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ORIGINAL PAPER

HtrA1 may regulate the osteogenic differentiation of human periodontal ligament cells by TGF-β1

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Abstract Periodontal ligament cells (PDLCs) in periodontal ligament (PDL) can differentiate into osteoblasts, while physiologically PDL remains non-mineralized space although located two hard tissues. But the exact mechanism of which is still unclear. High-temperature requirement protein A1 (HtrA1) is a key mineralization regulator and could inhibit the osteogenesis by transforming growth factor-β (TGF-β) signaling. However, the role of HtrA1 in PDLCs osteogenic differentiation has yet to be clarified. We assume HtrA1 may play an important role in maintaining the balance of PDL mineralization, and may regulate human periodontal ligament cells (hPDLCs) osteogenic differentiation by TGF-β1. Firstly we confirmed the mRNA expression of HtrA1 and TGF-β1 in hPDLCs by RT-PCR, then QDs-based immunofluorescence demonstrated the co-localization of them in the cytoplasm, and co-immunoprecipitation further confirmed the interaction between them. Lentivirus-mediated HtrA1 overexpression enhanced the osteogenic differentiation of hPDLCs, as well as up-regulation of TGF-β1. In contrast, knockdown of HtrA1 suppressed the osteogenic differentiation with down-regulation of TGF-β1. These findings suggested that HtrA1 plays a positive role in hPDLCs osteogenic differentiation and may regulate this process by TGF-β1.

Keywords Human periodontal ligament cells (hPDLCs) · High-temperature requirement protein A1 (HtrA1) · TGF-β1 · Osteogenic differentiation · Lentivirus

Introduction

The periodontal ligament (PDL) is a thin connective tissue located between the alveolar bone and cementum, in which PDLCs can differentiate into osteoblasts and cementoblasts and play a crucial role in the maintenance and regeneration of periodontal tissue (Seo et al. 2004; Sonoyama et al. 2006; Zhou et al. 2011). Interestingly, in physiological conditions, PDL maintains the non-mineralized space although it is located these two hard tissues. Although some factors have been found to be involved in periodontal tissue homeostasis, the exact mechanism of PDL free of mineralization is still unclear (Sun et al. 2014).

HtrA1, a member of the human HtrA serine protease family, is involved in multiply biological and pathological processes, such as growth, apoptosis, Alzheimer's disease (AD), age-related macular degeneration, osteoarthritis, and tumor development (Chien et al. 2004; Dewan et al. 2006; Grau et al. 2005). More importantly, HtrA1 is a key regulator of matrix mineralization. It can prevent mineral deposition by inhibiting TGF-β signaling depending on its proteolytic activity (Oka et al. 2004; Hadfield et al. 2007), and can also negatively regulate osteoblast differentiation partly by suppressing BMP2-induced activation of Smad1/5/8, ERK1/2 and p38 (Wu et al. 2014). Besides, loss of

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HtrA1 in vivo was found to increase the bone formation (Graham et al. 2013). Therefore, these findings suggested HtrA1 is a significant mineralization inhibitor. But to date, the effect of HtrA1 on PDL free of mineralization has not been reported.

TGF-β superfamily is an important signaling pathway, which controls cell growth, differentiation, bone-remodeling and regeneration (Blobe et al. 2000; Kajdaniuk et al. 2013). It consists of TGF-βs, bone morphogenic proteins (BMPs), growth/differentiation factors (Gdfs) and activins in mammals (Oka et al. 2004; Massague and Chen 2000). TGF-β1 is a member of the TGF-β family and is very important for periodontal reorganization and regeneration (Wang et al. 2014; Maeda et al. 2013; Markopoulou et al. 2011). Unfortunately, the precise mechanism of TGF-β1 in PDL has not been fully clarified. Previous study has showed HtrA1 could bind TGF-β1 in vitro (Oka et al. 2004). Particularly, HtrA1 could also regulate several physiological and pathological processes by TGF-β1, including neuronal maturation and developmental survival, human cerebral small vessels disease (Launay et al. 2008; Shiga et al. 2011). But whether HtrA1 regulates TGF-β1 to control hPDLCs osteogenic differentiation is hitherto unknown.

Therefore, we assume HtrA1 may play an important role in preventing PDL mineralization, and may regulate hPDLCs osteogenic differentiation by TGF-β1. This study was designed to investigate the role of HtrA1 in hPDLCs osteogenic differentiation, and by focusing on TGF-β1, to preliminarily explore the regulatory mechanism of HtrA1 in this process.

