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ABSTRACT

Twist1 plays a crucial role in dentinogenesis, and its activity depends on both a dimerization partner selection and phosphorylation. Other factors, like Id proteins, can affect the availability of dimerization partners for TWIST1, subsequently leading to diverse biological outcomes. The purpose of this study was to evaluate an impact of *Id1* expression on differentiation of dental pulp stem cells (DPSCs). The altered expression of *Id1* was achieved by transfection of human DPSCs with lentiviral vectors either driving an entire sequence of *Id1*, hence leading to *Id1* overexpression, or carrying the *Id1* silencing sequence. We observed that both overexpression and silencing of *Id1* modulated human DPSC differentiation. *Id1* overexpression resulted in a prevailing formation of TWIST1 homodimer and increased expression of genes encoding dentin sialophosphoprotein and dentin matrix protein 1, which confirm an enhanced odontogenic differentiation of DPSCs. Concurrently, *Id1* silencing produced an opposite effect, slowing DPSC differentiation. These results highlight *Id1* as an important modulator of molecular events during DPSC commitment and differentiation, which should be considered in dental research on tissue engineering. Moreover, we assume that the balance between TWIST1 dimerization forms in DPSCs might function in a cell-type-specific manner.

KEY WORDS: basic-helix-loop helix proteins, dentin sialophosphoprotein, protein dimerization, dentin, cell differentiation, odontoblast.

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Id1 Expression Level Determines the Differentiation of Human Dental Pulp Stem Cells

INTRODUCTION

Twist1 is a transcription factor belonging to the Class B bHLH family, which modulates mesenchymal cell allocation and behavior (Rice *et al.*, 2000; O'Rourke and Tam, 2002). Twist1 is activated by dimerization, and Twist1 homodimers (T/T) and heterodimers with E2 proteins (T/E) co-exist in the cells, although they regulate the expression of different genes (Connerney *et al.*, 2006). Accumulated evidence indicates that the T/E heterodimer maintains an immature phenotype and represses both chondrogenesis and osteogenesis (Isenmann *et al.*, 2009), while recent studies documented that both partner dimer selection and Twist1 phosphorylation are responsible for diverse biological functions of Twist1 (Connerney *et al.*, 2006; Firulli and Conway, 2008). The picture becomes even more complicated by the involvement of Id proteins affecting the availability of Twist1 dimerization partners. The Id proteins (Inhibitors of differentiation), which cannot bind to DNA, form the non-functional dimers principally with E2 proteins (Benezra *et al.*, 1990; Massari and Murre, 2000), thus sequestering the pool of E2 proteins available for dimerization with Twist1 (Sun and Baltimore, 1991; Sun *et al.*, 1991). Consequently, the Twist1 homodimer prevails over the heterodimer, which changes the ratio between T/E and T/T and subsequently initiates a production of transcription drivers for diverse downstream genes (Connerney *et al.*, 2008). Bhattacharya and Baker (2011) showed that the magnitude of *Id1* and E2a expression acts in the specific feedback loop and thus might define the precise threshold for differentiation in response to signaling from class B proteins.

Besides a developmental profile of Twist1 expression in dental mesenchyme (Rice *et al.*, 2005), there are limited data indicating the precise function of Twist1 in dentin formation. An *in vitro* study showed that TWIST1 overexpression in dental pulp stem cells (DPSCs) resulted in the increased expression of dentin sialophosphoprotein (DSPP), which implicates TWIST1 function in DPSC commitment to mineralization (Li *et al.*, 2011). Moreover, analysis of the the newest developmental data suggests that TWIST1 is indispensable in odontogenesis and cannot be compensated for (Zhang *et al.*, 2012).

In evaluation of the sophisticated pattern of Twist1 and Id1 expression in the cranial suture, palate, and a tooth, it becomes clear that these 2 proteins acting in concert mandate diverse downstream signaling and initiate the expression of diverse downstream genes in cells of mesenchymal origin (Rice *et al.*, 2005; Connerney *et al.*, 2006). There is no information concerning the impact of Id1 expression change on DPSC behavior. Since it has been shown, in osteoblasts, that Id1 forces Twist1 to form homodimers, which subsequently change the expression profile of downstream genes, we initiated this

study to elucidate whether the same mechanism works in mesenchymal dental cells or displays a cell-specific pattern.

