IPO7 Promotes Odontoblastic Differentiation of Mouse Dental Papilla Cells by Selectively Importing Odontogenic Transcription Factors

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Research

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Abstract

**Background:** An important biological process for dentin formation and mineralization is odontoblast differentiation, which is precisely governed by a series of transcription factors (TFs). Importin 7 (IPO7) is a member of the Karyopherin β-superfamily mediating nucleocytoplasmic transport of proteins. In this study, we aimed to study the mechanisms by which IPO7 participates in odontoblastic differentiation.

**Methods:** The expression patterns of IPO7 was investigated by immunofluorescence staining. Besides, mouse dental papilla cells (mDPCs) were extracted and cultured. After silencing *Ipo7* in mDPCs, odontoblastic differentiation and the effect on odontogenic TFs were evaluated. Using nuclear and cytoplasmic extraction and co-immunoprecipitation assay, we aimed to confirm that IPO7 imports some odontogenic transcriptions factors to promote odontoblastic differentiation.

**Results:** We found that IPO7 was increasingly expressed from the pre-odontoblasts to mature odontoblasts from PN2 to PN9. IPO7 enhanced odontoblast differentiation in mDPCs and imported essential TFs for odontoblastic differentiation, such as Distal-less homeobox 3 (DLX3), Osterix (OSX), Krüppel-like factor 4 (KLF4) and P-SMAD1/5 except for RUNX2. Besides, only RUNX2 showed no obvious interaction with IPO7.

**Conclusion:** In conclusion, our data demonstrated that IPO7 enhances odontoblast differentiation in mDPCs by selectively importing odontogenic TFs.

Introduction

Tooth development in mammalian depends upon epithelial-mesenchymal interactions. In mice, the mesenchymal stem cells from mouse dental papilla can differentiate into odontoblasts [1, 2]. Odontoblast differentiation is essential for tooth development by secreting predentin which then undergoes biomineralization into dentin [3]. Progress in the studies of odontoblastic differentiation will provide a basis for stem cell-based tooth regeneration. Numerous TFs, including DLX3, OSX, KLF4, and P-SMAD1/5, play pivotal roles in odontoblastic differentiation [1, 4, 5]. During dentinogenesis, the nuclear localization of these TFs is a prerequisite for target odontogenic gene expression.

Since TFs function in the nucleus, they have to be translocated to the nucleus after translation in the cytoplasm. Nuclear pores allow facile exchange of molecules smaller than 20–40 kDa, whereas the transport of greater macromolecules requires soluble transport factors or carrier molecules [6, 7]. Importin α and Importin β were identified as two types of nucleocytoplasmic transport receptors, which may import half of proteins that need to function in the nucleus in human [8]. The importin β family (20 members in humans), also known as importin β1 or karyopherin β (KPNB), includes bidirectional receptors, importins and exportins, involved in mediating nucleocytoplasmic transport [9]. Importin β family participates in differentiation, organism development and also contributes to lineage specification through regulating nuclear levels of TFs [10].
IPO7 and importin 8 (IPO8) are important members of importin β-superfamily. IPO8 exhibits significant changes during osteoblast differentiation and plays vital functions for osteoblast differentiation [11]. Both osteoblasts and odontoblasts are originated from cranial neural crest and are able to form hard tissues [12]. Thus, they share many similarities regarding the molecular mechanisms for cell differentiation. Furthermore, the positive expression of IPO8 was detected specifically in odontoblasts in healthy human pulps [13]. All these studies indicate that IPO8 is involved in the odontoblastic differentiation. IPO7 and IPO8 are quite similar in sequence identity (61%) and protein structure, and function redundantly in many cases [13, 14]. For example, IPO7 and IPO8 can both import SMAD3 and SMAD4 [15]. However, the involvement of IPO7 in odontoblastic differentiation has not been extensively described.

Here, we demonstrated the role IPO7 plays in odontoblastic differentiation in mDPCs. Our results suggest that IPO7 promotes odontoblastic differentiation in mDPCs through importing odontogenic TFs and induction of odontoblast-related genes.

