High-temperature Requirement Protein A1 Regulates Odontoblastic Differentiation of Dental Pulp Cells via the Transforming Growth Factor Beta 1/Smad Signaling Pathway

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Abstract

Introduction: Dentinogenesis includes odontoblast differentiation and extracellular matrix maturation as well as dentin mineralization. It is regulated by numerous molecules. *High-temperature requirement protein A1* (*HtrA1*) plays crucial roles in bone mineralization and development and is closely associated with the *transforming growth factor beta* (*TGF-β*) signal in osteogenesis differentiation. Simultaneously, the *TGF-β1/small mother against decapentaplegic* (*Smad*) signaling pathway is an important signaling pathway in various physiological processes and as a downstream regulation factor of *HtrA1*. However, the role of *HtrA1* and its relationship with the *TGF-β1/Smad* signaling pathway in dentin mineralization is unknown. **Methods:** We detected the role of HtrA1 and its relationship with the TGF-β1/Smad signaling pathway in odontoblastic differentiation of human dental pulp cells (hDPCs) in this study. First, hDPCs were cultured in mineralized medium, and odontoblastic differentiation was confirmed by investigating mineralized nodule formation, *alkaline phosphatase* (*ALP*) activity, and the expression of mineral-associated genes, including *ALP*, *collagen I*, and *dentin sialophosphoprotein*. Then, the expression of HtrA1 and *TGF-β1/Smad* in hDPCs was investigated in hDPCs during mineralized induction. After *HtrA1* knockdown by lentivirus, the mineralized nodule formation, *ALP* activity, and expression of mineral-associated genes and *TGF-β1/Smad* genes were investigated to confirm the effect of *HtrA1* on odontoblastic differentiation and its relationship with the *TGF-β1/Smad* signaling pathway. **Results:** The expression of HtrA1 and TGF-β1 was increased during odontoblastic differentiation of hDPCs along with the messenger RNA expression of downstream factors of the *TGF-β1/Smad* signaling pathway. In addition, lentivirus-mediated *HtrA1* knockdown inhibited the process of mineralization and the expression of *HtrA1* and TGF-β1/Smad genes. **Conclusions:** These findings suggest that HtrA1 might positively regulate odontoblastic differentiation of hDPCs through activation of the TGF-β1/Smad signaling pathway. *(J Endod 2018;■:1–8)*

Key Words

High-temperature requirement protein A1, human dental pulp cells, odontoblastic differentiation, *transforming growth factor beta 1/Smad* signaling pathway

Significance

Our study indicated the role of HtrA1 in odontoblastic differentiation of hDPCs and its potential associated signaling pathway, explored the specific mechanism of reparative dentin formation, and provided new visual on protection of pulp in clinical.

Dentinogenesis occurs to protect living dental pulp when it endures the stimulation of caries, abrasion, and traumatism. The precursor cells in dental pulp differentiated into odontoblasts play an important role in reparative dentin formation, which is important to protect vital pulp from early dental pulp disease. This type of dentinogenesis begins with odontoblast differentiation from human dental pulp cells (hDPCs) and then extracellular matrix maturation and dentin mineralization. Dentinogenesis is strictly regulated by numerous different transcription factors, growth factors, and signaling pathways (1, 2).

High-temperature requirement protein A1 (*HtrA1*) is a member of the *HtrA* protein family, which is involved in various physiological processes by its interaction with various extracellular and intracellular matrices (3) and plays an important role in the pathological mineralization process as well as the formation of reparative dentin (2). *HtrA1* could negatively regulate osteoblast differentiation partly by suppressing *bone morphogenetic protein 2*–induced activation of small mother against decapentaplegic (Smad) 1/5/8, extracellular regulated protein kinases 1/2, and p38 mitogen-activated protein kinase (4). In addition, the loss of *HtrA1 in vivo* is found to increase bone formation (5). Interestingly, it has been recently reported that *HtrA1* positively regulated osteogenesis of human bone marrow–derived mesenchymal stem cells and mineralization of differentiating bone-forming cells (6), and *HtrA1* is newly identified as a positive regulator of mouse adipose-derived stromal cell osteogenesis (7). These findings suggest that *HtrA1* is a significant mineralization regulator.

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The *transforming growth factor beta* (*TGF-β*)/Smad signaling pathway is involved in various physiological processes, such as fibrosis, differentiation, and mineralization (8–10). It consists of *TGF-β*, bone morphogenetic proteins, growth/differentiation factors, and activins in mammals (11, 12). The signaling transduction of the *TGF-β1/Smad* signaling pathway begins with *TGF-β1* ligand binding to *type II receptor* (*TβR II*), leading to constitutively active *TβR II* and *phosphorylate type I receptor* (*TβR I*) (13). *Smad2* and *Smad3* are phosphorylated by the activated *TβR I*, and then a trimeric complex is formed by combining with *Smad4*, which translocates into the nucleus and subsequently regulates downstream gene expression (14–16). *Smad6* and *Smad7* are intracellular inhibitors of the *TGF-β/Smad* signaling pathway; they are induced by members of the *TGF-β1* family and provide a negative feedback loop (17, 18). It has been shown that *HtrA1* could bind to *TGF-β1 in vitro* (11) and could regulate several physiological and pathological processes by *TGF-β1*, such as neuronal maturation, human cerebral small vessel disease, and osteogenic differentiation of periodontal ligament cells (19–21).

Dentin is a living connective tissue with biomechanical properties similar to bone; the essential inorganic substance is hydroxyapatite, and a series of epithelial-mesenchymal interactions regulate its differentiation as bone tissue (22). In addition, many factors play the same role in regulating mineralization of dentin and bone tissue (23, 24). Because *HtrA1* is involved in the mineralization of bone tissue, it may also regulate dentin mineralization. However, whether *HtrA1* participates in odontoblastic differentiation of hDPCs is unclear. The relationship between *HtrA1* and the *TGF-β1/Smad* signaling pathway during that process has not been defined yet.

