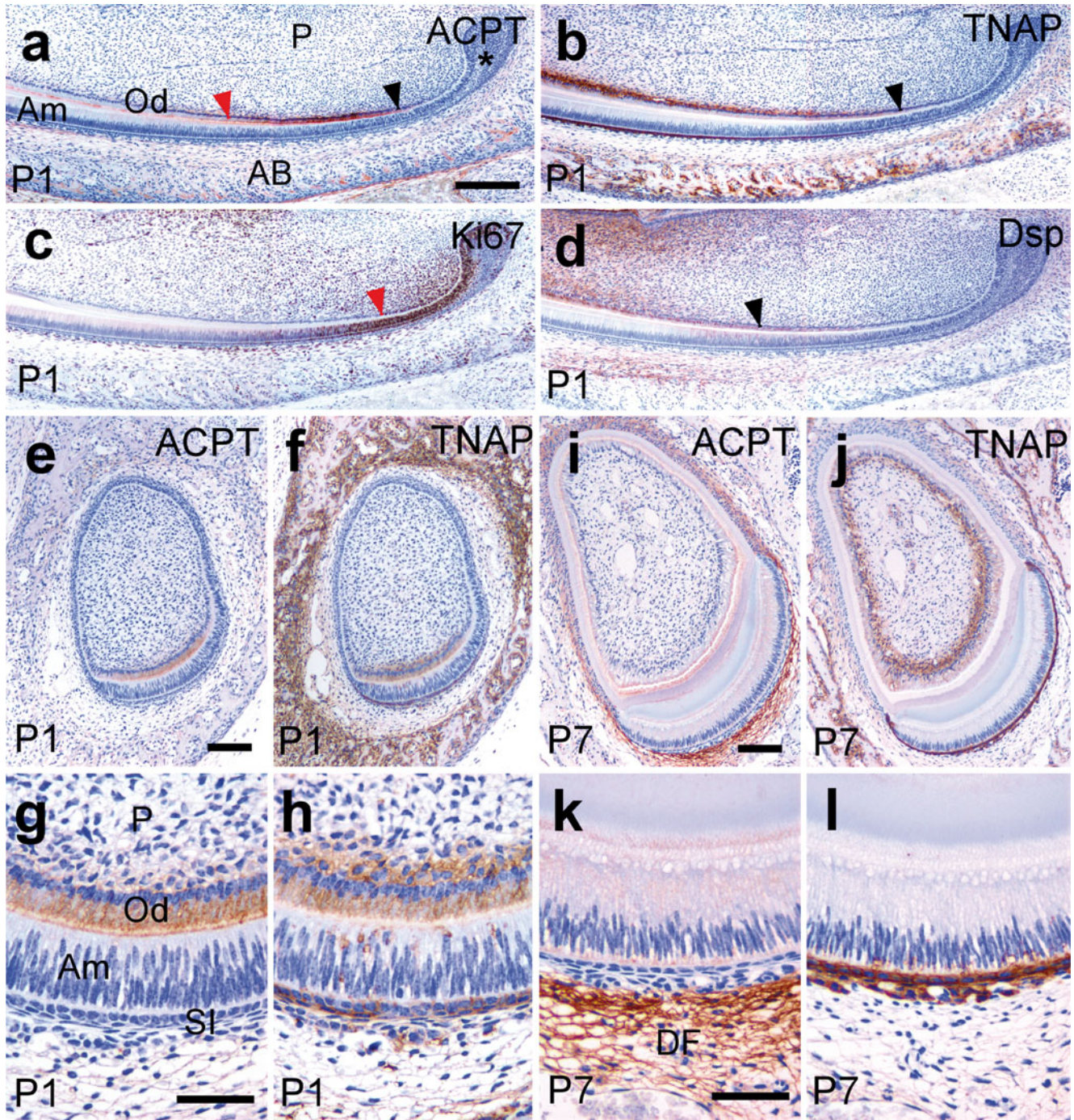


Fig. 1 Temporo-spatial localization of testicular acid phosphatase (ACPT) in developing mouse incisors. **a, b** ACPT is locally restricted in the early stage of differentiating odontoblasts but not in mature secreting odontoblasts or in proliferating cells in the dental mesenchyme. In contrast, tissue-nonspecific alkaline phosphatase (TNAP) is localized in the late stage of differentiating odontoblasts and matured secreting odontoblasts. **c, d** Ki67 is detected in the proliferating cells in the dental mesenchyme near the cervical loop, whereas Dsp localizes in the mature secreting odontoblasts. **e–h** In frontal sections,