Materials and methods

Cell culture and osteogenic induction

The isolation of hPDLCs was approved by the Institutional Ethics Committee, and informed consent was obtained from each patient. The cell culture, identification and osteogenic induction were performed as described previously (Hayami et al. 2007; Li et al. 2012). hPDLCs were obtained from healthy premolars from three healthy patients (two female and one male, age: 12–25 years) extracted for orthodontic reasons. Cells were incubated in Dulbecco's modified medium (DMEM, HyClone, USA) at 37 °C in 5 % CO2 in humidified air, and passages 3–6 were used in all experiments. For osteogenic induction, hPDLCs were cultured in mineralization medium (MM) supplement with 10 mM β-glycerophosphate (β-GlyP), 10^{-8} M dexamethasone and 50 mg/ml asorbic acid (Sigma, USA), while hPDLCs remained in the normal culture medium (CM) were used as control.

RNA isolation and RT-PCR analysis

Total RNA samples were isolated from hPDLCs at passage 3, and the isolation of total RNA, cDNA synthesis and PCR analysis were performed as our previous study (Li et al. 2012). Briefly, cells were lysed with TRIzol reagent (Invitrogen, USA). Then chloroform was added in the lysate and centrifuged at 12,000×*g*, 4 °C for 15 min. RNA in the upper aqueous phase were transferred to a new Eppendorf microcentrifuge tube, and precipitated by isopropanol. Finally, RNA pellets were washed with 75 % ethanol, dried, and dissolved in RNase-free water. Then 500 ng RNA was used for cDNA synthesis and PCR analysis (TaKaRa, Japan). The primer sequences and products sizes are shown in Table 1.

Co-localization analysis with QDs-based immunofluorescence

QDs-based double Immunofluorescence Staining Kit, with Qdots Streptavidin Conjugate (QDs-SA, 605 nm) and Qdots IgG Conjugate (QDs–IgG, 545 nm) probes, was obtained from Wuhan Jiayuan Quantum Dots Co., Ltd (Wuhan, China). hPDLCs were fixed and incubated with 0.1 % Triton-X 100 at 37 °C for 15 min, and then cells were blocked with serum. To avoid the cross-reactivity, hPDLCs were incubated with primary rabbit anti-HtrA1 antibody (ab38610, Abcam Inc, Cambridge, MA) and anti-TGFβ1 antibody (Santa Cruz sc-146, USA) respectively. Firstly, cells were incubated with primary anti-HtrA1 antibody (1:100) at 4 °C overnight. Next day the cells were washed, blocked, incubated in biotinylated secondary antibody and QDs-SA. After washing, cells were then blocked and incubated in primary anti-TGFβ1 (1:200) at 4 °C overnight. Then next day the slides were washed, blocked and incubated with QDs-IgG secondary antibody. The nuclei were stained by 4′, 6-diamidino-2-phenylindole (DAPI). Finally, the complex of HtrA1-biotinylated secondary antibody-QDs-SA and the complex of TGFβ1-QDs-IgG was detected by Nuance spectral imaging system (CRI, USA).

Co-immunoprecipitation assay

hPDLCs were lysed in ice-cold buffer for 15 min. Proteins were incubated with 1 μg of anti-HtrA1 monoclonal antibody (Santa Cruz sc-377050, USA) or anti-TGFβ1 antibody (Santa Cruz sc-146, USA) shaking overnight at 4 °C. Then the immunoprecipitates were obtained by conjugation to 20 μl Protein A/G–Agarose (Beyotime Biotechnology, P1012, China) for 3 h at 4 °C. Immunoprecipitates were then collected by centrifugation at 2500 rpm for 5 min. After washing five times in the above lysis buffer, samples

Table 1 Primer sequence of target gene

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Tm (°C)	Length (bp)
HtrA1	CAGACATCGCACTCATCAA	ACTTCACCGTCCAGGTTTAC	58	271
TGFβ1	GAGCCTGAGGCCGACTACTA	TGAGGTATCGCCAGGAATTG	58	256
ALP	ACGTGGCTAAGAATGTCATC	CTGGTAGGCGATGTCCTTA	60	486
Runx2	AACCCTTAATTTGCACTGGGTCA	CAAATTCCAGCAATGTTTGTGCTAC	60	145
OCN	GGTGCAGCCTTTGTGTCCAA	CCTGAAAGCCGATGTGGTCA	60	174
GAPDH	TCATGGGTGTGAACCATGAGAA	GGCATGGACTGTGGTCATGAG	60	146

