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NOTCH1 signaling regulates the BMP2/DLX-3 directed osteogenic differentiation of dental follicle cells

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ABSTRACT

Dental follicle cells (DFCs) are dental stem/progenitor cells and the genuine precursors of alveolar osteoblasts and dental cementoblasts. A previous study showed that the transcription factor DLX3 (distal less homeobox 3) supports the osteogenic differentiation in DFCs via a positive feedback loop with the bone morghogenetic protein (BMP) 2. Until today, however, the control of this BMP2/DLX3 pathway by additional signaling pathways remains elusive. Previous studies also suggested that the NOTCH signaling pathway plays a role in the osteogenic differentiation of DFCs. In this study we showed that DLX3 overexpression and the initiation of the osteogenic differentiation by BMP2 or dexamethasone induced the NOTCH signaling pathway in DFCs. However, the induction of NOTCH-signaling impaired not only the osteogenic differentiation (ALP activity and mineralized nodules) but also the expression of the transcription factor DLX3 and the activation of the BMP-signaling pathway. So, NOTCH signaling plays a regulatory role for the osteogenic differentiation of DFCs. In conclusion, results of our study suggest that the NOTCH-signaling pathway, which is activated during the osteogenic differentiation of DFCs, regulates the BMP2/DLX3 directed differentiation of DFCs via a negative feed-back loop.

1. Introduction

For more than 10 years dental stem/progenitor cells can be isolated from human dental tissues [1–3]. Since that time dental stem cells are highly suggested for cellular therapies in dentistry and a clinical pilot study with patients suffering from periodontitis have shown the feasibility of such a therapy [4]. However, undifferentiated dental cells are also very useful for studies about cellular and molecular processes under *in vitro* conditions. Dental follicle cells (DFCs) are a good example [5–9], because they are the genuine precursors of the periodontium including alveolar osteoblasts, dental cementoblasts and periodontal fibroblasts [5,6,10,11]. Thus, DFCs were used for molecular studies about the osteogenic differentiation of periodontal precursor cells. Here, genome wide gene expression profiles were compared during different stages of the osteogenic differentiation and regulated genes were identified among others, which are linked to signaling pathways such as TGFβ/BMP, WNT or NOTCH [12–14]. Moreover, we have already shown that the transcription factor DLX3 (distal less homeobox 3) supports the osteogenic differentiation in DFCs via a BMP2 positive feedback loop [15]. Until today, however, the control of this BMP2/DLX3 pathway by further signaling pathways remain elusive.

Among other signaling pathways, which coordinate the proliferation and differentiation of mesenchymal stem cells, the NOTCH-signaling plays an important role. This pathway mediates the communication of adjacent cells that express either a NOTCH receptor or a membrane-bound NOTCH ligand such as Jagged1. The signaling of the NOTCH-pathway is activated by a receptor-ligand binding and a cleavage of NOTCH by γ secretase and translocation of the intracellular domain of NOTCH (NICD) into the nucleus, where it binds to transcription factors such as RBP-JK. The NOTCH signaling pathway regulates for example the odontogenic differentiation of dental pulp stem cells (DPSCs). Zhang et al. found that overexpression of the NOTCH ligand Jagged1 or the overexpression of the NICD inhibited the odontogenic differentiation of DPSCs [16]. Interestingly, NOTCH1 is a marker of DFCs and its expression is regulated during the osteogenic differentiation of DFCs [5,17]. Moreover, the NOTCH target genes were up-regulated after overexpression of DLX3 in DFCs [15]. These previous studies suggested that the NOTCH signaling pathway plays also a decisive role for the regulation of the osteogenic differentiation in DFCs. In this study we investigated the relation between NOTCH and the osteogenic differentiation of DFCs that is supported by the BMP2/DLX3 pathway.