Materials And Methods

Cell culture and isolation

mDPCs were isolated from Kunming mice (China) at postnatal (PN) day 0.5 and were cultured in DMEM (Dulbecco's modified Eagle’s medium) (HyClone) containing 10% FBS (fetal bovine serum) (Gibco) and 1% penicillin/streptomycin (HyClone) at 37 °C in 95% air and 5% CO₂. Cell culture medium was changed every 2–3 days.

Preparations for Tissue and section

Animal samples were collected from Kunming mouse. Tissues for immunofluorescence staining were fixed in 4% PFA (paraformaldehyde), followed by embedding. 5 µm thick sections were prepared from each tissue block.

Immunofluorescence staining

Tissue sections were deparaffinized, dehydrared and then antigen retrieval was done in citric acid buffer (pH 6.0). The tissues were blocked with bovine serum albumin (BSA) for 1 hour before incubation overnight with the anti-IPO7 monoclonal antibody (1:100, sc365231, Santa Cruz) at 4 °C. The sections were then incubated with fluorescent secondary antibodies (1:200, A23210, Abbkine) for 1 hour. Then the tissues were washed 3 times in phosphate buffer saline (PBS), after which they were mounted in fluorescent mounting medium with DAPI (ZSGB-BIO). For cell immunofluorescence, the cells were fixed with 4% PFA at 4 °C, then permeabilized with 0.1% Triton X100 (BioFroxx), blocked by 3% BSA at 37 °C for 1 hour and were incubated with rabbit-anti-RUNX2 (YT5356, Immunoway) and mouse-anti-IPO7 (sc365231, Santa Cruz) antibodies overnight at 4 °C, then washed 3 times with PBS and incubated with fluorescent secondary antibodies for 1 hour (A23220, Abbkine; ANT034, Antgene). The fluorescence images were observed under fluorescence microscopy (Olympus 1 × 83, Japan).
IPO7 knockdown

Ipo7 siRNA oligonucleotides were purchased from GenePharma. The sequences are 5′-GCACACAAGUCUCGAAUUTT-3′ (sense) and 5′-AAUUCAGAGACUUGUGUGCTT-3′ (anti-sense). A negative scrambled control siRNA (GenePharma) was used.

mDPCs were seeded in plates and cultured in DMEM supplemented with 10% FBS. After 3–4 days, siRNA was transfected into cells with Lipofectamine 2000 (Life Technologies) with an additional incubation for 48–72 h. Then cells were harvested.

Odontoblastic differentiation of mDPCs, Alizarin Red Staining (ARS) and quantification

When the cells reached approximately 80% confluence, mDPCs were cultured with DMEM supplemented with 10 nM dexamethasone (Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich), 50 µg/mL ascorbic acid (Sigma-Aldrich), 10% FBS and 1% penicillin/streptomycin for odontoblastic differentiation induction. For calcification evaluation, cells incubated with odontoblastic differentiation medium for 9 days were fixed at 4 ° in PFA for 15 minutes and stained with 0.5% Alizarin Red S (Alladin) at 37 ° for 5–30 minutes. The cell matrix layers were washed 3 times with PBS to remove non-specific staining. Then the cells were observed under a microscope and photographed. 10% cetylpyridinium chloride (CPC) (Solarbio) was used to destain the cells for 2 hour with constant rotation at room temperature for quantification, the liquid was transferred to a 96-well plate and measured at 562 nm.

Alkaline phosphatase activity (ALP)

Cells were fixed in 4% PFA and stained using BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, China).

Total RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using HP Total RNA Kit (Omega Biotech). The cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) and were subsequently amplified using Fast Start Universal SYBR Green Master (Rox) with primers in Table 1 and assayed with quantitative RT-qPCR using the CFX Connect RealTime PCR Detection System (Bio-Rad). GAPDH primers were used for normalization.