were resolved in 20 μl 1X SDS loading buffer and heated at 100 °C for 5 min. Finally, to detect the bound proteins, samples were separated in SDS–polyacrylamide gels and transferred to the PVDF membranes. The membranes were blocked in 5 % nonfat milk at room temperature for 2 h, incubated with primary anti-HtrA1 antibody (1:200) and anti-TGFβ1 antibody (1:200) at 4 °C overnight. After washing three times, membranes were incubated with secondary antibodies (anti-mouse IgG or anti-rabbit IgG) at room temperature for 1 h. Finally, the membranes were visualized by enhanced chemiluminescence (ECL). Input and mouse IgG served as positive and negative control, respectively.

Alizarin red staining, alkaline phosphatase (ALP) activity assay

Alizarin red staining and ALP activity were measured according to the procedure of our previous study(Li et al. 2012). For alizarin red staining, hPDLCs were fixed in 4 % (w/v) paraformaldehyde for 15 min at room temperature. Then the cells were rinsed and incubated in 0.1 % alizarin red (Sigma, USA) at room temperature for 30 min. Finally, cells were washed and photographed.

ALP activity was measured according to the manufacturer's protocol (Nanjing-Jiancheng, China). hPDLCs were lysed by M-PER Reagent (Pierce 78501, USA) and centrifuged at 14,000×*g*, 4 °C for 10 min. Then the supernatant was mixed with the ALP substrate solution and incubated at 37 °C for 15 min. Finally, the reaction was stopped and the absorbance was measured at 410 nm. ALP activity was calculated as nanomoles of p-nitrophenol/μg of protein/30 min.

Quantitative real-time PCR (QPCR)

Real-time PCR was performed with SYBR green PCR mix (TaKaRa, Japan) on ABI 7500 machine (Applied Biosystems, USA), with amplification conditions as follows: 95 °C/10 min for denaturation, followed by 95 °C/5 s, 58 °C/20 s, and 72 °C/34 s for 40 cycles. The relative gene expression was calculated by the $2^{(-\triangle\triangle Ct)}$ method, and normalized to GAPDH. The primer sequences and product sizes are shown in Table 1.

Western blot analysis

The method of protein extractions was performed as our previous study (Li et al. 2012). hPDLCs were washed with cold PBS for three times. 200 μl M-PER Reagent (Pierce 78501, USA) with protease inhibitor was added with shaking gently for 5 min. The lysate was collected and centrifuged at 14,000×*g*, 4 °C for 10 min. The supernatant was carefully collected, and then equal amounts of protein were loaded in SDS–polyacrylamide gels (SDS-PAGE), electrophoresed, and transferred to PVDF membranes. The samples were detected with primary antibodies, including anti-FLAG(1:5000, Abmart, Shanghai, China) antibody, anti-HtrA1 antibody (1:200), anti-TGFβ1 (1:200), and anti-GAPDH antibody (1:1000, ProMab, Mab-2005079), and goat anti-mouse IgG or goat anti-rabbit IgG secondary antibodies. Finally, the membranes were visualized via ECL substrate (Pierce 32209, USA).

Lentivirus-mediated overexpression of HtrA1

HtrA1-overexpression lentivirus (NM_002775, pGC-FU-3FLAG-HTRA1) was provided by Genechem (Shanghai, China). hPDLCs were transduced by HtrA1-overexpression lentivirus (HtrA1 group) or negative control lentivirus (NC group), puromycin (1 μg/ml) was added for 3 days to select the resistant clones. Finally, HtrA1 expression was evaluated by QPCR and western blot analysis.

RNA interference

Short hairpin RNA (shRNA) lentivirus for the human HtrA1 gene (shHtrA1) (pGLV/H1/GFP + Puro Vector) and negative control (NC) were purchased from GenePharma (Shanghai, China). Following the above similar protocol, shRNA lentivirus was transduced into hPDLCs and resistant clones were selected by puromycin.

Statistical analysis

All data were presented as mean ± standard deviation. Statistical analyses were performed by using independent sample Student's t test. $p < 0.05$ was considered statistically significant.

