Upstream stimulatory factor 1 regulates OPNmRNA expression in odontoblasts

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Purpose: To detect the effects of upstream stimulatory factor 1 (USF1) on the expression of osteopontin (OPN) in odontoblasts, and explore its biological functions in tooth development.
Materials and Methods: Odontoblasts MDPC-23 were cultured and stably transfected with PCMV-USF1 or A-USF expression plasmids. The mountings of odontoblast coverslips in each group were prepared and total RNA was extracted. Immunofluorescence staining was performed with specific anti-USF1 and anti-HA tag antibodies. Semi-quantitative RT-PCR was carried out to detect the expression of OPN and β-actin in each group. Gray value of OPN and β-actin in each group was calculated and statistically analyzed.
Results: Clones of stable PCMV-USF1 and A-USF plasmids transfection were achieved. Positive staining of HA was shown in the cytoplasm of odontoblasts in A-USF transfection group. Compared with the control, PCMV-USF1 transfection group appeared stronger staining. Electrophoresis of semi-quantitative RT-PCR showed that OPN was upregulated in PCMV-USF1 transfection group, while it was downregulated in A-USF transfection group.
Conclusion: USF1 could regulate the expression of OPN in odontblasts, which could be blocked partially by A-USF. **(Int Chin J Dent 2007; 7: 59-63.)**
Key Words: immunofluorescence staining, odontoblast, OPN, RT-PCR, USF1.

Introduction

Osteopontin (OPN) is an important component in dentinal matrix and involved in matrix mineralization, cell transformation, and bone absorption. Upstream stimulatory factors 1 (USF1) belongs to the eucaryotic evolutionary conserved basic-Helix-Loop-Helix-Leucine Zipper transcription factor family, can activate the transcription of multiple growth and metabolism related genes, and regulate cell proliferation and differentiation.[1] In previous researches,[2,3] we cloned the specific coding sequence of USF1 from the tooth germ of 5-day postnatally balb/c mouse and detected the expression of USF1mRNA in an odontoblastic cell line MDPC-23. This experiment was aimed to detect the effects of USF1 on OPN mRNA expression in MDPC-23 by gene transfection and semi-quantitative PCR, and explore the biological function of USF1 during tooth development.

Materials and Methods

Cell culture

Odontoblasts MDPC-23 were cultured in aMEM medium (Gibco, Grand Island, NY, USA) with 10% fetal calf serum.

Plasmid transformation, extraction and quantitation

DH5a competent cells were transformed by PCMV-USF1 and A-USF plasmids, which contained anti-Neomycin genes both but HA tag only in A-USF, were kindly gifted by Prof. Michele Sawadogo and Dr. Charles Vinson respectively. After spread and culture, blue-white screening was carried out. Positive clones were chosen to expand culture, and plasmids were extracted by plasmid extraction kit (Clonetech, Palo Alto, CA, USA) and dissolved in TE solution. Four μL were taken out and detected by ultraviolet spectrophotometer to determine the purity and contents.

Gene transfection and screening

Odontoblasts were transfected with PCMV-USF1 and A-USF plasmids with Lipofectamine 2000 according to the instruction. Twenty-four hours after transfection, cells were passaged to a 60 mm flat plate at the ratio of 1:10. Three hundred μg/mL G418 was added to the cell culture medium one day after passage. Cell culture medium and G418 were replaced everyday. After 10 days of screening, all cells in the control died, then G418 was reduced to 150 μg/mL until resistant clones emerged. Filter paper method was used to transfer the resistant clones to a 24 well culture plate and macro-culture was progressed further. Then cell creeping slides and total RNA extraction were carried out as previously described.[2,3]

Immunofluorescence staining

In order to confirm the integration and expression of transfected plasmids by screened clones, the following immunofluorescence staining was undertaken on the cell creeping slides respectively according to the kit instruction: anti-USF1 staining in USF1 transfection group (antibody dilution 1:150), anti-HA staining in A-USF1 transfection group (antibody dilution 1:200). The secondary antibody was fluorescence-conjugated goat anti-rabbit-Texas Red (dilution 1:500). After incubation with the secondary antibody at 4˚C for 6 to 8 hours, cell creeping slides were rinsed with PBS, mounted with glycerine containing anti-quench agents, and examined with fluorescence microscopic scope.