Total cell extract Preparation

Cells were lysed in NP40 Lysis Buffer (Beyotime) for 15 minutes on ice. After the cells were collected, the total proteins in the supernatant were extracted by centrifugation for 15 minutes at 16,000 g.

Nuclear and cytoplasmic extract isolation
The nuclear extraction was prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). The concentration was measured by the Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific).

Co-immunoprecipitation analysis

mDPCs were lysed with NP40 Lysis Buffer for 10 minutes. Ten percent of the volume of the precleared cell extracts were taken as input. Cell lysates were incubated with anti-IPO7 antibody or normal IgG (A7016, Beyotime) at 4 °C overnight with constant rotation and incubated at room temperature for 1 hour with magnetic beads with protein A/G (Bimake). After immunoprecipitation, beads were washed with NP40 Lysis buffer for 6 times and were boiled for 10 minutes. The eluent was analyzed by Western blot. The specific secondary antibody used were EasyBlot anti-Mouse IgG (GTX22166701, GeneTex) and EasyBlot anti-Rabbit IgG (GTX22166601, GeneTex).

Western blot

Cells were lysed in NP40 Lysis Buffer with protease inhibitor cocktail (MCE). The cell lysis were analyzed with electrophoresis and then transferred to PVDF membrane (Millipore). The filter was blocked for 1–2 hours by 5% nonfat milk in TBST (Tris buffered saline containing Tween 20) and then incubated with the primary antibodies at 4 °C overnight. The primary antibodies used were: anti-IPO7 monoclonal antibody (sc365231, Santa Cruz), anti-RUNX2 polyclonal antibody (YT5356, Immunoway), anti-KLF4 polyclonal antibody (4038, Cell Signaling technology), anti-Phospho-SMAD1/5 monoclonal antibody (9516, Cell Signaling technology), anti-OSX monoclonal antibody (sc393325, Santa Cruz), anti-DLX3 polyclonal antibody (132613AP, Proteintech), anti-DMP1 polyclonal antibody (ab103203, Abcam), anti-DSP polyclonal antibody (NBP191612, NOVUS), anti-β-ACTIN monoclonal antibody (660091G, Proteintech) and anti-PCNA polyclonal antibody (102052AP, Proteintech). After incubation with the corresponding antibodies, the filter was washed 3 times for 5 minutes each with TBST. The filter was then incubated with HRP (horseradish peroxidase) conjugated goat anti-Mouse or anti-Rabbit IgG (Biofly, China) for 1 hour. The filter was washed and developed by a chemiluminescence assay (GE Healthcare). We used ImageJ software for further analysis.

Statistical analysis

All experiments were repeated at least 3 independent times. Data are presented as mean ± standard deviation (SD), analyzed with GraphPad Prism 5.0 software (San Diego, CA, USA) and differences between two groups were analyzed with a 2-tailed t test. In all analyses, \( p < .05 \) indicates statistically significance.

Results

Immunofluorescence of IPO7 in the murine teeth at embryonic and postnatal days
IPO7 is a nuclear protein that is ubiquitously detected in most tissues, such as bone marrow, brain, placenta and 24 other tissues in human [16]. To determine whether IPO7 plays roles in tooth development, we examined the expression of IPO7 in teeth from E12.5 to PN 9 using immunofluorescence staining. In the murine lower incisors at PN2 (Fig. 1a-d), IPO7 seldom expressed in the pre-odontoblasts and papilla cells (Fig. 1a) but displayed moderate in the polarizing odontoblasts (Fig. 1b) and intense in the secretory and mature odontoblasts (Fig. 1b, c), whereas it decreased gradually from pre-ameloblasts, polarizing ameloblasts, secretory ameloblasts to mature ameloblasts (Fig. 1a, b, c). In lower molars, from E12.5 to E18.5, IPO7 was slightly expressed in the dental mesenchymal cells, but highly expressed in dental epithelial cells (Fig. 1e-h); from PN1 to PN9, IPO7 was dramatically decreased in ameloblasts compared with that in epithelial cells of prenatal stages (Fig. 1i–l). While the expression of IPO7 was strong in the differentiated odontoblasts of PN1, 3, 7 and 9 molars (Fig. 1i–l).