Results

Expression patterns of HtrA1 and TGFβ1 in hPDLCs

RT-PCR and immunofluorescence staining were used to detect mRNA and protein expressions of HtrA1 and TGFβ1 respectively. As shown in Fig. 1a, mRNA expression of HtrA1 and TGFβ1 was confirmed by RT-PCR. Then to visualize the co-localization of HtrA1 and TGFβ1 in the same cells, QDs-based double immunofluorescence staining was performed. Figure 1b indicated that HtrA1 (red) and TGFβ1 (green) were both expressed in the cytoplasm of hPDLCs, and the co-localization was also found in the cytoplasm (yellow).

Co-immunoprecipitation assay

To further defect the interaction between endogenous HtrA1 and TGFβ1 in hPDLCs, co-immunoprecipitation was applied. As shown in Fig. 1c, anti-HtrA1 or anti-TGFβ1 antibodies were used to pull down the immunoprecipitates, and we found HtrA1 could bind to TGFβ1.

Osteogenic induction and ALP activity

As described before, the process of osteogenic induction was performed. To investigate osteogenic differentiation of hPDLCs, ALP activity and the formation of mineralized nodule were detected. Similar with our previous research, after 21-day osteogenic induction the ALP activity showed a time-dependent increase and reached a peak on day 14