MATERIALS & METHODS

Patients and Samples

Teeth for isolation of dental pulp stem cells (DPSCs) were obtained from patients (13-22 yr old) undergoing routine extractions in the Department of Oral Surgery, Medical University of Gdansk. The Institutional Review Board at the Medical University of Gdansk approved all procedures (NKEBN/427/2009-2010), and written consent was obtained from all patients.

Cell Isolation and Culture

DPSCs were isolated from unerupted wisdom teeth as described previously (Gronthos *et al.*, 2000). Obtained cells were cultured in DMEM supplemented with 15% fetal bovine serum, 2% L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin under standard conditions.

Cell Selection

DPSCs with the phenotypes STRO-1^{+/+}, CD146^{+/+}, CD34^{-/-}, and CD45^{-/-} were obtained by positive selection with anti-STRO 1 and anti-CD146 antibodies (R&D Systems, Minneapolis, MN, USA), followed by a negative selection with mouse anti-human CD34 and CD45 antibodies (R&D Systems) with magnetic nanoparticles (MagCelect™) coated with goat anti-mouse IgG ferrofluid according to the manufacturer's protocol (R&D Systems).

Lentivirus Transfection-driven *Id1* Overexpression

The human *Id1* cDNA (GenBank, Accession No. X77956) was obtained by polymerase chain reaction (PCR) with the primers AGCTTTGTTTAAACGGCGCGCCGGAGAATCATGAAA GTCGCCAGT (forward) and CGCGGATCCGCGGGCGCT TCAGCGACA CAAGAT (reverse), which introduced the cleavage sites (underlined) for restriction enzymes *PmeI* and *BamHI*, respectively. cDNA was cloned into the pWPI-GFP lentiviral vector. The empty pWPI-GFP vector was used as a negative control. Packaging cells (HEK 293T) were co-transfected with either empty pWPI-GFP or pWPI-GFP-*Id1* lentiviral plasmid, an envelope plasmid pMD2.G, and a packaging plasmid psPAX2 with X-treme Gene 9 (Roche, Mannheim, Germany), according to the manufacturer's protocol. The

Figure 1. Lentivirus-mediated expression of *Id1* in human DPSCs. **(A)** DPSCs transfected with the pWPI-GFP-*Id1* lentiviral vector (*Id1*, above) and in DPSCs transfected with the pLL3.7-*shId1* vector carrying the *Id1* silencing sequences (*shId1*) visible in normal light (upper panels) and under a fluorescent microscope (lower panel). **(B)** Quantitative real-time polymerase chain reaction analysis of *Id1* transcript levels in DPSCs. Data were normalized to *GAPDH* mRNA levels and are presented as fold-change relative to control DPSCs transfected with an empty vector or vector carrying non-targeting shRNA. The data represent the mean values $\pm$ SD ($n = 5$). * $p < .05$ vs. control. **(C)** The *Id1* protein level in DPSCs. The proteins in the cell extract were separated by SDS-PAGE and immunoblotted with appropriate antibodies. β -actin was used as a reference protein. The blots presented are representative of those obtained in 4 experiments performed on unique cell cultures derived from four different patients.

culture medium was used as the source of lentiviral particles for DPSC transfection. The efficiency of DPSC transfection was assessed by the observation of a GFP expression, under a fluorescent microscope (Leica, Wetzlar, Germany). The DPSCs were cultured with the infectious medium until at least 80% of the population became GFP-positive (Fig. 1A).

Lentivirus Transfection-driven *Id1* Knockdown

The *Id1* silencing sequence (sh RNA) TGACGTGCTG CTCTACGACATTTCAGAGAATGTCGTAGAGCAGCAC GTCTTTTTTC (Oligos, Warsaw, Poland) was subcloned into the 3.7 pLL lentiviral vector. The empty 3.7 pLL vector was used as a negative control. Each construct, together with pMD2.G plasmid and a packaging plasmid psPAX2, was used to transfect the HEK 293T cells as described above. The culture media were used for DPSC transfection. The DPSCs were used for analysis after at least 80% of the cell population became GFP-positive (Fig. 1A). All backbone vectors were gifts from Dr. Trono and/or Dr. Parijs, Addgene, Cambridge, USA.