Semi-quantitative PCR

PCR primers of OPN were designed according to mouse OPNcDNA sequence (AF5157081) in Genebank. The forward and backward primers were 5'TCACCATTCGGATGAGTCTG3' and 5'ACTTGTGGCTCTGATGTTCC3' (437bp), and the primers of β-actin were 5'TGGTGGGTATGGGTCAGAAGGACTC3' and 5'CATGGCTGGGGTGTTGAAGGTCTCA3' (265bp). Blast analysis revealed that both primers were specific. Reverse transcription PCR was progressed as described previously with the same amount of RNA (171 ng each).[3] PCR condition of OPN was 94˚C for 5 minutes, then 94˚C for 30 s, 55˚C for 30 s, 72˚C for 1 minute, repeated 27 cycles and finally 72˚C for 7 minutes. PCR condition of β-actin was 93˚C for 5 minutes, then entered the 33 cycles as 93˚C for 1 minute, 56˚C for 30 s, and 72˚C for 1 minute, and finally stayed at 72˚C for 7 minutes.

Statistical analysis

The gray scale value of electrophoresis bands in each PCR reaction was measured by Labworks software; the ration of OPN/β-actin in each group was calculated and compared by statistic software SPSS 11.0.

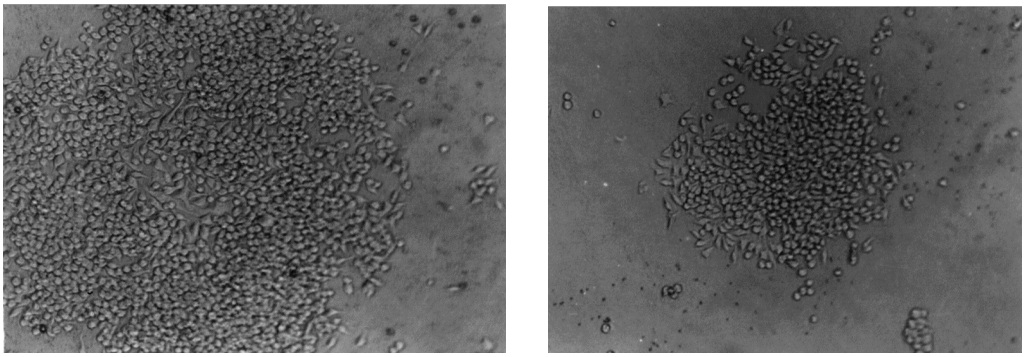
Results

Purity and contents of plasmids

The A260/A280 ratio of PCMV-USF1 and A-USF plasmids was 1.96 and 1.87, which indicated high purity and suitable for gene transfection. The final concentration of both plasmids was adjustment to 0.3 μg/μL.

Stable transfection and clone screening

After MDPC-23 odontoblastic cells were transfected with the plasmids, both grew in good condition. The day after adding G418, cell death began. Up to the 10th day, cells in untransfected group all died, while there remained some scattered cells in transfected groups. Then maintaining screening at 150 μg/μL for another two weeks until resistant cell clones emerged (Figs. 1 and 2).

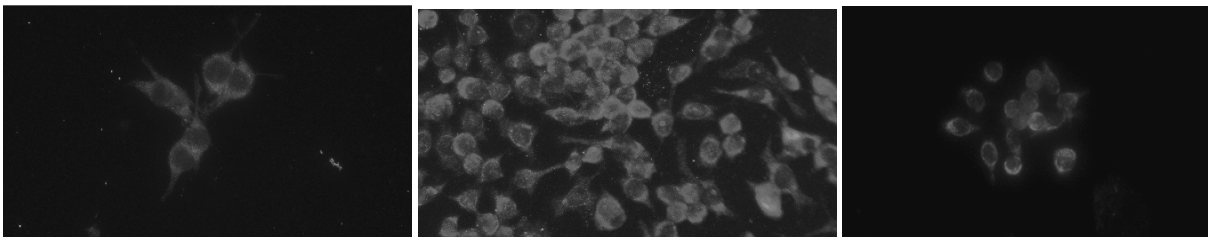


1 2

Fig. 1. Clone stably transfected with PCMV-USF1 plasmids (left, x20).
Fig. 2. Clone stably transfected with A-USF plasmids (right, x20).