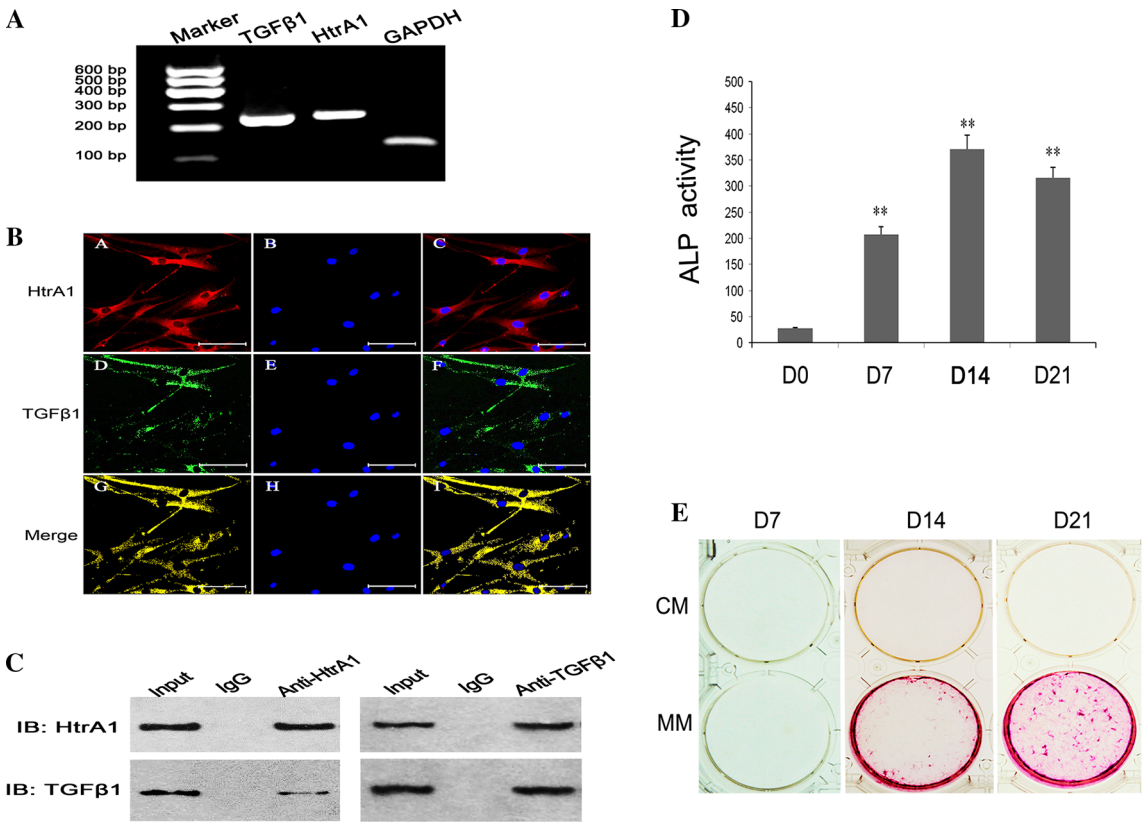

Fig. 1 **a** HtrA1 and TGF-β1 mRNA expression in hPDLCs by RT-PCR. **b** QDs-based immunofluorescence staining of HtrA1 and TGF-β1 in hPDLCs. Positive anti- HtrA1 staining (*red*, **a**–**c**) and anti-TGF-β1 staining (*green*, **d**–**f**) were in cytoplasm. Merged QDs signal of HtrA1 and TGF-β1 (*yellow*, **g**–**i**). DAPI staining for nuclei (*blue*, **b**, **e** and **h**). *Scale bar* 30 μm. **c** Co-immunoprecipitation assays of HtrA1 and TGF-β1. HtrA1 interacted with TGF-β1 in hPDLCs by using specific antibodies of anti-HtrA1 or anti-TGF-β1. Input: positive control, IgG: negative control. **d** ALP activity of hPDLCs for osteogenic induction. *Bars* represent mean ± SD (n = 3), $^{**}p < 0.01$. **e** *Alizarin red* staining for mineralized nodules during hPDLCs osteogenic differentiation. *MM* mineralization medium group, *CM* culture medium group. (Color figure online)

(Fig. 1d, ** $p < 0.01$). The mineralized nodules were first found on day 14 in the mineralization medium group (MM), but there were none mineral deposits in the normal culture medium group (CM) (Fig. 1e).

Overexpression of HtrA1 promotes osteogenic differentiation of hPDLCs

HtrA1 expression was confirmed at mRNA and protein levels by QPCR and western blot. In Fig. 2a, western blot result indicated FLAG tag was detected in HtrA1 group, while QPCR showed mRNA expression of HtrA1 was significantly increased 6.5-fold compared with NC (Fig. 2b, $p < 0.01$), suggesting pGC-FU-3FLAG-HTRA1 vector was successfully transduced. After osteogenic induction, more mineralized nodules were observed in HtrA1 overexpression group (Fig. 2c). Consistent with this finding, osteogenic marker genes expressions, including ALP, Runx2 and OCN, were up-regulated in HtrA1 on day 14 compared with NC (Fig. 2d, * $p < 0.05$, ** $p < 0.01$).

Knockdown of HtrA1 inhibits hPDLCs osteogenic differentiation

To further study whether down-regulation of HtrA1 affects hPDLCs osteogenic differentiation, HtrA1 was stably knockdown by lentivirus-mediated shRNA (shHtrA1). First, expression of HtrA1 was verified, and Fig. 3a, b demonstrated the expression of HtrA1 was dramatically decreased at both of mRNA and protein levels in shHtrA1 ($p < 0.01$). Next, the alizarin red staining showed that knockdown of HtrA1 delayed mineralized nodules formation (Fig. 3c). During 14-day osteogenic induction, the ALP activity showed significantly decreased in shHtrA1 group (Fig. 3d, $p < 0.01$). Meanwhile, the mRNA expression of osteogenic marker genes was also down-regulated compared with the control, such as ALP, Runx2 and OCN (Fig. 3e, * $p < 0.05$, ** $p < 0.01$).

Effect of HtrA1 on the expression of TGFβ1

To preliminarily investigate the mechanism of HtrA1 in regulating hPDLCs osteogenic differentiation, we