qRT-PCR

The level of *Id1* expression in the transfected DPSCs was assessed with qPCR. Total RNA was isolated from DPSCs with STAT-60 (AMS Biotechnology, Abingdon, UK) according to the manufacturer's protocol. The gene expression level in the transfected DPSCs was assessed by quantitative PCR with a QuantiTect® SYBR Green PCR kit (Qiagen, Hilden, Germany). The primer sequences used are shown in the online Appendix Table. Transcript levels were normalized to that of *GAPDH* by the $2^{-\Delta C_t}$ method. All reactions were performed in triplicate and validated by the presence of a single tip in the melting curve.

Western Blot

Immunoblotting was performed on a cell extract (Sakowicz-Burkiewicz *et al.*, 2013) subjected to 4 to 20% resolving SDS-PAGE gel electrophoresis. The proteins separated were electrophoretically transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 3% bovine serum albumin, incubated overnight with an appropriate primary antibody, *i.e.*, polyclonal anti-DSP LF-154 (1:500), polyclonal anti-DMP1 LF-148 (1:500), mouse anti-Periostin (R&D) (1:500), mouse anti-osteocalcin (R&D) (1 µg/mL), rabbit anti-TWIST1 (Abcam, Cambridge, MA, USA) (2.5 µg/mL), or goat anti-ID1 (1:500) (R&D), followed by washing and incubation with a secondary antibody. Both the anti-DSP and anti-DMP1 antibodies were kindly provided by Dr. Larry Fisher, National Institutes of Health/National Institute of Dental and Craniofacial Research (Bethesda, MD, USA). β -actin (1:10,000) (Sigma-Aldrich, St. Louis, MO, USA) was used as an internal reference protein.

Proliferation Analysis

Cell proliferation was measured with a Quick Cell Proliferation Assay Kit (Gentaur, Gdansk, Poland) according to the manufacturer's instructions. The experiment was done in triplicate and repeated 3 times.

Alkaline Phosphatase Activity Analysis

The activity of alkaline phosphatase (ALP) was determined with the QuantiChrom™ Alkaline Phosphatase Assay Kit (Gentaur) according to the manufacturer's recommendation. The absorbance (405 nm) was measured right after mixing and 5, 10, and 15 min thereafter with the Victor 3 plate reader (Perkin Elmer, Waltham, MA, USA). ALP activity was calculated according to an equation given by the manufacturer. The experiment was done in triplicate and repeated twice.

Calcium Concentration Analysis

For the analysis of intracellular calcium concentration, the cell extract was used. The procedure was performed with the QuantiChrom™ Calcium Assay Kit (Gentaur) according to the manufacturer's recommendation. Calcium concentration was calculated according to the slope derived from standards included in the kit. The experiment was performed in triplicate and repeated twice.

Determination of Mineral Deposit Formation by von Kossa Staining

DPSCs were cultured for 2 wk in DMEM supplemented with dexamethasone (10 nM), β -glycerophosphate (10 mM), and ascorbic acid (50 µg/mL). Next, DPSCs were fixed with ice-cold methanol and stained with 5% silver nitrate at a 50-cm distance from the UV lamp for 30 min. The background was counterstained with Alizarin Red.

Statistical Analysis

Values are reported as means $\pm$ SD. 'N' indicates the number of experiments performed on unique cell cultures derived from different patients. Differences between and among groups were assessed by Student's *t* test. $p < .05$ was considered as significant.

RESULTS

Id1 Level Affects the Expression of Dentin-related Genes

Our analyses showed that transfection of DPSCs with pWPI-GFP-Id1 lentiviral plasmid increased the level of *Id1* transcript over 55-fold, followed by elevated content of Id1 protein. Conversely, the *Id1* silencing procedure reduced the *Id1* transcript level by ten-fold (Figs. 1B, 1C). The qPCR analysis showed that *Id1* overexpression caused an insignificant increase of *TWIST1* and remarkable increases of dentin matrix protein 1 gene (*DMP1*) and dentin sialophosphoprotein gene (*DSPP*) expression, along with a noticeable fall of osteocalcin (*OC*) and periostin (*OSF2*) gene expression (Figs. 2A, 2B). In contrast to *Id1* overexpression, *Id1* shutdown significantly diminished the expression of all genes but not *OC*. These alterations were discernible at the mRNA level and a little less noticeable at the protein level (Figs. 2A, 2B).

