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The WNT inhibitor APCDD1 sustains the expression of β-Catenin during the osteogenic differentiation of human dental follicle cells

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ABSTRACT

In hair follicle cells APCDD1 inhibits the canonical WNT/β-Catenin pathway and its inactivation is associated with an autosomal dominant form of hair loss. We analyzed the role of APCDD1 for the osteogenic differentiation in dental follicle cells (DFCs) and identified a new and surprising function. Contrarily to hair follicle cells APCDD1 was crucial for the expression of β-Catenin and for the activity of the TCF/LEF reporter assay in DFCs. In addition, a depletion of APCDD1 inhibits the expression of osteogenic markers such as RUNX2 and decreased the matrix mineralization. However, similar to hair follicle cells in previous studies a control cell culture with oral squamous carcinoma cells showed that APCDD1 inhibits the expression of β-Catenin and of typical target genes of the canonical WNT/β-Catenin pathway. In conclusion, our data disclosed an unusual role of APCDD1 in DFCs during the osteogenic differentiation. APCDD1 sustains the expression and activation of β-Catenin.

1. Introduction

Adenomatosis Polyposis Coli Down-Regulated 1 (APCDD1) is a membrane protein that is associated with the WNT pathway [1,2]. A protein complex of β-Catenin and TCF4 regulates transcriptionally APCDD1 in human tumor cells [1]. Moreover, Shimomura and co-workers described APCDD1 as a novel WNT inhibitor mutated in hereditary hypotrichosis simplex, a rare autosomal dominant form of hair loss [1].

APCDD1 is expressed during tissue development and in diverse adult tissue cell types [3,4], but the specific role of APCDD1 remains elusive. Interestingly the expression of *APCDD1* is induced in neural-crest derived precursor cells from the dental follicle (DFCs), if gene expression is compared to that of mesenchymal stem cells (MSCs) [5]. Moreover, *APCDD1* was significantly induced in DFCs in osteogenic differentiated DFCs after overexpression of the transcription factor DLX3 [published microarray study GSE59949]. DLX3 is a homeobox containing transcription factor and plays an essential role in skeletal development of vertebrates and during the osteogenic differentiation of mineralizing tissue cells such as osteoblasts [6–8]. Moreover, this transcription factor is regulated in DFCs through a BMP2-feedback mechanism during the osteogenic differentiation [9].

Previous studies suggested that regulatory mechanism for the osteogenic differentiation of DFCs are unique in comparison to that of MSCs, which were derived from bone marrow or from other dental tissues such as stem cells from human exfoliated deciduous teeth (SHED) [10–12]. For example, previous studies reported that the canonical WNT-signaling pathway inhibits the osteogenic differentiation in DFCs, while WNT induces the osteogenic differentiation in bone marrow derived stem cells [13,14]. Silverio et al. showed that β-Catenin was crucial for the osteogenic differentiation in DFCs, but the WNT independent induction of β-Catenin remains elusive [14]. Interestingly, an analysis of a previous microarray study revealed that APCDD1 is also up-regulated in DFCs after the osteogenic differentiation with dexamethasone [15]. Our new study showed that APCDD1 sustains the expression of β-Catenin during the osteogenic differentiation of DFCs.

2. Materials and methods

2.1. Cell culture

Human dental follicle cells (DFCs) were isolated from the dental follicle, obtained from impacted human third molars surgically removed and collected from patients with informed consent.

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The isolation of DFCs was performed as described previously [16]. Briefly, the follicle tissues were digested with a medium containing collagenase type I, hyaluronidase (Sigma–Aldrich, Taufkirchen, Germany), and DNase I (Roche, Mannheim, Germany) for 1 h at 37 °C in 5% CO_2. The digested tissue suspension was seeded into T25 flasks in standard culture medium (see below) with antibiotics at 37 °C in 5% CO_2.

Oral squamous carcinoma cells were cultivated as previous described [17].