localization of ACPT overlaps with that of TNAP in differentiating odontoblasts of neonatal mice. **i–l** At P7, ACPT is not localized in the odontoblasts but appears in the dental follicle. In contrast, TNAP is localized in the odontoblasts and in the stratum intermedium (black arrowheads initiation point of expression, AB alveolar bone, Od differentiated odontoblasts, Am ameloblasts, SI stratum intermedium, P pulp, DF dental follicle, asterisk cervical loop). Bars 200 μm (**a–d**), 50 μm (**e–l**)

loop. Cells in the dental mesenchyme near the cervical loop were also positive for Ki67 (Fig. 1c). However, Dsp occurred in mature secretory odontoblasts and late differentiating odontoblasts (Fig. 1d). In the frontal section of the mandible of neonatal mice, ACPT was specifically localized only in the odontoblasts (Fig. 1e, g). In contrast, TNAP was localized in the cells of the stratum intermedium and subodontoblast layers and in the odontoblasts (Fig. 1f, h). Interestingly, ACPT was weakly expressed in alveolar bone, whereas TNAP was strongly localized in the alveolar bone around the mandibular incisor. In P7 mouse mandibular incisors, no ACPT expression was found in the odontoblasts (Fig. 1i) but TNAP was still strongly expressed (Fig. 1j). In addition, ACPT was expressed in the cells of the dental follicle adjacent to the cells of the stratum intermedium (Fig. 1k), where TNAP was also strongly expressed (Fig. 1l). The temporo-spatial localization of ACPT strongly suggests that ACPT is involved in early odontoblast differentiation during tooth development.

Expression of ACPT protein in vitro

To determine the functional role of ACPT in the physiology of odontoblast differentiation, we employed the MDPC-23 cell line derived from mouse dental papilla. MDPC-23 expresses both ACPT and odontoblast differentiation markers including Dsp and Phex (Ruchon et al. 2000; Suzuki et al. 2009). The level of

ACPT protein gradually increased during differentiation (over 10-fold at day 4 of differentiation) but disappeared at the later stages of differentiation, which was demonstrated by the strong expression of Dsp and Phex at day 6 (Fig. 2a, b). However, active β -catenin, which is not phosphorylated by glycogen synthase kinase 3 β , gradually decreased over the differentiation period. The level of ACPT protein was further compared in mouse primary pulp cells, odontoblastic OD11 cells and osteoblastic MC3T3-E1 cells. ACPT expression increased in all of those cells with OM treatment (Fig. 2c). Unexpectedly, the expression of ACPT protein was largely increased by OM exposure in primary cultured mouse pulp cells and MC3T3-E1, as a lower expression of ACPT was demonstrated in dental pulp and alveolar bone by immunohistochemistry (Fig. 1).

ACPT inhibits odontoblast proliferation

To investigate whether altered ACPT protein expression contributes to the physiology of odontoblasts, we investigated alterations in the gene expression of ACPT in MDPC-23 cells by using RNA interference and overexpression of *Acpt* via gene transfection technologies. The alteration of ACPT expression was confirmed by Western blot analysis (Fig. 3a) and was shown to affect gross acid phosphatase activity in MDPC-23 cells (Fig. 3b). We examined the proliferation rate

