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Regular paper

Overexpression of *ID1* reverses the repression of human dental pulp stem cells differentiation induced by *TWIST1* silencing

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Multiple studies showed that the cessation of *TWIST1* expression is the prerequisite for osteoblasts' maturation. However, recent reports revealed that the function of TWIST1 is different in the dental pulp stem cells (DPSCs), where a high level of *TWIST1* expression promoted DPSCs' differentiation. The aim of the study was to investigate the impact of TWIST1 and ID1 on the differentiation process in the human DPSCs. Methods: *TWIST1* and *ID1* expression in the DSPCs was modulated by lentivirus transduction. Genes expression was assessed with qRT-PCR. The proteins level was evaluated by Western blot. The DPSCs differentiation was assessed with the proliferation, alkaline phosphatase (ALP) activity, and calcium concentration assays. Results: *TWIST1* silencing suppressed the expression of *ID1* and both the early and late markers of odontoblasts' differentiation detected at the transcript and protein level. The forced overexpression of *ID1* increased the expression of the late markers of odontoblasts differentiation but diminished the expression of the early markers. DPCSs with the silenced *TWIST1* and subsequent *ID1* overexpression displayed an increase in the expression of the late markers of odontoblasts differentiation. Cells with silenced *TWIST1* and overexpressing *ID1* had increased activity of ALP, higher calcium concentration and decreased proliferation rate. The high level of *ID1* expression might be a critical factor stimulating DPSCs differentiation and it might compensate the repressed differentiation of DPSCs caused by *TWIST1* silencing. Conclusion: The mutual correlation between the expression level of *TWIST1* and *ID1* might be a critical factor driving the process of the human odontoblasts' differentiation.

Key words: dental pulp stem cells, TWIST1, ID1, odontoblast differentiation, DSPP, DMP1

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Abbreviations: DPSC, dental pulp stem cell; TWST1, twist family BHLH transcription factor 1; ID1, inhibitor of DNA binding 1

INTRODUCTION

The expression of *TWIST1* undergoes precise spatiotemporal regulation, which mediates the development of tissues and organs of the mesenchymal origin. The phenotype assigned to TWIST1 results from TWIST1 binding to its dimerization partners and might occur as a transcriptional repression and/or activation (Connerney *et al*., 2006; Connerney *et al*., 2008; Thisse *et al*., 1991; Zhang *et al*., 2008). *TWIST1* expression pattern was thoroughly examined in bone, where the cessation of *TWIST1* expression was claimed as the critical signal for osteoblasts differentiation (Bialek *et al*., 2004). It was shown that TWIST1 homodimers enhanced bone formation and mineralization while TWIST1 heterodimers with E12 protein repressed osteoblasts differentiation and subsequent bone mineralization (Connerney *et al*., 2006). This mechanism of TWIST1 dimerization was discovered during *Drosophila* mesoderm development (Castanon *et al*., 2001) but seems more complicated in mammals (Connerney *et al*., 2006). The complexity of TWIST1/E12 (T/E) heterodimer function in mice was reported by Laursen and coworkers who showed that murine T/E heterodimer drove the reporter construct coming from *Drosophila,* which originally was driven by TWIST1 homodimer (Laursen *et al*., 2007). Multiple studies on TWIST1 function showed that the cessation of *TWIST1* expression is the prerequisite for the final osteoblasts maturation and subsequent bone mineralization (Rice *et al*., 2000; Rice *et al*., 2003; Rice *et al*., 2005), but the recent reports suggest that the high level of both *TWIST1* and *ID1* expression works differently in the dental pulp stem cells (DPSCs), where the overexpression of both genes pushed DPSCs into the final differentiation into odontoblasts-like cells (Li *et al*., 2011; Zhang *et al*., 2012). The *in vivo* study confirmed this observation proving that the stable expression of *TWIST1* is critical for the dental organ development including odontoblasts differentiation and dental crown development (Meng *et al*., 2015).

Thus, considering that TWIST1 and ID1 are acting in concert to modulate the differentiation process of cells originating from mesenchyme and observing that these two genes might act in a cell specific manner we examined if the forced overexpression of *ID1* in the DPSCs might rescue the repression of their differentiation caused by *TWIST1* silencing.