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2. Materials and methods

2.1. Cell culture

Impacted human third molars were surgically removed and collected from patients with informed consent. Dental follicle cells (DFCs) were isolated as described previously [17]. Briefly, the follicle tissues were digested in a solution of collagenase type I, hyaluronidase (Sigma–Aldrich, Munich, Germany), and DNAse I (Roche, Mannheim, Germany) and seeded in cell culture dishes. DFCs were initially cultivated in Mesenchym Stem Medium (PAA, Pasching, Austria) at 37 °C in 5% CO_2. The standard cell culture medium (DMEM) was Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum, FBS (Invitrogen) and 100 μg/mL Penicillin/Streptomycin. For experiments DFCs were used at cell passage 6.

2.2. Osteogenic differentiation

DFCs were cultivated in standard cell culture medium before they were stimulated with the dexamethasone-based osteogenic differentiation medium (ODM) and with a BMP2 containing osteogenic differentiation medium (BMP2) as described previously [18]. The alkaline phosphatase activity was evaluated after 7 days of treatment with ODM, BMP2 and standard cell culture medium. DFCs were washed, lysed and a 100 mM p-nitrophenyl phosphate (Sigma) diluted 1:1 in 1× PBS were added to each sample thereafter. The liberated p-nitrophenol was measured spectrophotometrically at 405 nm. ALP activity values were normalized to total DNA concentration determined by Quant-iT PicoGreen dsDNA Assay (Invitrogen). Calcium staining of differentiated cells was made with alizarin red.

2.3. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

The RNeasy Plus Mini kit (Qiagen, Hilden, Germany) was used for the isolation of total RNA and QuantiTect Reverse Transcriptase Kit (Qiagen) was performed for cDNA synthesis. Quantitative PCR was performed with Fast Start DNA Master SYBR® Green I kit (Roche) and the LightCycler PCR 2.0 System (Roche) with the LightCycler 4.05 software for estimation of threshold cycles (Ct-value). Primer sequences can be obtained from the authors. Quantification was done with the delta/delta calculation method as previously described [19]. DFCs in standard cell culture medium was used for calibration for each qRT-PCR (relative gene expression = 1).

2.4. Transfection experiments

Two days before transfection, DFCs were seeded at a cell density of $7 \times 10^3/cm^2$. For transfection, the cells were grown until subconfluence and cultivated in standard basal medium comprising antibiotics. DFCs were transiently transfected with the plasmid pTAN1-cDNA containing the NICD1 fragment (a kind gift from Dr. Herbert Chen, Dept. of Surgery, University of Wisconsin) [20], or the DLX3 expression plasmid pCMV-V5DLX3 (pDLX3) (kindly provided from Dr. Maria I. Morasso, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland) [21]. The transfection was performed with the FUGEN HD Transfection Reagent (Roche) according to the manufacturer's recommendations. As control, an empty vector without an insert (pEV) was used. The expression of NICD1 or DLX3 in DFCs was determined by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) or Western blot after 48 h.

2.5. Luciferase assay for the RBP-JK promoter

The activity of Notch signaling pathway was measured using the transcriptional activity of the RBP-JK transcription factor, a direct downstream modulator of Notch signaling. The luciferase assay was performed with the RBP-JK Reporter Assay Kit (Qiagen, Hilden) following the reverse transfection protocol described by the manufacturers. For transfection, DFCs were seeded at a cell density of $2 \times 10^4/cm^2$ and cultivated in standard medium containing 5% FBS without antibiotics. The constructs were diluted in 25 μL Opti-MEM (Invitrogen) and combined with the Lipofectamine 2000 Transfection reagent (Invitrogen) and incubated at room temperature for 20 min to form the transfection complex. Subsequently, the latter was added to 96 well culture plates. 24 h after transfection, the signals were assayed using the Dual-Luciferase ® Reporter Assay System (Promega). The Notch activity was measured as the ratio of the RBP-JK induced Firefly luciferase and the constitutively expressed Renilla luciferase activity. Three biological replicates were analyzed for each condition and the data were presented as the means ± S.E. ($\sigma/\sqrt{n}$).