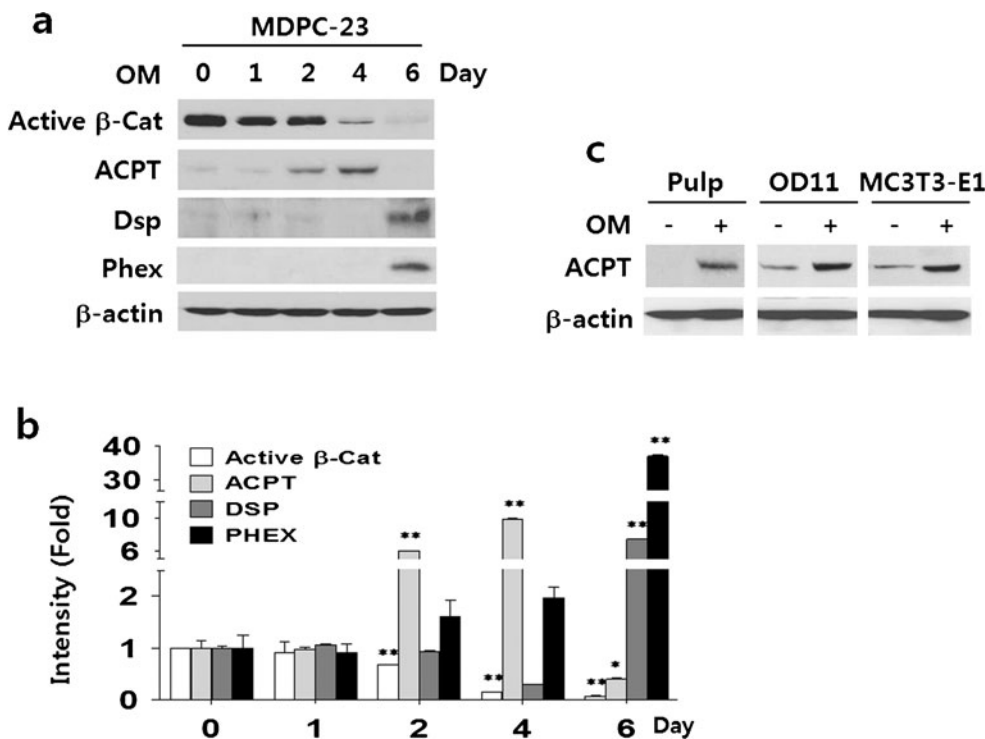


Fig. 2 Expression of ACPT protein in vitro. **a** MDPC-23 cells were treated with osteogenic medium (OM) for the indicated time to induce odontoblastic differentiation. Protein levels of MDPC-23 treated with OM were determined by Western blot analysis with specific antibodies as indicated (β -Cat β -catenin). **b** Data from Western blot analysis were quantified by densitometry after normalizing bands to β -actin levels and

are presented as fold-change (means \pm standard errors; $n \geq 3$). *P*-values were analyzed by one-way analysis of variance with Tukey’s procedure for multiple comparisons. *******P*<0.001, ******P*<0.05 vs. Day 0. **c** Protein levels in mouse primary pulp cells, OD11 and MC3T3-E1 treated with OM for 7, 4 and 7 days, respectively, were determined by Western blot analysis with specific antibodies as indicated