MATERIAL AND METHODS

Patients and samples. Teeth for isolation of dental pulp stem cells (DPSCs) were obtained from 8 patients (13–22 years 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.

Cells isolation and culture. DPSCs were isolated from the interrupted wisdom teeth as described previously (Gronthos *et al*., 2000). Obtained cells were cultured at density of $1 \times 10^3/cm^2$ in DMEM supplemented

with 15% FBS, 2% L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin under standard conditions. The culture medium was replaced twice a week and cells were passaged at 70% confluence.

Cells selection. DPSCs with the phenotype STRO-1/+/; CD146/+/; CD34/−/; CD45/−/ were obtained by the positive selection with anti-STRO1 and anti-CD146 antibodies followed by a negative selection with mouse anti-human CD34 and CD45 antibodies employing the magnetic nanoparticles (MagCellect™) coated with goat anti-mouse IgG ferrofluid accordingly with the manufacturer protocol (all antibodies and MagCellect™ were from R&D Systems, Minneapolis, MN, USA). The number of viable cells was determined by a hemocytometer-based Trypan Blue dye exclusion method. Cells were passaged at 80% confluence (usually the number of passages ranged from 3 to 4).

Lentivirus transduction driven *TWIST1* knockdown. The *TWIST1* silencing constructs: Twist-siRNA7 (#8457), Twist-siRNA3 (#1784) and Twist-siRNA5 (#8456) by Bob Weinberg were from Addgene (Cambridge, MA, USA). The empty 3.7 pLL vector was used as a negative control. The culture media were used for DPSCs transduction. The packaging cells (HEK 293T) were co-transfected with either mix of all three constructs in the ratio 1:1:1 or empty 3.7 pLL vector, an envelope plasmid pMD2.G and a packaging plasmid psPAX2 using X-treme Gene 9 (Roche, Germany) according to the manufacturer's protocol. The culture medium was used as the resource of lentiviral particles for DPSCs transduction. The efficacy of DPSCs transduction was assessed by the observation of a GFP expression using a fluorescent microscope (Leica, Wetzlar, Germany). The DPSCs were cultured with the infectious medium until at least 80% of the population showed the presence of GFP and thereafter used for the further analysis. The silencing of *TWIST1* expression was confirmed with the qPCR analysis.

Lentivirus transduction driven *ID1* overexpression. The human *ID1* cDNA (Gen Bank, Accession No. X77956) was obtained by PCR and cloned into the pWPI-GFP lentiviral vector as described previously (Maciejewska *et al*., 2014). The empty pWPI-GFP vector was used as a negative control. The pWPI-GFP-*ID1* lentiviral vector, together with pMD2.G plasmid and a packaging plasmid psPAX2, or the empty pWPI-GFP vector was used to transfect the HEK 293T cells. The medium with virus particles was used to transduce the wild DPSCs and DPSCs with silenced *TWIST1* expression (siDPSCs). The everyday exposition of siDPSCs to the viral particles driving *ID1* overexpression proceeded for a week, and the level of the *ID1* overexpression was confirmed with the qRT-PCR.

Cultures of transduced cells. Transduced DPS cells were cultured in DMEM supplemented with 10% FBS, 2% L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin under standard conditions. For experiments DPSCs at 5 or 6 passage were plated in 100 mm dishes at a density of 2×10^4 cells and were cultured for 14 days with medium exchange every 3–4 days. The representative photographs of cultured cells are presented in Fig. 1.

Quantitative real-time PCR. Total RNA was isolated from DPSCs using STAT-60 (AMS Biotechnology, Abingdon, UK) according to the manufacturer's protocol. The levels of genes expression in the DPSCs were assessed with Quantitative PCR using a QuantiTect® SYBR Green PCR kit (Qiagen, Germany). The primer sequences used are shown in Table 1S (at www.actabp.pl). Transcript levels were normalized to that of *GAPDH* using the $2^{\Delta Ct}$ method. All reactions were performed in triplicate and validated by the presence of a single pick in the melting curve.