**Up-regulation of IPO7 during the odontoblastic differentiation of mDPCs**

Some odontogenic marker genes including Alkaline phosphatase (Alp), Dentin matrix acidic phosphoprotein 1 (Dmp1), Dentin sialophosphoprotein (Dspp) display upregulation during odontoblast differentiation. Osteoblastic differentiation can be assessed by the increased expression of Osteocalcin (Ocn), Osteopontin (Opn) and Runx2 [17–19]. When cultured in differentiation medium for 9 days, mRNA levels of the odontogenic and osteogenic genes displayed an increased tendency, however, significant differences were found only in Alp, Dmp1 and Dspp, which indicated the differentiation of mDPCs towards the odontogenic lineage (Fig. 2a). With the odontoblastic differentiation of mDPCs, the Ipo7 expression was increased significantly on day 9 (Fig. 2b). The protein levels of DMP1, DSP and IPO7 showed similar trends to their mRNA levels (Fig. 2c).

To detect the subcellular localization of IPO7, immunofluorescence staining was carried out. IPO7 was present in both the nuclear and cytoplasm of mDPCs in growth medium, but after 9 days’ culture in differentiation medium, IPO7 showed obvious localization in the nucleus of mDPCs (Fig. 2d).

**Knockdown of Ipo7 disrupts odontoblastic differentiation in mDPCs**

To determine the role of IPO7 on odontoblastic differentiation in mDPCs, IPO7 was silenced via Ipo7 siRNA transfection. Immunofluorescence revealed that IPO7 expression was not only decreased but also restricted to the cytoplasm in Ipo7 siRNA group compared with the control (Fig. 3a). mRNA level of IPO7 in the Ipo7 siRNA group was 87.9% less than the control and the mRNA levels of Alp, Dmp1 and Dspp were also substantially down-regulated (95.5%, 72.2%, 84.5% separately) (Fig. 3b). Furthermore, Western blot showed lower DMP1, DSP and IPO7 protein levels in the Ipo7 knockdown group (Fig. 3c).

ARS assay revealed that mDPCs exhibited a decreased capacity of calcium deposition in Ipo7 knockdown group (Fig. 3d). ALPase activity was also down-regulated in Ipo7 siRNA group (Fig. 3e).

**Knockdown of Importin 7 reduces the nuclear import of odontogenic TFs but does not affect RUNX2**
Since the main function of IPO7 is to import proteins into nucleus, to further elucidate the mechanism of IPO7 promoting odontoblastic differentiation of mDPCs, we detected the cytoplasmic and nuclear distribution of the odontogenic TFs including DLX3, OSX, KLF4, P-SMAD1/5 and RUNX2 with knockdown of Ipo7. Effective knockdown of Ipo7 in vitro would be predicted to contribute to a more cytoplasmic distribution of TFs essential for odontoblastic differentiation over time.

As shown in Fig. 4a, knockdown of Ipo7 had almost no significant influence on the total protein expressions of the TFs mentioned above. While DLX3, OSX, KLF4, and P-SMAD1/5 were mainly localized in cytoplasm and prominent reduction in the nucleus was found with knockdown of Ipo7 (Fig. 4b and c). However, subcellular distribution of RUNX2 was not influenced by knocking down of Ipo7 (Fig. 4b and c), which was further confirmed by immunofluorescence staining (Fig. 4d).

**IPO7 is able to bind to DLX3, OSX, KLF4, P-SMAD1/5 but not RUNX2 in mDPCs**

Based on the selectively importing of IPO7 to odontogenic TFs, we sought to figure out whether this selection is associated with protein-protein interactions. Thus, co-immunoprecipitation assays in mDPCs were performed to detect the binding ability of IPO7 to these odontogenic TFs. As shown in Fig. 5a, IPO7 could bind to DLX3, OSX, KLF4, and P-SMAD1/5 but not RUNX2 in mDPCs.