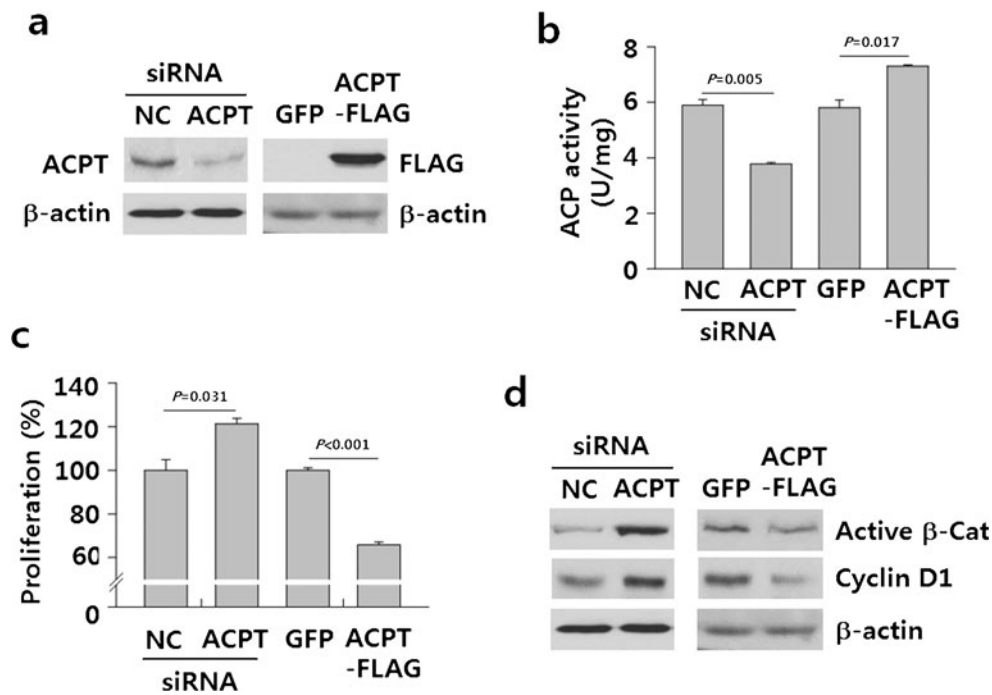


Fig. 3 ACPT inhibits odontoblast proliferation. **a** MDPC-23 cells were transfected with short interfering RNA (*siRNA*) as a negative control (*NC*), with ACPT and a control green fluorescent protein (*GFP*) vector, or with the vector driving expression of ACPT fused with FLAG (*ACPT-FLAG*) and protein expression was confirmed by Western blot analysis. **b** MDPC-23 cells were transfected with *siRNA* and vectors for *GFP* or ACPT for 6 h and after 36 h, acid phosphatase activity in MDPC-23 cells was analyzed by using an Acid Phosphatase Activity Colorimetric

Assay Kit. **c** MDPC-23 cells were transfected under the same conditions as in **b** and cell proliferation was analyzed by using Cell Counting Kit-8. Data are presented as percentages (means±standard error; $n \geq 3$). *P*-values were analyzed by Student's *t*-test and are indicated in **b**, **c**. **d** MDPC-23 cells were transfected under the same conditions as in **b** and protein levels were determined by Western blot analysis with specific antibodies as indicated

of MDPC-23 cells with altered ACPT expression. Interestingly, the reduction of ACPT protein by RNA interference significantly increased the proliferation of MDPC-23 cells ($P=0.031$), whereas ACPT overexpression significantly decreased it ($P<0.001$; Fig. 3c). To examine whether molecular change in proliferation-associated proteins followed the same pattern as ACPT expression, Western blot analysis was performed for active β -catenin and cyclin D1 (Fig. 3d). Active β -catenin was significantly increased upon ACPT *siRNA* transfection and decreased with ACPT overexpression. Similar results were obtained with cyclin D1, a marker of cell proliferation (Datta et al. 2007) and a known β -catenin downstream target. Collectively, the levels of active β -catenin and cyclin D1 demonstrated a negative correlation with ACPT expression.