Western blot. Immunoblotting was performed on cell extract (Sakowicz-Burkiewicz *et al*., 2013) subjected to 4–20% resolving SDS-PAGE gel electrophoresis. The proteins separated were electrophoretically transferred onto PVDF membranes. The membranes were blocked with 3% bovine serum albumin, incubated overnight with an appropriate primary antibody (Table 2S at www.actabp.pl), followed by washing and incubation with a secondary antibody. β-actin was used as an internal reference protein.

Proliferation analysis. After 13 days of the cell culture DPSCs were plated at a density of 1×10^3 cells per well in 96 wells plate for 24 hours. Then, cells proliferation was measured with a Quick Cell Proliferation Assay Kit (Gentaur, Sopot, Poland) according to the manufacturer's instruction. The experiment was done in triplicate and repeated 5 times.

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

Calcium concentration analysis. For the analysis of intracellular calcium concentration the cell extract

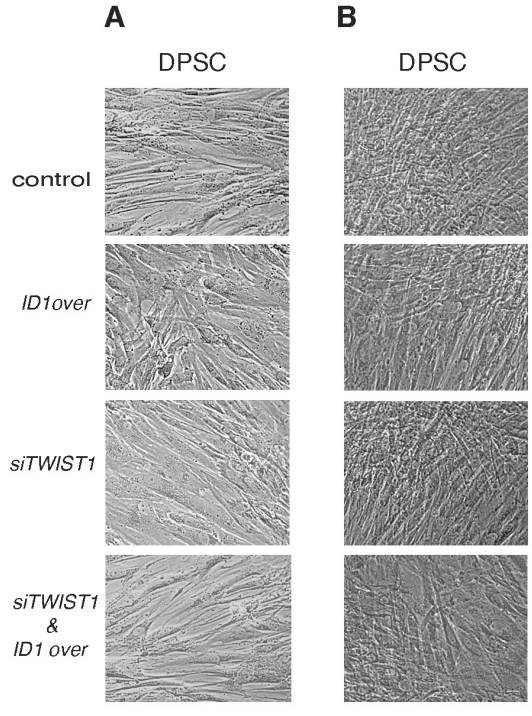

Figure 1. Light microscopy images of transduced DPSCs in culture.
The cells were transduced with an empty 3.7 pLL vector (control), with pWPI-GFP-*ID1* (*ID1*over), with 3.7 pLL vector carrying the *TWIST1* siRNAs (si*TWIST1*), with 3.7 pLL vector carrying the *TWIST1* siRNAs and subsequently with pWPI-GFP-*ID1* vector (si*TWIST1*&*ID1*over). (**A**) Representative microphotographs of 7-day-old cultures of transduced DPSC (20x magnification), (**B**) Representative microphotographs of 14-day-old cultures of transduced DPSC (10x magnification).

was used. The procedure was performed using QuantiChrom™ Calcium Assay Kit (Gentaur, Sopot, Poland) according to the manufacturer's recommendation. Calcium concentration was calculated according to the slope driven from standards included in the kit. The experiment was performed in triplicate and repeated 3 times.

Statistical analysis. Values are reported as means ±S.D. Differences between groups were assessed using a Student's *t*-test. $P<0.05$ was considered as significant.

RESULTS

ID1 overexpression compensates *TWIST1* silencing related restriction in the expression of differentiation-related genes in DPSCs

qRT-PCR analysis showed that transduction of DPSCs with the equal mix of the three *TWIST1*-siRNA (#3, 5, 7) lentiviral vectors resulted in the stable decrease of the *TWIST1* expression down to 15%, which last throughout the entire period of experiment regardless the subsequent modifications in the expression of *ID1*. We also observed that the transduction of DPSCs with the pWPI-GFP-*ID1* resulted in the ~5-fold increase in the expression of *ID1* regardless of *TWIST1* expression status. This was visible both at the mRNA and protein level (Fig. 2A, 2B).