Immunohistochemistry

Immunofluorescence staining showed that, compared with the control, anti-USF1 staining in USF1 transfection group was enhanced (Figs. 3 and 4), and that anti-HA staining was only positive in A-USF transfection group (Fig. 5), indicating the success of stable transfection and integration of both plasmids.



3 4 5

Figs. 3-5. Staining of USF1 in MDPC-23 (left, x40), staining of USF1 in stable USF1 transfection group (center, x40), and staining of HA in A-USF transfection group (right, x40).

RT-PCR

Gel electrophoresis displayed that there was only a specific band at 437bp and 265 bp in each group, consistent with what we expected. The band brightness of β-actin in each group was equal, indicating the same amount of template added to RT-PCR. Compared with the control, brightness of OPN band in USF1 transfection group was brighter, but darker in A-USF transfection group (Fig. 6). Mearued by Labworks software, the relative ratio of gray scale between OPN and β-actin in USF1, A-USF transfection group, and control was 60.33±4.51%, 229.33±7.09%, and 110.00±15.62% respectively, which differed significantly from each other via statistical analysis.

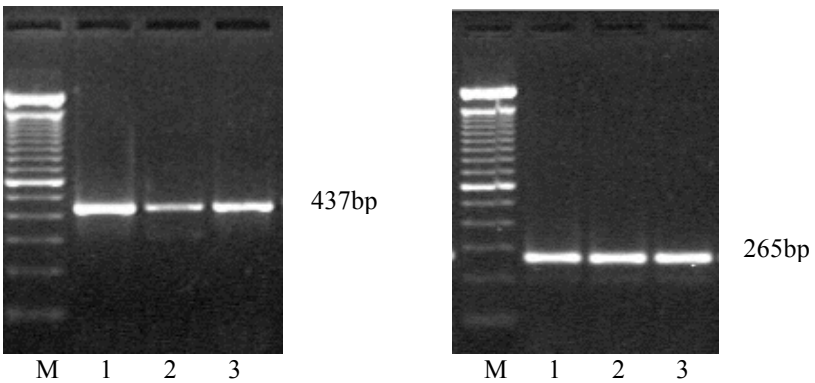


Fig. 6.
Electrophoresis of RT-PCR for OPN and β-actin.
M, Marker
Track 1, USF1 transcription group
Track 2, A-USF transcription group
Track 3, Control

Discussion

OPN, a prominent component of the extracellular matrix of mineralized tissues in teeth, is a secreted glycosylated phosphoprotein containing specific sequences of ARG-GLY-ASP that can adsorb hydroxyapatite, form bridges between cells and minerals in the matrix, and mediate cell adhesion and migration. OPN was first separated from bone matrix, and then detected in a variety of other tissues. It was found to be implicated in matrix mineralization, cell transformation and bone matrix absorption, and have very tight relationship with teeth. Wei et al.[4] detected the expression of OPN in rat tooth development via immunohistochemical staining method, and found that OPN presented in ameloblasts only on 1d postnatal rats and expressed in both ameloblasts and odontoblasts in the ensuing 1-6 week-old rats, indicating that OPN plays an important role in the development and mineralization of rat tooth. Zhang et al.[5] observed the persistent OPN expression in odontoblasts and reparative dentin within 10 days after direct pulp capping, and presumed that OPN may be concerned with the formation of reparative dentin. In addition, OPN is involved in tooth root development and tooth absorption process.[6]

The expression of OPN is tissue-specific and regulated by a variety of exogenous factors, growth factors, and transcripton factors. Ohishi et al.[7] noticed that retinoic acid could stimulate osteopontin synthesis by cultured dental pulp cells in a dose-dependent manner, and 6.2 fold increases were observed at the concentrations of 10^{-6} mol/L. High glucose also enhances the expression of osteopontin in aortic smooth muscle cells at the transcriptional level through the activation of protein kinase C-dependant pathway.[8] While genetic structure of OPN analyzed, several transcription factor recognition sites were identified, such as TATA-box, E-box, and GC-box in the promoter region. Transient transfection and gel-shift analyses of osteopontin promoter indicated that a region between -123 and +66 bp was crucial in the regulation of osteopontin expression, and that USF1 could specifically bind the E-box between -101 and -96 bp to be involved in the regulation of osteopontin expression on vascular smooth muscle cells.[9] An extensive deletion analysis of the 5'-flanking region of rat OPN gene revealed that a high glucose response element was present within -112 and -62 bp of the OPN promoter including an E-box and a GC-rich region. Mutation of the E-box or the GC-rich region resulted in at least 30% loss of high glucose induced promoter activity.[10] Bidder et al.[11] confirmed further that OPN gene transcription was regulated by USF and AP1 in aortic vascular smooth muscle cells, and that glucose up-regulated both AP1 and USF DNA binding and transactivation activities. However, whether USF1 in odontoblasts could also regulate the expression of OPN and mediate the effect of high-glucose, retinoic acid and other external factors on tooth development is still unknown.