ACPT induces odontoblast differentiation

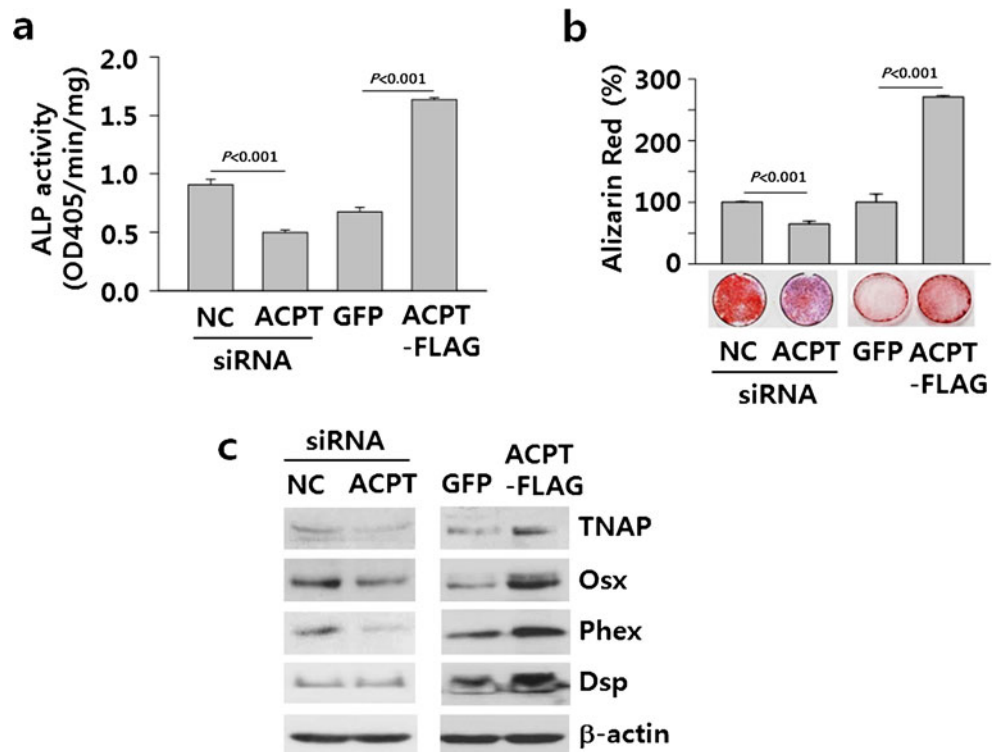
Based on the negative correlation of ACPT expression with cell growth in MDPC-23 cells, we hypothesized that ACPT might induce odontoblast differentiation for later matrix secretion and further mineralization. To investigate our hypothesis, we analyzed the activity of alkaline phosphatase (ALP), a well-known marker for osteogenic activity (Harrison et al.

1995) in response to knockdown or overexpression of ACPT in MDPC-23 cells. Interestingly, ACPT *siRNA* significantly decreased ALP activity, whereas ACPT overexpression increased it ($p<0.001$) (Fig. 4a), demonstrating a clear correlation between ACPT expression and ALP activity in MDPC-23 cells. We determined whether this ALP-inducing ability of ACPT affected later mineralization of MDPC-23 cells. A significant correlation of ACPT expression with mineralization was also shown by alizarin red staining of OM-treated MDPC-23 cells with altered ACPT expression (Fig. 4b). Furthermore, we examined matrix proteins and differentiation markers of odontoblasts at the molecular level by Western blot analysis. The protein levels of osterix (*Osx*), a differentiation marker of odontoblasts and of TNAP were decreased by ACPT *siRNA* but were increased by ACPT overexpression in OM-treated MDPC-23 cells. The expression of *Dsp* and *Phex* were also changed in the same manner after OM treatment (Fig. 4c).

Extracellular catalytic activity of ACPT is important in odontoblast differentiation

Based on the location of ACPT at the cellular membrane within the extracellular catalytic domain, we treated MDPC-23

Fig. 4 ACPT induces odontoblast differentiation. **a** MDPC-23 cells were transfected with siRNA and vectors for GFP or ACPT for 6 h and after 36 h; alkaline phosphatase (ALP) activities were analyzed as described. **b** MDPC-23 cells were transfected under the same conditions as in **a**, further differentiated with OM for 4 days and stained with alizarin red S. The quantitative data from alizarin red S staining are presented as percentages (means \pm standard error; $n \geq 3$). *P*-values were analyzed by Student's *t*-test and are indicated. **c** Protein levels of MDPC-23 cells transfected and differentiated by using the same conditions as in **b** were determined by Western blot analysis with specific antibodies as indicated (*Osx* osterix)



cells with polyclonal anti-ACPT IgG to neutralize the extracellular domain, thus blocking ACPT catalytic activity. ACPT IgG treatment did not have any effect on proliferation (Fig. 5a), suggesting that the extracellular catalytic domain is not involved in cellular proliferation. To examine whether the extracellular catalytic domain of ACPT is involved in ALP activity, MDPC-23 cells were cultured for 4 days in OM containing ACPT IgG. Polyclonal anti-ACPT IgG treatment significantly decreased the ALP activity of MDPC-23 cells (Fig. 5b), which is in contrast to the effect of ACPT on proliferation. These results indicate that the extracellular domain of ACPT is involved in odontoblast differentiation but not in odontoblast proliferation. In addition, the activation of ALP by ACPT overexpression was completely blocked by ACPT IgG treatment (Fig. 5c), which strongly suggests that the extracellular catalytic activity of ACPT might be responsible for odontoblast differentiation through ALP activation.