2.6. Western blotting

For protein extraction DFCs were harvested with trypsin and treated with lysis buffer (1 mM Na-Orthovanadate, 150 mM NaCl, 1 mM EDTA and 1% NP-40, Protease-Inhibitor tablets (complete mini, Roche)). Aliquots of 25 μg protein extracts in SDS sample buffer were separated by SDS–polyacrylamide electrophoresis in 12% Tris–Glycine gels (Invitrogen) and transferred to nitrocellulose membranes. The membranes were blocked with skimmed milk and were incubated with primary antibody either for β-Actin (Novus Biologicals), cleaved NOTCH1, NOTCH1, Phospho-Smad1/5 (pSMAD1) (Cell Signaling) or DLX3 (Abnova) at 4 °C overnight. After washing membranes were incubated either with a biotinylated anti-mouse IgG or a biotinylated anti-rabbit IgG with avidin-conjugated horseradish peroxidase (HRP). The detection was performed by chemiluminescence (Pierce).

3. Results and discussion

3.1. DLX3 induces NOTCH signaling

NOTCH1 is a specific marker for DFCs and Chen et al. disclosed that NOTCH1 signaling regulates the proliferation and self-renewal of DFCs by modulating the G1/S phase transition and telomerase activity [22]. Previous studies also suggested that NOTCH1 plays a role during the osteogenic differentiation of DFCs [5,17], but little is known about the role of this important signaling pathway. Therefore we investigated the NOTCH signaling pathway in DFCs during the osteogenic differentiation, which is directed by the BMP2/DLX3 pathway, for the first time.

DFCs were transfected with the pDLX3 plasmid to evaluate whether the NOTCH signaling pathway is induced down-stream from the transcription-factor DLX3. Typical markers for the activated NOTCH-signaling such as cleaved NOTCH1 protein were induced after DLX3 overexpression (Fig. 1A and B). These results showed that DLX3 induced the NOTCH signaling pathway in DFCs. This was expected, because DLX3 induced NOTCH signaling pathway associated genes in a previous study with DFCs [15]. Moreover, the NOTCH signaling pathway was slightly activated in DFCs during the osteogenic differentiation (Fig. 1C). These results are in accordance with that of a previous study of our group [17] and support the assumption of that NOCTH1 signaling plays a role in DFCs during the osteogenic differentiation.

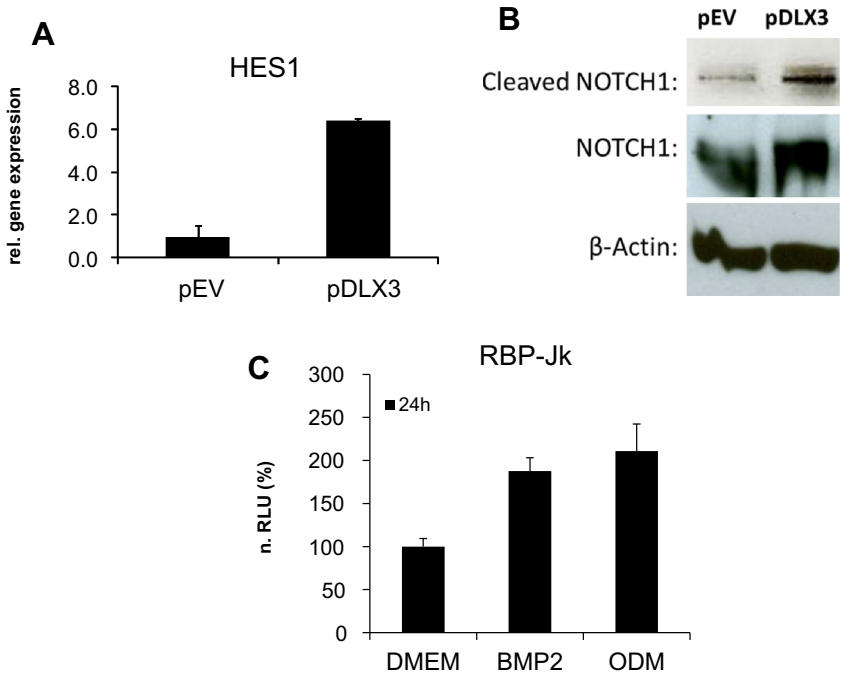

Fig. 1. NOTCH-signaling activation in DFCs after overexpression of DLX3 (A and B) and after the induction of the osteogenic differentiation (C). (A) Real-Time RT-PCR analysis of the NOTCH target gene HES1 performed after 48 h after transfection with the empty vector (pEV) and a DLX3 expression plasmid (pDLX3). (B) Western blot analysis with specific antibodies for cleaved NOTCH1, NOTCH1 and β-Actin, which was used as a housekeeper protein, after 48 h after transfection with pEV and pDLX3. (C) Luciferase assay for the RBP-JK promoter after 24 h after induction of the osteogenic differentiation with BMP2 and ODM. The luciferase activity of DFCs in a standard cell culture medium (DMEM) was used for calibration.