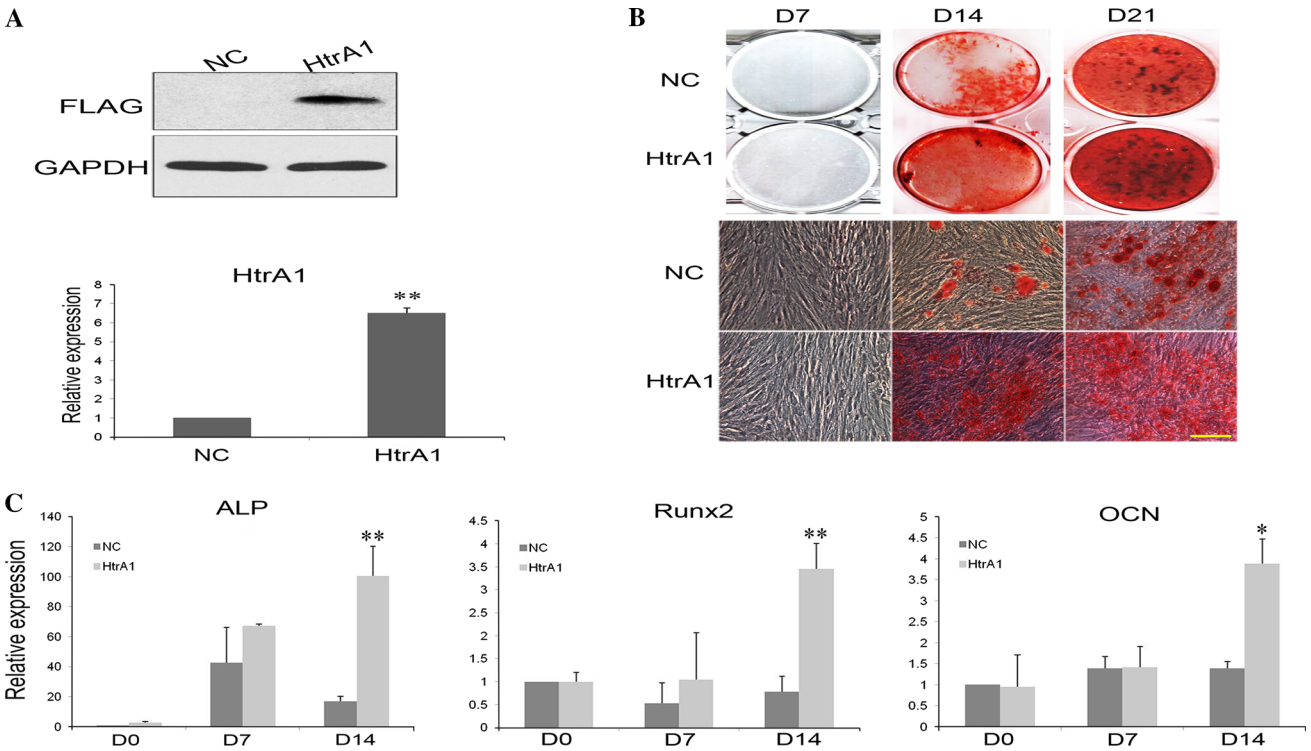

Fig. 2 Overexpression of HtrA1 promotes osteogenic differentiation of hPDLCs. **a** FLAG tag was detected in HtrA1 group by western blot, mRNA expression of HtrA1 was significantly increased compared with NC, **b** overexpression of HtrA1 led to more mineralized nodules formation, **c** expression of osteogenic genes including ALP, Runx2 and OCN were increased after HtrA1 was up-regulated. Values are present as mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$. *HtrA1* the group of overexpression of HtrA1, *NC* negative control group. *Scale bar* 100 μm