Discussion

The molecular and cellular events required for odontoblasts to produce mineralized dentin are only partially known, whereas the temporal and spatial coordination of events are even less well understood. In this study, we investigated the functional significance of ACPT in odontoblast differentiation during tooth development. Our investigation was specifically focused on the significance of ACPT expression during odontoblast differentiation as a possible mechanism that may

temporally coordinate cellular and molecular events switching cell proliferation to odontoblastic differentiation for dentin formation.

Reciprocal- and functionally-coupled relationships occur between the decline in proliferative activity and the subsequent induction of genes associated with matrix secretion and maturation during mineralized tissue development (Stein et al. 1990). Our immunohistochemical data in mouse mandibular incisors revealed lower ACPT expression in mature secretory odontoblasts and in proliferating cells in the dental mesenchyme near the cervical loop but this is locally restricted in early differentiating odontoblasts. The reciprocal relationship between proliferation and differentiation is supported by a temporal sequence of events in which enhanced expression of alkaline phosphatase occurs immediately after the proliferative period, as indicated by Ki67 staining (van Oijen et al. 1998) and later as increased expression of Dsp at the onset of dentin matrix secretion and maturation. Based on these findings, we believe that ACPT expression follows the early expression of Ki67 and the later expression of TNAP in the odontoblasts of the developing mouse incisor; the temporo-spatial expression of ACPT seems to be locally restricted in the transition from proliferation to differentiation of odontoblasts during tooth development. From these results, we hypothesized that ACPT is involved in the transition from proliferation to differentiation for further matrix secretion and mineralization. In our in vitro experiments, MDPC-23 cells follow similar paths of differentiation and matrix secretion to the odontoblast differentiation model by exhibiting