Id1 Changes DPSC Commitment and Differentiation

Searching for the symptoms of DPSC commitment and differentiation, we measured the activity of alkaline phosphatase (ALP). ALP activity was comparable in the controls and DPSCs that overexpressed *Id1* (Fig. 3A). Interestingly, the DPSCs with silenced *Id1* demonstrated significantly less ALP activity (Fig. 3A). We observed that the transcript levels of genes encoding osteopontin (*OPN*), bone sialoprotein (*BSP*), osteonectin (*ON*), and collagen 1 (*COL1A1*) increased in DPSCs overexpressing Id1 over time, whereas such changes were not observed in DPSCs with silenced *Id1* (Fig. 3B). The assumption of odontogenic commitment of DPSCs overexpressing *Id1* was reinforced by the calcium concentration determined, which was significantly higher compared with that from both the controls and DPSCs with silenced *Id1* (Fig. 3C). Moreover, the high level of calcium concentration in DPSCs that overexpressed *Id1* was associated with the noticeable deposition of mineral, which was not observed in the controls and DPSCs with silenced *Id1* (Fig. 3D).

Assuming that cell differentiation attenuates their potential for proliferation, we assessed the impact of *Id1* expression status on DPSC proliferation and observed that the DPSCs with *Id1*

overexpression exhibited lower growth capacity. Simultaneously, the DPSCs with knocked-down *Id1* demonstrated viability comparable with that of the controls (Fig. 3E).

The Level of *Id1* Expression Affects TWIST1/E2 Complex Formation in DPSCs

Co-immunoprecipitation analysis showed that DPSCs with *Id1* overexpression completely lacked TWIST1/E2 complexes, while in the controls and DPSCs with silenced *Id1*, the amount of TWIST1 coupled to E2 proteins was comparable (Fig. 4).

DISCUSSION

Following others' suggestions that by mediating antagonistic effects on tooth development, Twist1 and Id1 produce a regulatory network controlling mesenchymal cell differentiation (Rice *et al.*, 2005; Hayashi *et al.*, 2007), we examined whether the changed expression of *Id1* altered the expression of *TWIST1* and selected dentin- and bone-related genes. We showed that both *Id1* overexpression and silencing modulated a capacity for differentiation of human DPSCs. Raised expression of *DMP1* but specifically *DSPP* (Qin *et al.*, 2003) confirms both odontogenic commitment and odontoblastic-like phenotype of DPSCs permanently overexpressing *Id1*, while a diminished expression of *OC* could reflect a direct inhibition of *OC* promoter by an abundantly formed Id/E2 complex (Huang *et al.*, 2008; Zhang *et al.*, 2008). This seems very likely considering our observation from DPSCs with silenced *Id1*, which displayed an increased expression of *OC* while the expression of other genes of interest decreased substantially.

Our observation that ALP activity was comparable in the controls and DPSCs overexpressing *Id1* and significantly lower in DPSCs with knocked-down *Id1* supports an assumption of the inductive effect of *Id1* overexpression on DPSC commitment. This observation suggests the existence of differences in a development of DPSCs and osteogenic cells, since, in the MC3T3-E1 cell line, a forced overexpression of *Id1* resulted in increased ALP activity (Peng *et al.*, 2004). Moreover, one should consider that ALP is an early marker of osteogenic cell commitment, so our result seems to confirm that DPSCs with *Id1* overexpression have already overcome a specific threshold of maturation, and thus commenced their life function of ECM formation, which resulted in an elevated expression of the late markers *DMP1*, *DSPP*, and *BSP*. Earlier studies showed that, acting in concert with BMP-9 and BMP-2, Id1 stimulated the osteogenic commitment in osteoblast progenitors, which accompany the elevated activity of ALP; however, to maintain the BMP-9-induced terminal differentiation of osteoblasts, the expression of *Id1* must cease (Peng *et al.*, 2004). Our study shows that this regulatory loop does not necessarily function in DPSCs, since the DPSCs that overexpressed *Id1* exhibited an elevated expression of late-mineralization markers but not increased ALP activity. Compared with controls and DPSCs with knocked-down *Id1*, the odontogenic commitment of DPSCs overexpressing *Id1* was confirmed by a raised calcium concentration followed by an increased capacity for mineral deposition. When these data are combined, it seems reasonable to conclude that the more mature cells, already involved in the process of