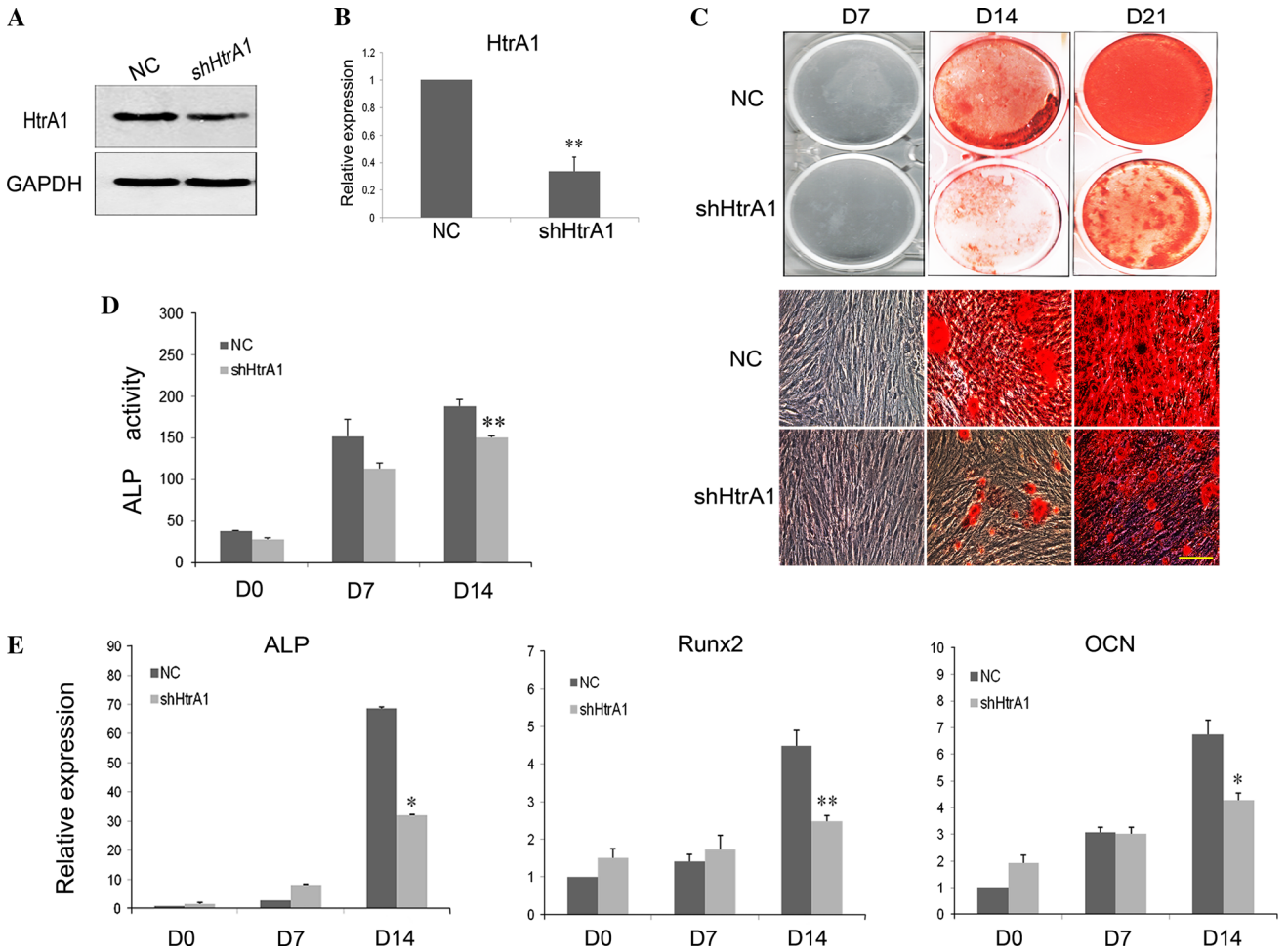

Fig. 3 Knockdown of HtrA1 inhibits hPDLCs osteogenic differentiation. When HtrA1 was knockdown, (**a**, **b**) Western blot and QPCR showed the expression of HtrA1 was significantly decreased, respectively. **c** Down-regulation of HtrA1 repressed mineralized nodules formation, **d** ALP activity was significantly decreased in shHtrA1 group. **e** Expression of osteogenic genes of ALP, Runx2 and OCN were decreased. Values are present as mean ± SD (n = 3). $*p < 0.05$, $**p < 0.01$. *shHtrA1* the group of HtrA1 knockdown, *NC* negative control group. *Scale bar* 100 μm

examined the expression profile of TGFβ1. In Fig. 4a, it is shown that, HtrA1 overexpression led to a significant increase in TGFβ1 mRNA expression on day 14 ($p < 0.01$). Consistent with this, the protein expression of TGFβ1 was also increased compared with the control (Fig. 4b). Then, we further determine the effect of knockdown of HtrA1 on TGFβ1 expression. QPCR and western blot results demonstrated that when HtrA1 was knockdown by shRNA, TGFβ1 expression was also decreased at both mRNA and protein levels (Fig. 4c, d, $p < 0.01$).

Discussion

PDL plays crucial roles for homeostasis of periodontal tissues, inculuding remodeling, wound healing and tissue regeneration (Hou et al. 2012; Wang et al. 2013). In periodontal defect, the osteogenic differentiation of PDLCs plays an important role in periodontal regeneration (Markopoulou et al. 2011). However, interestingly, under normal physiological conditions, PDL remains non-mineralized although located between two hard tissues, but the exact mechanism of which is still unknown. Many previous studies showed that HtrA1 is an essential mineralization inhibitor and could regulate bone homeostasis by TGF-β signaling (Oka et al. 2004; Hadfield et al. 2007; Wu et al. 2014; Graham et al. 2013). Unfortunately, the role of HtrA1 in PDL remains unclarified. Therefore, this study aimed to investigate the effect of HtrA1 on hPDLCs osteogenic differentiation and preliminarily explore its signaling pathway.