The standard culture medium (DMEM) was Dulbecco's Modified Eagle Medium (Sigma–Aldrich) supplemented with 10% fetal bovine serum, FBS (Sigma–Aldrich) and 100 μg/mL Penicillin/Streptomycin. DFCs at passage 6 were used for experiments.

2.2. Osteogenic differentiation

DFCs were cultivated until sub-confluence (>80%) in standard cell culture medium before the osteogenic differentiation was stimulated with 10 ng/ml BMP2 (Biomol) in a Dulbecco's Modified Eagle Medium (DMEM) (Sigma–Aldrich) based cell culture medium comprising 1% fetal bovine serum (Sigma–Aldrich), 100 μmol/l ascorbic acid 2-phosphate, 10 mmol/l KH_2PO_4, HEPES (20 mmol/l) and 100 μg/ml Penicillin/Streptomycin. Differentiation of DFCs was determined by quantification of Alkaline Phosphatase (ALP) activity (see below) and Alizarin Red staining, which was previously described [15].

2.3. Transfection experiments

The siRNAs targeting human APCDD1 mRNA (Gene Bank No. NM_153000) or non specific mRNAs (NS) were purchased from Qiagen or non specific (NS) for controls. DFCs were seeded at a cell density of $7 \times 10^3/cm^2$ and cultivated until sub-confluence in standard cell culture medium 1 day before transfection with siRNAs. The cells were transfected with 50 nM of the respective siRNAs using Hiperfecta (Qiagen) reagent according to the manufacturer's instructions. After 48 h, RT-qPCR and Western Blot analyses determined the expression of APCDD1 in DFCs.

2.4. Luciferase assay

The activation of the WNT/β-Catenin signaling was measured using the transcriptional activity of the TCF/LEF transcription factor, a direct downstream modulator of WNT signaling. The luciferase assay was performed with the TCF/LEF Reporter Assay Kit (Qiagen, Hilden), which contains a TCF/LEF luciferase reporter construct as wells as a negative control (non-inducible firefly luciferase reporter construct), a positive control (constitutively expressing firefly luciferase construct), following the reverse transfection protocol described by the manufacturers. For co-transfection with APCDD1 siRNA, DFCs were seeded at a cell density of $1.5 \times 10^4/cm^2$ and cultivated in standard basal 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. After 24 h of transfection signals were assayed using the Dual-Luciferase® Reporter Assay System (Promega). The ratio of luciferase activity in the control (NS siRNA) was used for calibration. Three biological replicates were analyzed for each condition and the data were presented as the means ± S.E. ($\sigma/\sqrt{n}$).