Figure 2. Impact of altered expression of *Id1* on dentin- and bone-related markers of human DPSC differentiation. **(A)** Quantitative real-time PCR analysis of *TWIST1*, *OC*, *DSPP*, *DMP1*, and *OSF2* transcript levels in DPSCs transfected with the pWPI-GFP-*Id1* lentiviral vector (*Id1*, above) and in DPSCs transfected with the vector carrying the *Id1* silencing sequences (*shId1*). Total RNA was isolated from cells cultured for 14 days after transfection. Data are normalized to *GAPDH* mRNA level and are presented as fold-change relative to control DPSCs transfected with an empty vector or a vector carrying non-targeting shRNA. Data represent the mean values $\pm$ SD ($n = 4$). * $p < .05$ vs. control. **(B)** The *TWIST1*, *OC*, *DSPP*, *DMP1*, and *OSF2* protein levels in DPSC with altered expression of *Id1*. The proteins in the cell extract (from cells cultured for 14 days after transfection) were separated by SDS-PAGE and immunoblotted with appropriate antibodies. β -actin was used as a reference protein. The blots presented are representative of those obtained in 5 experiments performed on unique cell cultures derived from five different patients.

extracellular matrix formation, recruited more calcium, which was necessary for mineral deposition.

Cell differentiation usually results in an altered potency for proliferation. Accordingly, the DPSCs with *Id1* overexpression exhibited lower vitality, which is consistent with cell senescence, while DPSCs with knocked-down *Id1* demonstrated viability comparable with that of the controls. This was surprising, to some extent, considering that DPSCs with knocked-down *Id1* concurrently showed a significant fall in *TWIST1* expression. A previous study on *TWIST1* documented its pro-proliferative and anti-apoptotic role, which makes *TWIST1* a critical contributor to and marker of cancer invasiveness (Laursen *et al.*, 2007).

Since *Id1* cannot bind DNA, analysis of our data implied that, by sequestering E2 proteins, *Id1* produced a space for some other transcription factor to overtake. Multiple comprehensive studies emphasized the critical role of *TWIST1* in the development of tissues of mesenchymal origin, including muscle and bone (Thisse *et al.*, 1991; Seto *et al.*, 2007; Vincentz *et al.*, 2008). On the one hand, a primary role of *TWIST1* was claimed to prevent premature differentiation of myoblasts (Rohwedel *et al.*, 1995; Hamamori *et al.*, 1997) and osteoblasts (Rice *et al.*, 2003; Bialek *et al.*, 2004), thus protecting against pre-mature

Figure 3. *Id1* overexpression-induced differentiation of human DPSCs. DPSCs were transfected with the *pWPI-GFP-Id1* lentiviral vector (*Id1*, above) or *pLL3.7-shId1* vector carrying the *Id1* silencing sequences (*shId1*). The control DPSCs were transfected with an empty vector or vector carrying non-targeting shRNA. The cells were cultured in DMEM for 14 days after transfection. The cell viability was 90% or greater, as determined by Trypan Blue dye exclusion. **(A)** The activity of alkaline phosphatase (ALP) in DPSC extracts was assayed by a colorimetric method. The data are the mean values $\pm$ SD ($n = 5$). $*p < .05$ vs. control. **(B)** Transcript levels of genes encoding osteopontin (OPN), bone sialoprotein (BSP), osteonectin (ON), and α chain of collagen 1 (*COL1A1*). Data were normalized to *GAPDH* mRNA level and are presented as mean fold-change relative to control (0 time) $\pm$ SD ($n = 3$). $*p < .05$ vs. control. **(C)** Calcium content in DPSCs with altered expression of *Id1*. Data are presented as mean values $\pm$ SD ($n = 3$). $*p < .05$ vs. control. **(D)** A representative ($n = 3$) photograph of von Kossa staining of DPSCs cultured in a medium supplemented with 10 nM dexamethasone, 10 mM β -glycerophosphate, and 50 μ g/mL ascorbic acid. Mineral deposits (white arrow) are visible exclusively in DPSCs overexpressing *Id1* (*Id1*, above), while control cells and DPSCs with silenced *Id1* (*shId1*) are void of mineral deposits. **(E)** Cell number after 14 days in culture. The data are the mean values $\pm$ SD ($n = 5$). $*p < .05$ vs. control.

