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# NOTCH/WNT CROSS-SIGNALLING REGULATES STEMNESS OF DENTAL PULP STEM CELLS THROUGH EXPRESSION OF NEURAL CREST AND CORE PLURIPOTENCY FACTORS

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**Abstract**

Dental pulp stem cells (DPSCs) from adult teeth express neural crest (NC) markers together with core transcriptional factors associated with stem cell pluripotency, such as *Oct4a*, *Sox2*, *c-Myc*, *Rex1*, *Stella/Dppa3*, *Ssea1/Fut4*, *Lin28* and *Nanog*. The possibility to boost the natural stemness features of DPSCs by mild methods, that do not involve gene and/or chromatin modification or gene transfection, is highly desirable for cell therapy. Canonical Wnt and Notch are two highly conserved developmental signalling pathways that are involved in NC emergence and stem cell self-renewal. We determined that both pathways coordinate to regulate the expression of core pluripotency and NC factors in DPSCs. Pharmacological inhibition of the Notch pathway for 48 h, by the γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), abolished the expression of NC and core factors. In addition, it induced a silencing of the canonical Wnt signalling and a clear reduction in the stemness potential of DPSCs, as shown by a reduced ability to generate mature, fully differentiated osteoblasts and adipocytes. Conversely, pharmacological activation of the Wnt pathway for 48 h, by either the glycogen synthase kinase 3 beta (GSK3-β) inhibitor 6-bromoindirubin-3′-oxime (BIO) or the human recombinant protein Wnt-3a, not only largely increased the expression of NC and core factors, but also increased the efficiency of DPSCs to differentiate into mature osteoblasts and adipocytes. These results showed that a short preconditioning activation of Wnt/Notch signalling by small molecules and/or recombinant proteins enhanced the stemness and potency of DPSCs in culture, which could be useful for optimising the therapeutic use of these and other tissue-specific stem cells.

**Keywords**: Dental pulp stem cells, multipotency, self-renewal, pluripotency core factors, neural crest, stemness and differentiation, osteogenesis, adipogenesis, Notch, Wnt, BIO, DAPT, Wnt-3a.

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## Introduction

During embryogenesis, neural crest (NC) cells generate most craniomaxillofacial tissues, including all major tissues of the teeth, except the enamel. The remarkable abilities of the NC prompted its designation as "the fourth embryonic layer" (Shyamala *et al.*, 2015; Thomas *et al.*, 2008). Importantly, some NC stem cells with a non-differentiated phenotype remain in the dental pulp of mature teeth and are known as dental pulp stem cells (DPSCs) (Aurrekoetxea *et al.*, 2015; Gronthos *et al.*, 2002; Gronthos *et al.*, 2000; Ibarretxe *et al.*, 2012; Janebodin *et al.*, 2011; Kaukua *et al.*, 2014; Liu *et al.*, 2015). Other stem cell types with similar properties may be obtained from gingival and periodontal tissues (Abe *et al.*, 2012; Huang *et al.*, 2009; Ibarretxe *et al.*, 2012; Lima *et al.*, 2017; Liu *et al.*, 2015; Petrovic and Stefanovic, 2009) and even from periodontal inflammatory lesions, such as human periapical cysts (Marrelli *et al.*, 2013; Marrelli *et al.*, 2015; Tatullo *et al.*, 2015). Similarly to NC-derived cells, dental stem cells are characterised by the expression of neural crest factors, such as Snail/Snai1, Slug/Snai2, Twist1, Hnk1, Pax3, Neurogenin2 and Sox10 (Kiraly *et al.*, 2009; Schiraldi *et al.*, 2012), and core factors including Oct4a, Sox2, Klf4, Lin28, stage specific embryonic antigen 3 antibody (Ssea1) and Nanog (Atari *et al.*, 2012; Ferro *et al.*, 2012; Janebodin *et al.*, 2011; Kerkis *et al.*, 2006; Rosa *et al.*, 2016). Core factors are fundamental in maintaining stem cell pluripotency (Chambers and Tomlinson, 2009; Takahashi *et al.*, 2007; Yu *et al.*, 2007), thus suggesting that dental stem cells may present some superior features with