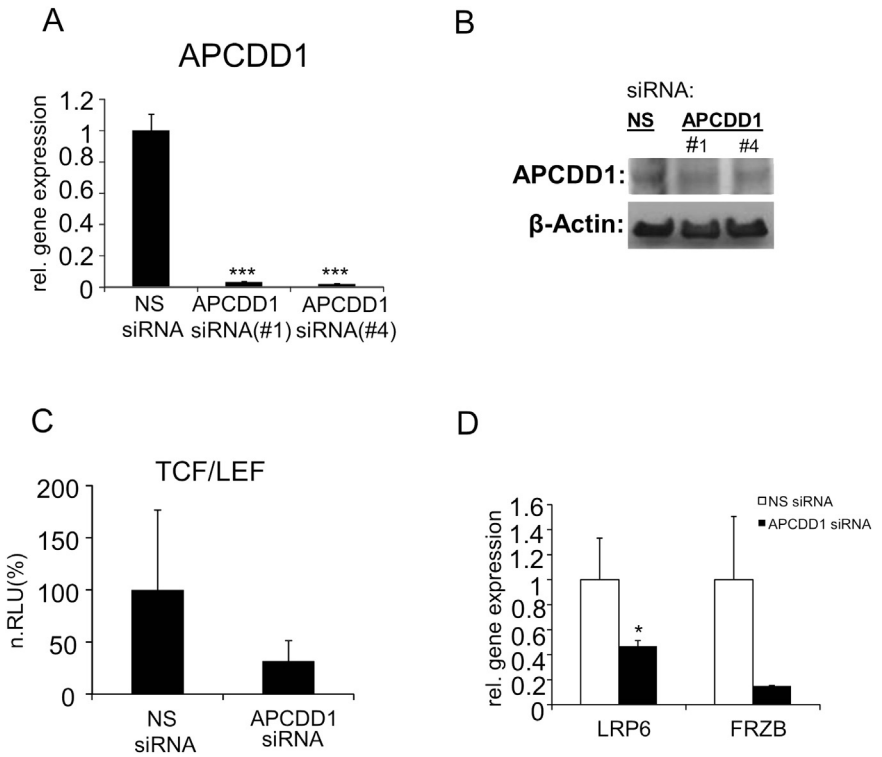

Fig. 1. APCDD1 induces LEF1/TCF activity and WNT-target genes. Depletion of APCDD1 in DFCs was verified with RT-qPCRs after 48 h (A) and by immunoblotting after 72 h (B) of transfection with APCDD1 siRNAs (1 or 4) or NS siRNA. WNT-signaling was evaluated with a luciferase LEF1/TCF-reporter assay in APCDD1 depleted DFCs after 48 h of transfection (C). Evaluation of WNT-target genes in APCDD1 depleted DFCs by RT-qPCRs after 48 h (D) of transfection with the siRNAs (Control = NS siRNA). For calibration served control DFCs (transfected with NS siRNA). All values are means ± SEM of three biological replicates. Significant differences are shown with the Student's t test (*: $p < 0.05$ and ***: $p < 0.0125$).

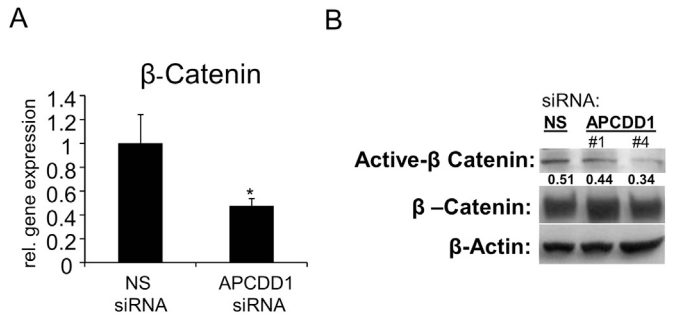

Fig. 2. APCDD1 induces the WNT/β-Catenin pathway in DFCs. Analysis of *β-Catenin*-expression by RT-qPCRs after 48 h (A) of transfection with APCDD1 siRNA (# 4) or NS siRNA. All values are means ± SEM of three biological replicates. Significant differences are shown with the Student's t test (*: $p < 0.05$). Western blots with whole lysates of DFCs after 72 h depletion of APCDD1 with specific siRNAs (# 1 and # 4) and antibodies direct against active-β-Catenin, β-Catenin, and β-Actin (B).

2.5. Measurement of alkaline phosphatase (ALP) activity

The osteogenic differentiation potential was evaluated after 10 days of cultivation in differentiation medium with BMP2 or standard culture medium (s. above). DFCs were washed with PBS buffer, disrupted by freezing −80 °C and thereafter 60 μl of each sample were mixed with 60 μl of 1.5 M alkaline buffer (Sigma) and 100 μl of 100 mM p-nitrophenylphosphate (Sigma). After incubation at 37 °C for 60 min, the reaction was stopped by adding 300 μl of 0.3 M NaOH and the liberated p-nitrophenol was measured spectrophotometrically at 405 nm. ALP activity values were normalized to total DNA concentrations determined by Quant-iT PicoGreen dsDNA Assay (Invitrogen). At least three biological replicates were analyzed for each condition and the data were presented as the means ± S.E. ($\sigma/\sqrt{n}$).