ossification of the entire skeleton, including mid-suture mesenchyme, and maintaining the multi-potential character of human mesenchymal stem cells (Isenmann *et al.*, 2009). Recently, we reported that stable overexpression of *TWIST1* promotes DPSC differentiation, leading to overexpression of both bone- and tooth-related late-differentiation markers (Li *et al.*, 2011). This discrepancy between opinions on *TWIST1* functions compelled us to evaluate whether the detected changes in DPSC maturity might reflect the *Id1*-dependent alteration in *TWIST1/E2*

interaction. Our observation that DPSCs with *Id1* overexpression totally lacked *TWIST1/E2* dimers indicates that altered *Id1* expression disrupted the sophisticated balance between *TWIST1* homodimer (T/T) and heterodimer (T/E). Previous reports (Rice *et al.*, 2005; Connerney *et al.*, 2006, 2008) showed that *Id1* strongly competes with *TWIST1* for dimer-partner attraction, so the bulky quantity of *Id1* constrained a pool of free *E2*, and thus T/T prevailed over T/E. Consequently, T/T-dependent pro-osteogenic signaling was initiated. In our study, the enforced expression of *Id1* in DPSCs enhanced expression of *DMP1* and *DSPP*, both late markers of odontoblast differentiation. Contrary to the previous study (Connerney *et al.*, 2006), we observed a diminished expression of periostin in DPSCs overexpressing *Id1*, but we assume that, in terminally differentiated DPSCs, *TWIST1* no longer stimulated the *OSF2* promoter, as has been proven in differentiated osteoblasts (Bhattacharya and Baker, 2011).

It has already been demonstrated that dental pulp is a reservoir of odontoblast precursors, which are able to differentiate into functional odontoblasts under appropriate signaling (Gronthos *et al.*, 2000). Thus, contemporary dental research focuses on defining methods of DPSC usage for reparative dentistry and tissue engineering. It seems that, among others, *Id1* might be a factor playing the critical role in directing the pattern of differentiation for odontoblast precursors. Since *Id1* is a downstream target of bone morphogenetic protein 2 (BMP2) signaling (Hollnagel *et al.*, 1999; Rice *et al.*, 2000), we speculate that the strictly monitored expression of *Id1* could become an administrator of DPSC differentiation into odontoblasts, which might be a cornerstone in dental tissue engineering.

To the best of our knowledge, this is the first report to show the responses of DPSCs to the modulation of *Id1* expres-

sion and to reveal the intriguing specificity of DPSC behavior compared with that of osteogenic cells. Thus, a search for factors/mechanisms responsible for cell-type *Id1* signaling specificity is necessary.

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Figure 4. Impact of *Id1* expression status on TWIST1/E12 complex formation in DPSCs. DPSCs were transfected with the pWPI-GFP-*Id1* lentiviral vector (*Id1* above) or pLL3.7-*shld1* vector carrying the *Id1* silencing sequences (*shld1*). The control DPSCs were transfected with an empty vector or vector carrying non-targeting shRNA. The presence of the TWIST1/E12 complex in DPSC protein extracts was determined by the co-immunoprecipitation method with the Pierce Direct IP Kit (Thermo Scientific, Waltham, MA, USA). Agarose beads were coupled to anti-TWIST1 polyclonal antibody. The proteins eluted from agarose were electrophoretically separated and immunoblotted with anti-E12 monoclonal antibody (upper panel). The equivalence of loaded proteins on agarose beads was controlled by β -actin immunoblotting performed on cell extract (lower panel). The blot presented is representative of those obtained in 4 experiments performed on unique cell cultures derived from four different patients.

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