**Discussion**

The importin β family involves in the nucleocytoplasmic transport processes of various proteins. IPO7, as a member of importin β family, participates in developmental processes [20], among which it functions as importing proteins. In this research, we focused on IPO7 and examined its potential function in regulating odontoblast differentiation in mDPCs. Our results revealed that IPO7 enhanced odontoblastic differentiation of mDPCs by selectively importing odontogenic TFs, such as DLX3, OSX, KLF4, P-SMAD1/5 instead of RUNX2 (Fig. 5b).

Mouse incisors and molars were used to investigate the expression pattern of IPO7 in all developmental stages during odontoblast differentiation and we found that IPO7 displayed an increasing trend from pre-odontoblasts to mature odontoblasts, which was in consistent with the results in vitro. mRNA knockdown assay showed that IPO7 increased both mRNA and protein levels of the odontogenic markers, as well as mineralized nodule formation and ALP activity. These results indicated that IPO7 enhanced the odontoblastic differentiation of mDPCs. Similarly, it has already been proved that IPO7 regulates nuclear import of activated ERK 1/2 (extracellular signal regulated kinase) and JNK/p38 (cJun-N-terminal kinase), dysfunctions of which results in cell differentiation disorders [21–24]. In Runx2/Osx double heterozygous embryos, Ipo7 was downregulated in calvaria with reduced mineralized bones which indicates its role in bone development [25]. Previous studies also found that Ipo7 is involved in different stages of NE (neural ectoderm) differentiation [10] and overexpression of Ipo7 in drosophila influences eye and wing development [26].
Nuclear translocation of TFs is an important event in different signaling pathway, during which TFs migrate into the nucleus and control gene expression. Transcription factors DLX3, OSX and KLF4 promote odontoblastic differentiation and are crucial regulatory TFs for dentin formation [4, 5, 27]. Smad1/5/8 signaling pathway is also associated with promoting odontoblastic differentiation [28]. During the earlier stages of tooth development (from E12 to E16), RUNX2 expressed in dental mesenchyme. However, as the development proceeds (from E18 to PN14), RUNX2 expression was down-regulated in odontoblasts [5], which means RUNX2 is not essential for later odontoblast differentiation. We found that there was a significant difference concerning the subcellular distribution of the tested TFs (DLX3, OSX KLF4 and P-SMAD1/5) between the lpo7 siRNA group and the control, while the localization of RUNX2 was not affected by IPO7.

The cargo proteins binding importins are highly conserved in sequence and structure. The classical nuclear protein import mechanism proposed that importin α recognize a specific motif known as the nuclear localization signal (NLS) of proteins in the cytoplasm and then importin β1 binds to importin α to form a nuclear pore-targeting complex [29]. After translocating across the nuclear pore, the protein complex are dissociated and triggered inside the nucleus where they play physiological roles [6, 7]. The co-immunoprecipitation assay revealed that DLX3, OSX, KLF4 and P-SMAD1/5 were the cargo proteins bound with IPO7. The NLSs of RUNX2, DLX3, SMAD1/5 have already been identified [30–32]. However, RUNX2 showed no obvious physical interaction with IPO7 in the present investigation. In one way, the downregulation of Runx2 expression and/or genomic occupancy as the odontogenic program progresses may contribute to part of the inability of RUNX2 binding to IPO7. In the other way, the binding may be too weak to detect using current experiment techniques. As NPC allows facile exchange of proteins smaller than 40 kDa [6, 7] and the molecular mass of the TFs mentioned above are larger than 40 kDa except for DLX3, DLX3 may be able to diffuse into the nucleus. However, the NLS identified in DLX3 was shown to be sufficient for nuclear localization [31] and our study found that DLX3 can be imported by IPO7. Two kinds of ways may help DLX3 maintain its biological activity: passive diffusion of DLX3 to the nucleus may be sufficient for regular cell activity; however, the requirements of high level of DLX3 in nucleus during the odontogenic differentiation of mDPCs may need additional DLX3 nuclear import provided by an active IPO7 mediated process [33]. In addition to NLSs, many cargos recognized by IPO7 have the specific structures such as the proline-rich homeodomain [34], zinc fingers [35], the PAS domain [36], the leucine zipper domain [37, 38] and the phosphorylated domain [39]. The similar domains appear in the KLF4, which contains zinc fingers DNA binding domain [40]. Similarly, OSX has also been identified as a transcription factor containing zinc finger [41]. Therefore, it is predicted that IPO7 binds to DLX3, P-SMAD1/5 through the NLSs and interacts with the specific structures in KLF4 and OSX.