2.6. Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cells by using RNeasy isolation kit (Qiagen). The cDNA synthesis was performed using ~500 ng total RNA and QuantiTect Reverse Transcriptase Kit (Qiagen). Quantitative RT-PCR (RT-qPCR) was performed with Fast Start DNA Master SYBR® Green I kit (Roche) or the Light Cycler TaqMan kit (Roche) and the Light Cycler PCR System (LightCycler 480 II, Roche). For normalization, GAPDH gene expression was used as a control. Primer pairs used for RT-qPCR are listed on (Table S1).

2.7. Western blotting

For protein extraction, DFCs were washed with PBS at indicated points in time and harvested by trypsin-EDTA treatment. After extensively washing with cell culture medium and PBS to eliminate trypsin, cells were treated with lysis buffer (20 mM Tris, 48 mM NaF, 150 mM NaCl, 2 mM Na-Orthovanadate, 1% NP-40 and 10% Glycerol) and freshly added protease (Roche) and phosphatase (Sigma) inhibitor cocktail on ice for 30 min. An aliquot of 20 μg protein extract was denatured and reduced by boiling in lithium dodecyl sulfate (LDS) sample buffer containing DTT, separated by SDS-polyacrylamide electrophoresis in NuPAGE® Novex® 4–12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes. The membranes were blocked with skimmed milk and incubated for 60 min at room temperature followed by incubation with primary antibody for APCDD1 (Abcam), Active-β-Catenin (Millipore), and β-Catenin (Cell Signaling) at 4 °C overnight. After washing the membranes were incubated either with a biotinylated anti-mouse IgG or a biotinylated anti-rabbit IgG for 60 min followed by incubation with avidin-conjugated horseradish peroxidase (HRP). Detection was performed by chemiluminescence (Pierce). For densiometric evaluation of immunoblots were used the ImageJ software.

3. Results and discussion

APCDD1 is an inhibitor of the WNT/β-Catenin signaling pathway and transcriptionally regulated by β-Catenin [1,2,18]. This WNT-inhibitor may play an important role during embryogenesis, is associated with the hereditary hypotrichosis simplex and differentially expressed in diverse tissues such as the embryonic eyelid epithelium [1,4].

We proposed that APCDD1 is involved in the BMP2/DLX3 directed osteogenic differentiation of DFCs. This assumption was supported by microarray studies of our group with differentiated DFCs ((GSE59949); [15]). A loss of function assay was done to determine the role of APCDD1 in DFCs. Here, we showed an almost completely inhibition of the mRNA expression (Fig. 1A) and an almost complete depletion at the protein level of APCDD1 (Fig. 1B) with two different specific siRNAs for APCDD1 (#1 and #4). Although APCDD1 was reported as a Wnt inhibitor [1,19], APCDD1 sustains the WNT/β-Catenin pathway in DFCs (Figs. 1C,D and 2). The TCF/LEF promoter activity in DFCs decreased after depletion of APCDD1 (Fig. 1C). Furthermore, the expression of WNT-target genes such as LRP6 and FRZB were down-regulated after inhibition of APCDD1 (Fig. 1D). However, for control, we investigated the APCDD1 depletion in squamous cell carcinoma cells (PCI). Here, the gene expression of the WNT-target genes LRP6, FRZB, and LEF1 was