Given these previous findings, we assumed *HtrA1* plays an important role in odontoblastic differentiation of hDPCs and might regulate this process via the *TGF-β/Smad* signaling pathway. The mineralized nodules, *alkaline phosphatase* (*ALP*) activities, and messenger RNA (mRNA) expression of *ALP*, *collagen I* (*COLI*), and *dentin sialophosphoprotein* (*DSPP*) were investigated to confirm odontoblastic differentiation of hDPCs. The expression of *HtrA1*, *TGF-β1*, *TβRI*, *TβRII*, *Smad2*, *Smad4*, *Smad6*, and *Smad7* were investigated during odontoblastic differentiation of hDPCs. In addition, we detected the effect of *HtrA1* knockdown on odontoblastic differentiation of hDPCs and the expression of *TGF-β/Smad* signals.

Materials and Methods

Ethics Statement

The entire study was performed with the approval of the Institutional Review Board of the College and Hospital of Stomatology, Guangxi Medical University, Guangxi, China. Written informed consent from the donors was obtained for the use of dental pulp tissue.

Cell Culture and Mineralized Induction

hDPCs were obtained from healthy premolars of healthy patients (12–20 years old); extraction occurred for orthodontic reasons. The extracted teeth were split immediately. The pulp tissues were harvested and cut into small pieces. Afterward, tissues and cells were maintained in normal growth medium consisting of Dulbecco-modified Eagle medium–high glucose (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Sigma-Aldrich, St Louis, MO) at 37°C under a humidified atmosphere containing 5% CO_2. Three to 6 passages were used in subsequent experiments.

To characterize the cell lineage of hDPCs, morphologic analysis by phase-contrast microscopy (Olympus, Tokyo, Japan) and immunocytochemical staining for vimentin and keratin were performed. The cells were seeded on a well plate, fixed with 4% paraformaldehyde, blocked with goat serum, and incubated with primary antibodies to vimentin and keratin (1:200; Boster, Wuhan, China) overnight at 4°C. Following the instructions of the SP kit (Zhongshan, Beijing, China), the immunoreaction was performed. Finally, diaminobenzidine tetrahydrochloride with hematoxylin counterstain was used to visualize the immunoreactivity. The negative control (NC) was treated with phosphate-buffered saline (PBS) instead of primary antibodies.

The hDPCs were seeded on a well plate; hDPCs were maintained in mineralized medium when cells reached 90% confluence. The mineralized medium was supplemented with 10 mmol/L beta glycerophosphate, 10^{-8} mol/L dexamethasone, and 50 mg/mL ascorbic acid (Sigma-Aldrich). Alizarin red staining and ALP activity assay were performed on days 0, 7, 14, 21, and 28, and the total RNA, protein, and supernatant were harvested from cells for subsequent experiments simultaneously.

RNA Interference

Oligonucleotides that encode short hairpin RNAs (shRNAs) directed against human *HtrA1* mRNA were constructed in a lentiviral plasmid. The plasmid contained an shRNA against HtrA1 or a nontargeting control. The separate small interfering RNA was used to produce small interfering RNA–expressing cell lines, denoted by *shHtrA1*. The oligonucleotides were identified by the library selection of accessible sites. The sequences were *shHtrA1* (5′-3′): CGGCCGAAGTTGCCTCTTT and NC (5′-3′): TTCTCCGAACGTGTCACGTTTC. Then, the lentivirus was constructed by GenePharma (Shanghai, China) (21). hDPCs were transfected with predesigned shRNA lentivirus against either *HtrA1* or a nonspecific NC. A preliminary experiment was performed to ensure the multiplication of infection and infection time according to the suggested protocol. In addition, resistant clones were selected by using puromycin (1.5 μg/mL). The cells were observed under fluorescence microscopy to evaluate transfection efficiency. Finally, *HtrA1* gene knockdown efficiency was assessed by real-time polymerase chain reaction (RT-PCR) and Western blot analysis.

Alizarin Red Staining

The amount of mineralized nodules was detected by alizarin red staining on days 7, 14, 21, and 28 during mineralized induction according to Matsuda et al (25). The supernatant was removed, and hDPCs were fixed in 4% paraformaldehyde for 20 minutes at room temperature and washed twice with PBS. Then, the hDPCs were stained with 0.1% alkaline alizarin red (Sigma-Aldrich) for 30 minutes at 37°C. Finally, hDPCs were washed 3 times with PBS and observed under an inverted phase contrast microscope.

ALP Activity Assay

During mineralized induction, hDPCs were collected to measure *ALP* activity on days 0, 7, 14, 21, and 28. First, hDPCs were lysed by radioimmunoprecipitation assay lysate (Beyotime, Jiangsu, China) collected into a 1.5-mL Eppendorf microcentrifuge tube for centrifugation at 12,000*g* at 4°C for 10 minutes. Next, a portion of the supernatant and *ALP* substrate solution, which included 50 μL buffer solution and 50 μL matrix solution (Nanjing-Jiancheng, Jiangsu, China), were incubated at 37°C for 15 minutes. After adding the developer, the absorbance of each sample was measured at 520 nm. Finally, the total protein was quantified using the BCA Protein Assay Kit (Beyotime), and *ALP* levels were normalized to the total protein content of cells. *ALP* activity was calculated as unit per gram of protein.

RNA Isolation and RT-PCR Analysis

The total RNA of the hDPCs was isolated on days 0, 7, 14, 21, and 28 using RNAiso Plus (Takara, Tokyo, Japan) according to the