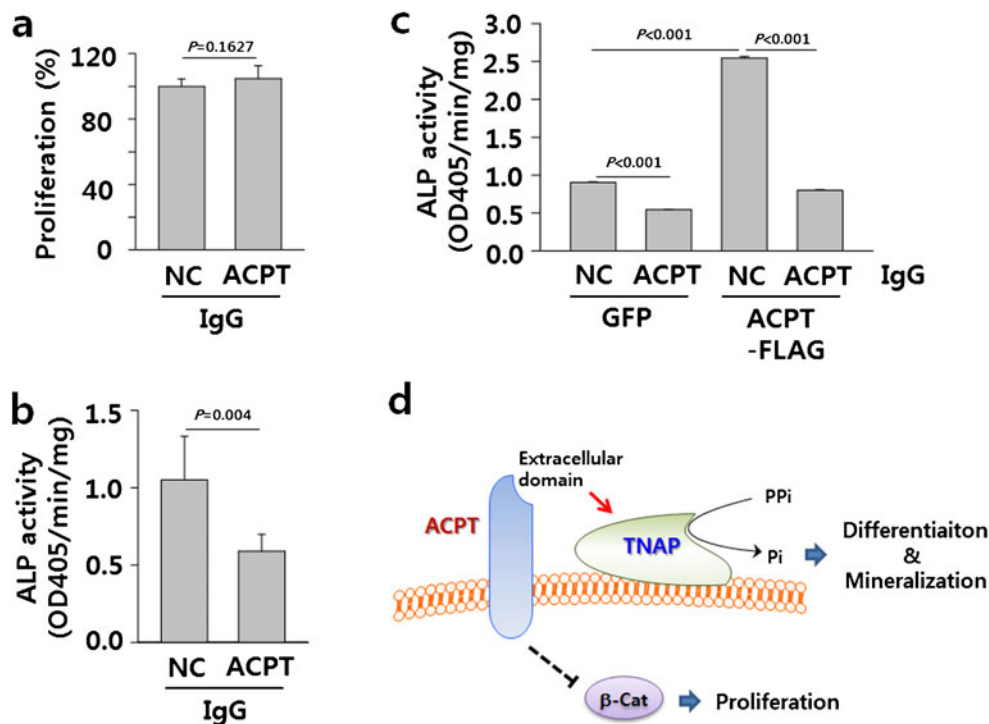


Fig. 5 Extracellular catalytic domain of ACPT is important for odontoblast differentiation. **a** MDPC-23 cells were treated with polyclonal anti-ACPT IgG or normal IgG (2 μ g/ml) in medium for 24 h and cell proliferation was analyzed by using Cell Counting Kit-8. **b** MDPC-23 cells were treated with polyclonal anti-ACPT IgG or normal IgG (2 μ g/ml) with OM for 4 days and ALP activities were analyzed as described. **c** MDPC-23 cells were transfected with vectors for GFP or ACPT for 6 h and treated with polyclonal anti-ACPT IgG or normal IgG

(2 μ g/ml) in the medium for 24 h and ALP activities were analyzed as described. *P*-values were analyzed by Student's *t*-test and are indicated. **d** ACPT induces odontoblast differentiation by inhibition of cell proliferation and ALP activation for further matrix secretion and dentin formation. Cell proliferation is inhibited by ACPT through inhibition of β -catenin signaling; the extracellular catalytic activity of ACPT is important for ALP activation in the odontoblastic differentiation process

a similar pattern and time frame of protein expression. Another odontoblastic cell line, OD11 and primary dental pulp cells and osteoblastic MC3T3-E1 cells also express ACPT upon differentiation by OM, implying that ACPT plays a common role in both osteogenesis and odontogenesis. In vitro cell-based assays involving the alteration of ACPT expression by RNA interference-mediated knockdown and overexpression of ACPT in MDPC-23 cells strongly suggest that ACPT acts as a regulator that promotes the switch from proliferation to differentiation in odontogenesis. The consequent alteration of active β -catenin and cyclin D1, one of the Wnt/ β -catenin-responsive genes required for cell cycle progression (Datta et al. 2007), by ACPT expression also supports our hypothesis with regard to the functional role of ACPT as a negative regulator of cell proliferation. Furthermore, we also specifically linked the induction of ACPT to the inhibition of cell proliferation and the enzymatic activity of alkaline phosphatase, which results in the elevation of matrix secretion and further mineralization in MDPC-23 cells. Currently, no reliable method is available for analyzing ACPT-specific activity. Thus, our in vitro system has not elucidated whether phosphate elevated by ACPT has a direct role in the inhibition of cell growth and ALP

activation. The complete blockage of induction of ALP activity together with the masking of the catalytic domain by treatment with ACPT-specific IgG in osteogenic media, implies that the phosphate generated by ACPT is involved in the differentiation process. However, proliferating MDPC-23 cells treated with ACPT-specific IgG do not show a significant change in cell proliferation, implying no involvement of the phosphate generated by ACPT or an alternative inhibitory mechanism for cell proliferation. Taken together, our results suggest that elevated phosphate from ACPT catalytic activity might have a role in ALP activation; meanwhile, proliferation is inhibited by ACPT, regardless of its activity through the inhibition of β -catenin signaling and the generation of inorganic phosphate by ACPT might be more important for the differentiation process than actual hydroxyapatite formation, which occurs later (Fig. 5d).

Interestingly, the gap junction protein, pannexin 3 (Panx3), has been reported to have similar roles to ACPT in the induction of osteoblast differentiation. Panx3 is involved in the transition from proliferation to differentiation of osteoprogenitors by regulating multiple signaling pathways through its hemichannel, endoplasmic reticulum channel and gap junctional activities (Ishikawa et al. 2011, 2014).

Transient expression of ACPT might be associated with such a cellular process. For a full understanding of the mechanism by which ACPT might regulate cell proliferation and odontoblastic differentiation, further investigation is needed. A more complete comprehension of ACPT regulation of functionality leading to hard tissue development might be defined in a knockout animal model.

In conclusion, ACPT inhibits cell proliferation and elicits odontoblast differentiation and mineralization during tooth development. These findings contribute to the further understanding of the molecular mechanisms underlying odontoblast differentiation and dentin formation, with the aim of improving dentin regeneration.

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