Silencing of *TWIST1* expression resulted in the dramatic suppression of *ID1* expression and expression of genes encoding both the early and late markers of odontoblasts differentiation i.e. *ALP* (alkaline phosphatase), *DSPP* (dentin sialophosphoprotein), *DMP1* (dentin matrix acidic phosphoprotein protein 1), *BSP* (bone sialoprotein), and *OSF2* (periostin). Concurrently, the mild decrease of the expression of *ON* (osteonectin) and *TSP1* (thrombospondin 1) was detected (Fig. 2A). The increase was observed only in the expression of *OCN* (osteocalcin). The analogous observations were made on the basis of the Western Blot analysis (Fig 2B). Conversely, the forced overexpression of *ID1* increased the subsequent expression of the late markers of odontoblasts differentiation i.e. *DSPP, DMP1, BSP, ON, TSP1* at the transcript and protein level but diminished the expression of early markers *OCN, ALP* and *OSF2* (Fig. 2A, 2B). Interestingly, in the DPSCs with the silenced *TWIST1* and subsequent *ID1* overexpression we observed an increase in the transcript level of *BSP, ON,* and *DSPP*. The changes in mRNAs correlated with the proteins levels (Fig. 2A, 2B).

ID1 cooperate with *TWIST1* in DPSCs differentiation into the odontoblast-like cells

To supplement our observation coming from the qRT-PCR and WB analyses we measured the proliferation potential of DPSCs. The proliferation test showed that DPSCs with silenced *TWIST1* and overexpressed *ID1* proliferated significantly slower compared to the other cells (Fig. 3A). Measurements of ALP activity showed that cells overexpressing *ID1* displayed the highest ALP activity regardless of the *TWIST1* expression status. The lowest activity of ALP was detected in the DPSCs with silenced *TWIST1* (Fig. 3B). Similarly, tests for calcium concentration showed that the DPSCs with forced overexpression of *ID1* had the highest and the DPSCs with silenced *TWIST1* the lowest calcium concentration. We noted significant difference in the level of calcium in DPSCs with silenced *TWIST1* and the DPSCs with silenced *TWIST1* and concomitant overexpression of *ID1* (Fig. 3C).