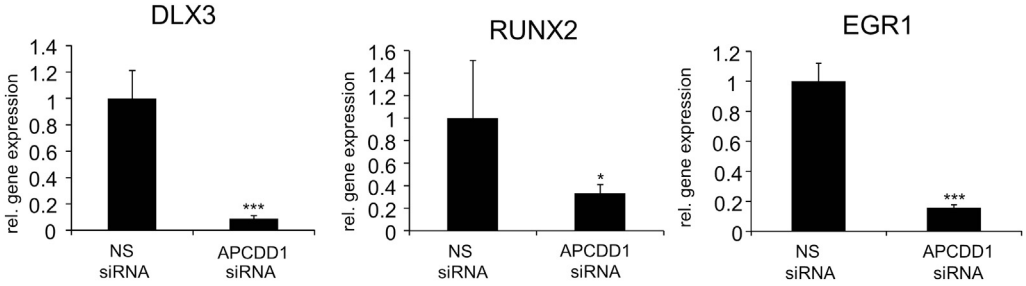

Fig. 3. APCDD1 induces the expression of osteogenic markers. The relative gene expression of DLX3, RUNX2 and EGR1 was down-regulated in DFCs after depletion of APCDD1. Total RNAs were assayed by RT-qPCRs after 48 h transfection with APCDD1 siRNA (4) or NS siRNA. Control cells (DFCs transfected with NS siRNA in DMEM) were used for calibration. All values are means ± SEM of at least three biological replicates. Significant differences are shown with the Student's t test (*: $p < 0.05$ and ***: $p < 0.0125$).

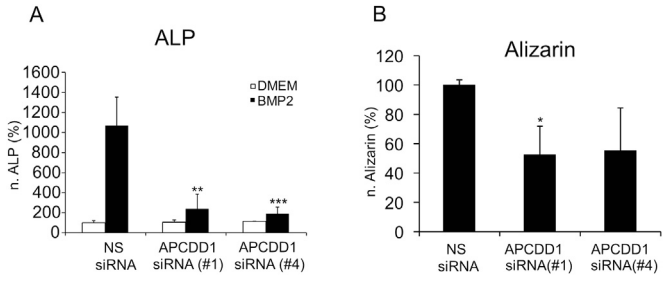

Fig. 4. APCDD1 controls osteogenic differentiation in DFCs. After depletion of APCDD1 the alkaline phosphatase activity (A) and mineralization by quantification of alizarin red (B) were measured in DFCs after ten days osteogenic differentiation without (DMEM) or with BMP2. Control cells (DFCs transfected with NS siRNA in DMEM) were used for calibration. All values are means ± SEM of at least three biological replicates. Significant differences are shown with the Student's t test (*: $p < 0.05$; **: $p < 0.025$ and ***: $p < 0.0125$).

increased (Fig. S1B). Interestingly, the depletion of APCDD1 increased the expression of β-Catenin and the active form of β-Catenin in PCI cells (Fig. S1A), but decreased the expression of β-Catenin and the coefficient active-β-Catenin/β-Catenin from 0.51 (for NS siRNA) to 0.44/0.34 (APCDD1 siRNA #1/#4) in DFCs (Fig. 2A). These data consider that in contrast to PCI cells or neurons, APCDD1 sustains the expression and/or activation of β-Catenin in DFCs.

Next we examined the role of APCDD1 for the osteogenic differentiation in DFCs (Figs. 3 and 4). Here we showed that a depletion of APCDD1 inhibits the expression of the osteogenic differentiation markers (Fig. 3). Furthermore, the loss of APCDD1 expression hampers the ALP activity (Fig. 4A) and the capability of matrix mineralization (Fig. 4B) of differentiated DFCs. Our results are in accordance with that of a previous with rat DFCs [14]. Here the expression of β-Catenin was crucial for the osteogenic differentiation of rat DFCs [14].

APCDD1 seems to have diverse functions in different cell types. A comparison between the microarray results from MSCs and DFCs indicated that APCDD1 showed a greater than 41-fold intensity in DFCs than in MSCs [5]. While in MSCs, APCDD1 was associated with osteoclastogenesis [20], our study suggest that APCDD1 can be associated with the osteogenic differentiation in DFCs. A depletion of APCDD1 decreased the expression of DLX3, RUNX2 as well as of EGR1, which are essential markers for the osteogenic differentiation of DFCs [21]. We concluded that APCDD1 plays an unusual role in DFCs during the osteogenic differentiation; it induces the osteogenic differentiation possibly via the activation of β-Catenin. However, further studies have to evaluate molecular processes of the activation of β-Catenin and the induction of osteogenic differentiation in DFCs by APCDD1.

Conflict of interest

None.

Transparency document

The transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrc.2014.12.107.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2014.12.107.

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