PCMV-USF1 and A-USF plasmids could code for full-length USF1 and A-USF protein. A-USF is a USF dominant-negative mutant.[12] In this construct, the basic region of the bHLH-zip domain of USF1 was replaced with an acidic sequence, which greatly stabilizes the heterodimers between A-USF and USF. Consequently, A-USF is a very efficient inhibitor of USF DNA binding and could effectively block the transcriptional control activity of USF1. In this research, odontoblast MDPC-23 was cultured; stably trasfected with PCMV-USF1 and A-USF plasmids, then the variance of OPNmRNA expression in each group was detected by semi-quantitative RT-PCR and compared statistically. The results showed that there was OPNmRNA expression in MDPC-23 odontoblasts, and that compared with the control, OPN mRNA was upregulated in PCMV-USF1 transfection group, but downregulated in A-USF transfection group. Thus it indicated that USF1 could regulate the expression of OPN in odontblasts, which could be blocked partially by A-USF. These results provide evidence

for further researches on USF1 function in the process of odontoblasts maturation and dentin formation, and indicate that USF1 might be implicated in dentin matrix secretion and mineralization during tooth development.

References

1. Corre S, Galibert MD. Upstream stimulating factors: highly versatile stress-responsive transcription factors. Pigment Cell Res 2005; 18: 337-48.
2. Wu LA, Wen LY, Yang FS, Fan SM. cDNA Cloning and partial sequencing of USF1 in mouse tooth germ. Chin J Conserv Dent 2002; 12: 588-90.
3. Wu LA, Wen LY, Yang FS, Liu Y, Wang XJ. In situ hybridization analysis of USF1 mRNA expression in odontoblasts. Int Chin J Dent 2004; 4: 15-8.
4. Wei SS, Cao CF, Meng HX. Expression of sialoprotein and osteopontin in rat tooth development. Chin J Stomatol 2002; 27: 47-9.
5. Zhang Q, Fan M, Bian Z, Chen Z, Zhu Q. Immunohistochemistry of bone sialoprotein and osteopontin during reparative dentinogenesis in vivo. Chin J Dent Res 2000; 3: 38-43.
6. Shimazu Y, Nanci A, Aoba T. Immunodetection of osteopentin at sites of resorption in the pulp of rat molars. J Histochem Cytochem 2002; 50: 911-21.
7. Ohishi M, Horibem, Ikedo D, et al. Effect of retinoid acid on osteopontin expression in rat clonal dental pulp cells. J Endod 1999; 25: 683-5.
8. Takemoto M, Yokote K, Yamazaki M, et al. Enhanced expression of osteopontin by high glucose in cultured rat aortic smooth muscle cells. Biochem Biophy Res Comm 1999; 258: 722-6.
9. Malyankar UM, Hanson R, Schwartz SM, Ridall AL, Giachelli CM. Upstream stimulatory factor 1 regulates osteopontin expression in smooth muscle cell. Exp Cell Res 1999; 250: 535-47.
10. Asaumi S, Takemoto M, Yokotek, et al. Identification and characterization of high glucose and glucosamine responsive element in the rat osteopontin promoter. J Diabetes Complications 2003; 17: 34-8.
11. Bidder M, Shao JS, Charlton-Kachigian N, Loewy AP, Semenkovich CF, Towler DA. Osteopontin transcription in aortic vascular smooth muscle cells is controlled by glucose-regulated upstream stimulatory factor and activator protein 1 activities. J Biol Chem 2002; 277: 44485-96.
12. QyangY, Luo X, Lu T, et al. Cell-type-dependent activity of the ubiquitous transcription factor USF in cellular proliferation and transcriptional activation. Mol Cell Biol 1999; 19: 1508-17.

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