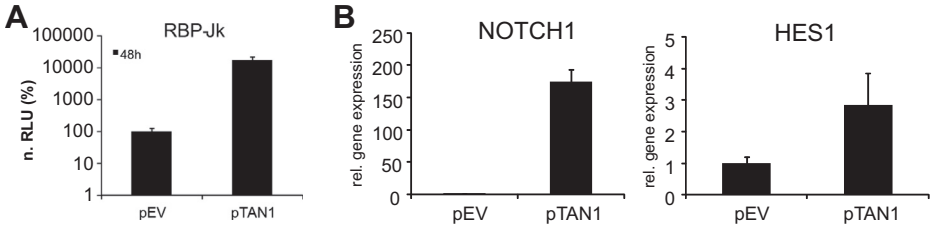

Fig. 2. NOTCH-signaling activation in DFCs after overexpression of NICD with the expression plasmid pTAN1. (A) Luciferase assay for the RBP-JK promoter after 48 h of transfection with the plasmid pTAN1 or pEV for control. The luciferase activity in a negative control with a non-inducible Firefly luciferase construct was used for calibration (not shown). (B) Real-Time RT-PCR analysis of NOTCH1 and HES1 performed 48 h after transfection with pEV and pTAN1.

3.2. Notch-signaling impairs the osteogenic differentiation of DFCs

In a next step of this study we investigated the impact of the NOTCH pathway on the osteogenic differentiation of DFCs. The plasmid pTAN1 was transferred into DFCs for the induction of the NOTCH signaling pathway (Fig. 2). This transfection induced the NOTCH signaling in DFCs that could be shown by the induction of the RBP-JK Reporter Assay and the expression of NOTCH1 and of HES1 (Fig. 2A and B). NOTCH signaling impaired the ALP activity and the formation of mineralized nodules 4 weeks after the induction of the osteogenic differentiation with both BMP2 and dexamethasone-based osteogenic differentiation media (Fig. 3).

These results suggest that NOTCH1 impairs the osteogenic differentiation of DFCs. This conclusion is in accordance with that of previous studies about the osteogenic differentiation of mesenchymal stem cells. The NOTCH-signaling pathway inhibits for example the osteogenic differentiation of the MC3T3 cell line, whose robust differentiation was documented cytochemically and molecularly before [23]. Here, Hey1, which is induced via the activation of Notch-signaling, abrogated the transcriptional activity of the main osteogenic transcription factor Runx2. In a different study, lipopolysaccharides from the oral pathogen *Porphyromonas gingivalis* inhibits of osteogenic differentiation of mesenchymal stem cells via the activation of NOTCH1 [24]. Here, the expression of HES1 and HEY1 was increased after cultivation in osteogenic differentiation medium supplemented with lipopolysaccharides.

3.3. Notch-signaling impairs the BMP2/DLX3 pathway

After we have shown that NOTCH inhibits the osteogenic differentiation of DFCs, we investigated whether this signaling-pathway inhibits also the activation of the BMP2/DLX3 pathway in DFCs. The activation of NOTCH impaired not only the expression of the transcription factor DLX3 but also the activation of the BMP-signaling pathway (phosphorylation of SMAD1, pSMAD1) in DFCs (Fig. 4A and B). This results stands in contrast to a previous study of Nobta et al. [25]. Here authors showed that a functional NOTCH signaling was essential for the BMP2 induced osteogenic differentiation of mesenchymal cell lines MC3T3 and C2C12. Our results suggest that the NOTCH-signaling pathway negatively regulates the BMP2/DLX3 pathway and probably via a negative feedback loop. However, further studies are required to disclose the exact