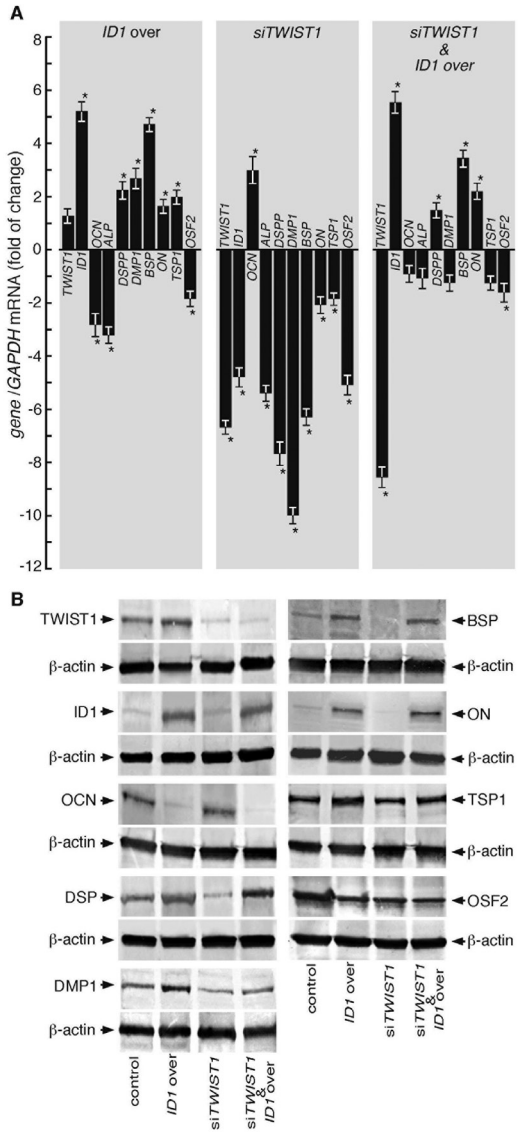

Figure 2. The effect of lentivirus-mediated alteration of *TWIST1* and *ID1* expression on markers of human DPSCs differentiation. (**A**) Quantitative RT-PCR analysis of *TWIST1*, *ID1*, *OCN*, *ALP*, *DSPP*, *DMP1*, *BSP*, *ON*, *TSP1*, and *OSF2* expression in: DPSCs transduced with pWPI-GFP-*ID1* (ID1over), DPSCs transduced with 3.7 pLL vector carrying the *TWIST1* siRNAs (si*TWIST1*), and DPSCs transduced with 3.7 pLL vector carrying the *TWIST1* siRNAs and subsequently with pWPI-GFP-*ID1* vector (si*TWIST1*&*ID1*over). Data are normalized to GAPDH mRNA level and are presented as fold-change relative to control DPSCs transduced with an empty vector or vector carrying scrambled sequence. Data present the mean values ± SD (n=6). *p<0.05 *vs.* control. (**B**) The TWIST1, ID1, OCN, DSP, DMP1, BSP, ON, TSP1, and OSF2 protein levels in: DPSCs with suppressed expression of *TWIST1* (*siTWIST1*), DPSCs with overexpression of *ID1* (*ID1*over), DPSCs with suppressed expression of *TWIST1* and overexpression of *ID1* (si*TWIST1*&*ID1*over). 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 3 experiments performed on unique cell cultures.

DISCUSSION

Our previous and current study showed that both genes modulate the dental pulp stem cells commitment

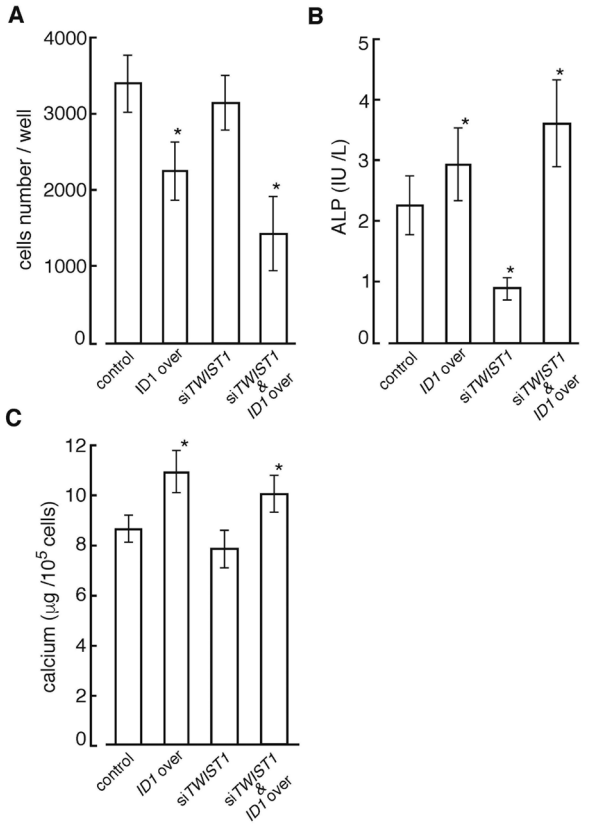

Figure 3. An impact of *TWIST1* and *ID1* expression level on DPSCs differentiation.
The cell proliferation (**A**), activity of alkaline phosphatase (ALP) (**B**), and calcium content (**C**) in cultures of: DPSCs with suppressed expression of *TWIST1* (si*TWIST1*), DPSCs with overexpression of *ID1* (*ID1*over), DPSCs with suppressed expression of *TWIST1* and overexpression of *ID1* (si*TWIST1*&*ID1*over) were determined as described in Materials and Methods. The data are the mean values ± S.D. (n=3). **p*<0.05 *vs*. control.