First of all, we confirmed the expression of HtrA1 and TGFβ1 in hPDLCs at both mRNA and protein levels. Previous study has showed that HtrA1 could bind TGFβ1 in vitro (Oka et al. 2004). Our results found HtrA1 and TGFβ1 were co-localized in the cytoplasm of hPDLCs, and then co-immunoprecipitation further confirmed the

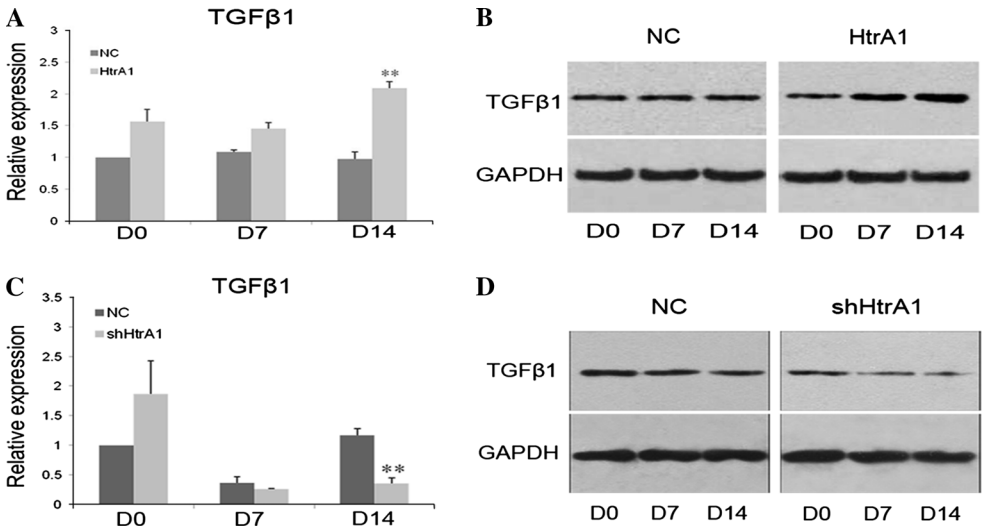

Fig. 4 Effect of HtrA1 on TGFβ1 expression. **a** Up-regulation of HtrA1 led to increased mRNA expression of TGFβ1, **b** up-regulation of HtrA1 increased TGFβ1 expression at protein level, **c**, **d** Knockdown of HtrA1 decreased the expression of TGFβ1 at both of mRNA and protein levels. Values are present as mean ± SD (n = 3), $^{**}p < 0.01$

interaction between HtrA1 and TGFβ1, showing they could bind each other in hPDLCs. These findings strongly suggested that there is a close relationship between HtrA1 and TGFβ1, and HtrA1 may regulate the TGFβ signaling by binding TGFβ1.

After that, to explore the role of HtrA1 in hPDLCs osteogenic differentiation, the model of hPDLCs osteogenic differentiation followed our previous study was firstly established by detecting ALP activity and the mineralized nodules (Li et al. 2012). Then lentivirus-mediated overexpression and reduction of HtrA1 was performed. Firstly, the up-regulated and down-regulated expression of HtrA1 was confirmed at both of mRNA and protein levels. Surprisingly, we found overexpression of HtrA1 enhanced the mineralized nodules formation and expression of mineralization-related genes ALP, Runx2 and OCN. Consistent with this finding, knockdown of HtrA1 inhibited the mineralized nodules formation, ALP activity and mineralization-related genes. Previous studies have shown HtrA1 is a negative regulator for mineralization (Hadfield et al. 2007; Wu et al. 2014; Graham et al. 2013). Conversely, our findings suggested that HtrA1 could positively regulate the osteogenic differentiation of hPDLCs. Similarly, a recent study has also demonstrated the positive role of HtrA1 in the osteogenesis of MSC from human bone marrow (Tiaden et al. 2012). Hence, we assume HtrA1 may possess dual roles in regulating osteogenesis, different results may be caused by different cell sources, experimental variations and other different conditions.

Finally, to further investigate the mechanism of HtrA1 in regulating hPDLCs osteogenic differentiation, we focused on TGF-β signaling, especially TGF-β1. It demonstrated that during 14-day osteogenic induction, the expression of TGF-β1 in HtrA1 overexpression group was significantly increased at both of mRNA and protein levels. While when HtrA1 is knockdown, the expression of TGF-β1 was also down-regulated. Previous studies showed HtrA1 could degrade and regulate the amount of TGF-β1 to control neural development and cerebral small-vessel disease (Launay et al. 2008; Shiga et al. 2011). Although the precise mechanism is still unclear, our present results implied that HtrA1 may regulate hPDLCs osteogenic differentiation by TGF-β1.

In conclusion, our work attempts to provide the role and mechanism of HtrA1 in regulating hPDLCs osteogenic differentiation. We found HtrA1 could promote hPDLCs osteogenic differentiation and may regulate this process by TGF-β1. Nevertheless, to illuminate the exact regulatory mechanism further work is still needed. Our study may provide new insight into the maintenance and regeneration of periodontal tissues.

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Conflict of interest None.

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