Conclusions

In summary, our research indicated that IPO7 promoted odontoblastic differentiation of mDPCs by selectively importing TFs essential for later odontoblastic differentiation. The future study will focus on the interaction between these TFs and IPO7 as well as the mechanism of their cooperative functions on odontoblast differentiation. Moreover, in vivo studies will be performed to further support this discovery.
**Abbreviations**

**TFs**: transcription factors  
**IPO7**: Importin 7  
**mDPCs**: mouse dental papilla cells  
**DLX**: Distal-less homeobox 3  
**OSX**: Osterix  
**KLF4**: Krüppel-like factor 4  
**KPNB**: karyopherin β  
**IPO8**: Importin 8  
**DMEM**: Dulbecco’s modified Eagle’s medium  
**FBS**: fetal bovine serum  
**PFA**: paraformaldehyde  
**BSA**: bovine serum albumin  
**PBS**: phosphate buffer saline  
**ARS**: Alizarin Red Staining  
**CPC**: cetylpyridinium chloride  
**ALP**: Alkaline phosphatase activity  
**RT-qPCR**: reverse transcription quantitative polymerase chain reaction  
**BCA**: bicinchoninic acid  
**TBST**: tris buffered saline containing Tween 20  
**HRP**: horseradish peroxidase  
**SD**: standard deviation  
**Alp**: Alkaline phosphatase  
**Dmp1**: dentin matrix acidic phosphoprotein 1
Declarations

Acknowledgments

Not applicable.

Author Contributions

Yue Zhang made contributions to data acquisition, analysis, draft and critical revision of the paper; Hao Zhang contributed to advice, analysis and discussions; G. Yuan conceived, analysed the experiments and critically revised the paper; G. Yang contributed to conception, design, analysis and critically revised the manuscript. All authors approved the manuscript and agree to be accountable for the work.

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Ethic Approval

The use of animal tissues was approved by the Animal Ethics Committee in Wuhan University (protocol 00266935).

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest related to this study.

Availability of data and materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Table 1
Oligonucleotide primer sequences utilized in RT-qPCR

| Gene | Forward primer | Reverse primer |
|------|---------------|----------------|
| Gapdh | F:TGTGTCCGTCGTGGATCTGA | R:TTGCTGGTTGAAGTCGAGGAG |
| Alp  | F:GCAGTATGAATTGAATCGGAACAAC | R:ATGCGCTGTGGCATCTCCAC |
| Dmp1 | F:ACCACAATACTGAATCTGAAAGCT | R:TGGCTGTCGTGTGGTCACTA |
| Dspp | F:GTGGGATGTCATCGCAGTCGAG | R:TGGCTTGTGGGACCTTTCA |
| Ipo7  | F:CCAGCTCAATGAAGCACAAG | R:AGTATATGGAGCGATGTCCCCT |
| Ocn  | F:CTTTGGTCACACCTAGCAGA | R:ACCTTCTTGCCCTCCTGCTT |
| Opn  | F:CCCCGTGAAAGTGACGATT | R:ATGGCTTTCTATTGGAATTGC |
| Runx2 | F:TGGTTACTGTCATGGCGGGA | R:TCTCAGATCGTTGAAACCTTGCTA |