and differentiation and are responsible for pushing the DPSCs' to differentiation into odontoblast-like cells (Maciejewska *et al*., 2014). A similar conclusion from *in vivo* study has been just announced by Meng and coworkers (Meng *et al*., 2015). These authors showed that the *Twist2*$^{cre/+}$;*Twist1*$^{fl/fl}$ mice embryos had smaller tooth germs with defective dentin and enamel compared to the controls and these defects reflected disturbances in odontoblasts differentiation. Our current *in vitro* study also showed that *TWIST1* silencing repressed human DPSCs commitment, which appeared as the diminished expression of both the early and late markers of odontoblasts differentiation with the exception of osteocalcin. This observation was enhanced by the fact that DPSCs with silenced *TWIST1* showed the diminished ALP activity and low concentration of calcium, which indicated the lack of their involvement in the mineralization process. The DPSCs with silenced *TWIST1* showed morphologic features of undifferentiated cells with the high rate of proliferation compared to the controls. Thus, our results seem to confirm that TWIST1 is an essential factor for both the odontoblasts commitment and final differentiation and that in the DPSCs TWIST1 might act in the cell type specific manner, and it differs from that observed in osteoblasts (Bialek *et al*., 2004).

Multiple studies on TWIST1 role in bone development emphasized that the cessation of *TWIST1* expression is the prerequisite for osteoblast differentiation (Bialek *et al*., 2004) but also suggested that osteoblasts and odontoblasts differentiation might be regulated by distinct mechanisms (Batouli *et al*., 2003). In bone it was shown that the outcome of *TWIST1* expression depends on the balance of the concentration of TWIST1 homodimers and heterodimers, which is regulated by the co-expression of the ID1 protein. This dimerization mechanism was thoroughly investigated in the cranial sutures, palate and tooth (Connerney *et al*., 2006; Rice *et al*., 2005). Our previous study showed that the forced expression of *ID1* in the DPSCs pushed the cells to differentiation into the odontoblast-like cells (Maciejewska *et al*., 2014). Thus, in the current study we intended to check whether the forced overexpression of *ID1* might compensate the repressed differentiation of DPSCs caused by *TWIST1* silencing. Indeed, the *ID1* overexpression resulted in the increase of the DPSCs maturity regardless the dramatic decrease in *TWIST1* expression. The modulated cells commenced the specific threshold of maturity, which appeared as the elevated expression of *DSPP*, *DMP1*, BSP, *ON* and *TSP1* in comparison to that observed in the controls. The finding was enhanced by the increased activity of ALP and calcium concentration in the DPSCs ($Twist1^{-}/Id1^{+}$) compared to the DPSCs with silenced *TWIST1*. The observation of ID1-dependent increase in ALP activity was reported in osteoblast by Peng and coworkers who concluded that ID1 stimulated osteoblasts maturation by up-regulation of *BMP9* expression (Peng *et al*., 2004). However, before the final osteoblasts maturation the *ID1* expression must cease (Peng *et al*., 2004; Song *et al*., 2011). Rice and co-workers showed that the similar mechanisms operate *in vivo* (Rice *et al*., 2005). These previous reports clearly indicate that TWIST1 and ID1 must directly or indirectly interact in the cell type specific loop that regulates DPSCs development. So far the only confirmed explanation for the reported results is the prevailed formation of TWIST1 homodimers over heterodimers in the presence of ID1 (Connerney *et al*., 2006; Connerney *et al*., 2008). The dimerization data came from the experiment in M3T3-E1 cells, which showed that the forced expression of *ID1* abrogated the TWIST1-dependent repression of BMP signaling (Hayashi *et al*., 2007). Since BMP signaling stimulates the expression of osteoblasts differentiation markers (Ju *et al*., 2000) in C3H10T1/2 cells, it seems likely that in our experiment the same mechanism could be involved. This assumption is strengthened by the fact that in both our and others' experiments (Ju *et al*., 2000; Zhang *et al*., 2008) *TWIST1* expression adversely modulated the expression of the osteocalcin gene. Thus, even if TWIST1 acts differently in the DPSCs and C3H10T1/2 cells the overexpression of *ID1* might have overcome the results in the TWIST1 activity in the both cell lines.

Concluding, in our experiment we showed that the high level of *ID1* expression might be a critical factor stimulating dental pulp stem cells to final differentiation and maturation. We also showed that the forced overexpression of *ID1* might compensate the repressed differentiation of DPSCs caused by *TWIST1* silencing. Since both proteins TWIST1 and ID1 are involved in the numerous signaling pathways, the further experiments should clarify the detailed direct or indirect connection between those two factors, which seems critical for the proper development of a tooth organ.

Conflict of interest

Authors deny any conflict of interest.

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