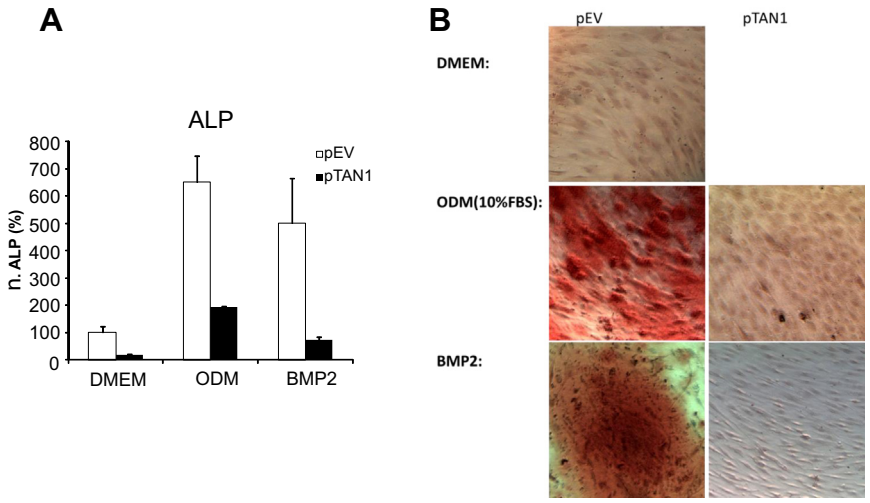

Fig. 3. NOTCH-signaling impairs the osteogenic differentiation of DFCs. (A) ALP-activity of DFCs 2 weeks after the induction of the osteogenic differentiation; bars are means of at least three biological replicates ± S.E. ($\sigma/\sqrt{n}$). (B) Alizarin red staining of DFC long-term cultures 4 weeks after the induction of the osteogenic differentiation. DFCs were transfected with pEV and pTAN1 72 h before the induction of the osteogenic differentiation. The osteogenic differentiation of DFCs was induced either with ODM or BMP2 differentiation medium.

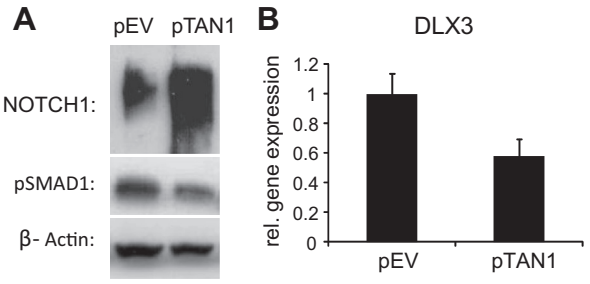

Fig. 4. NOTCH-signaling impairs the activation of BMP-signaling and the expression of DLX3. (A) Western blot analysis with specific antibodies for cleaved NOTCH-1, pSMAD1 and β-Actin, which was used as a housekeeper protein, 48 h after transfection with pEV and pTAN1. (B) Real-Time RT-PCR analysis of DLX3 performed after 48 h after transfection with pEV and pTAN1.

relationship between the NOTCH-signaling pathway and the regulation of the BMP2/DLX3 directed osteogenic differentiation of DFCs. A previous study for example showed that the differentiation of osteoblasts is regulated positive by NOTCH and that NOTCH could be an interesting target molecule for the treatment of osteoporosis [26]. Nonetheless this study contradicts our observations and that of previous studies about NOTCH signaling and the osteogenic differentiation [16,23,24]. We suggest that NOTCH plays a central role in the negative regulation of the osteogenic differentiation of somatic stem cells.

In conclusion, our study revealed that the NOTCH-signaling pathway is activated after the induction of DLX3. The activation of NOTCH in turn decreases the expression of DLX3 and the osteogenic differentiation of DFCs. However, further studies are required to reveal more details about NOTCH-signaling and the regulation of the osteogenic differentiation. These studies will also disclose new information about the regulation of the BMP2/DLX3 pathway.

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