respect to other multipotent stem cell populations of the adult human body (Atari *et al.*, 2011; Atari *et al.*, 2012; Rosa *et al.*, 2016). This could be very relevant for cell therapy because stem cells from dental tissues are known to be easily accessible for extraction and well-tolerated upon grafting, due to their immune-suppressive properties (Pierdomenico *et al.*, 2005). Furthermore, DPSCs are non-tumourigenic even after their immortalisation by telomerase overexpression (Wilson *et al.*, 2015). Finally, given that the dental pulp is rather well preserved in mid-to-advanced age patients, DPSCs are also suitable for autologous therapy (Ibarretxe *et al.*, 2012; Kellner *et al.*, 2014; Wu *et al.*, 2015).

The canonical Notch and Wnt signalling pathways are critical for the maintenance of the stem cell phenotype (Androutsellis-Theotokis *et al.*, 2006; Borghese *et al.*, 2010; Clevers *et al.*, 2014; Perdigoto and Bardin, 2013; Reya and Clevers, 2005). Both pathways also play an important role in the emergence of the NC (Hari *et al.*, 2012; Leung *et al.*, 2016; Rogers *et al.*, 2012; Stuhlmiller and Garcia-Castro, 2012). Dental stem cells present higher levels of core factors and Wnt/Notch activity compared to other mesenchymal stem cells in the adult body (Atari *et al.*, 2012; Huang *et al.*, 2009; Janebodin *et al.*, 2011; Vasanthan *et al.*, 2015). However, the role of these pathways in the maintenance of stemness and self-renewal in DPSCs is still unclear.

Activation of Notch signalling through ligand binding triggers the proteolytic cleavage of Notch receptors, by a disintegrin and metalloproteases (ADAM) followed by γ-secretases, which results in the cleavage and release of the Notch intracellular domain (NICD) from the membrane. NICD translocates to the nucleus where it directly interacts with CSL/RBPj/CBF-1 transcription factors to turn on the expression of Notch target genes, such as the hairy/enhancer of split (*Hes*) family (D'Souza *et al.*, 2010). In the canonical Wnt signalling, the interactions between Wnt protein ligands and Frizzled/LRP receptors lead to the recruitment of axis inhibition protein (AXIN), adenomatous polyposis coli (APC) and glycogen syntase kinase 3 beta (GSK3-β) to the membrane, thus preventing phosphorylation and degradation of β-catenin. As a result, β-catenin accumulates in the cytoplasm and translocates into the nucleus, where it interacts with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family factors and leads to the expression of Wnt signalling target genes (Clevers, 2006). Both pathways regulate each other at multiple points (Borggrefe *et al.*, 2016; Fukunaga-Kalabis *et al.*, 2015) and promote the maintenance of self-renewal and inhibition of differentiation in many stem cell types, including DPSCs (Mizutani *et al.*, 2007; Scheller *et al.*, 2008; Yiew *et al.*, 2017).

Pharmacological manipulation of the Notch and Wnt pathways is relatively simple using well-known drugs, such as N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (γ-secretase inhibitor; Notch signalling blocker) and BIO (GSK3-β inhibitor; Wnt signalling activator), and recombinant activator proteins, such as Wnt-3a. The use of BIO and DAPT has already proven to be a valuable complementary strategy to induce either cellular reprogramming or cellular differentiation (Ichida *et al.*, 2014; Kitajima *et al.*, 2016). Therefore, a thorough understanding of the stemness and differentiation potential of DPSCs and their modulation by cell signalling pathways would be highly desirable to use these cells more efficiently in areas such as regenerative medicine, tissue engineering and drug screening. In this work, we used DAPT and BIO, as well as human recombinant Wnt-3a, as pharmacological modulators to investigate the role of Notch/Wnt in maintaining the stemness and expression of pluripotency core factors in DPSCs, with the goal of optimising existing protocols for differentiation to somatic cells.

## Materials and Methods

### DPSC culture

DPSCs were isolated from human third molars obtained from healthy donors between 15 and 30 years of age. The pulp tissue was isolated by fracture and enzymatic digestion for 1 h at 37 °C with 3 mg/mL collagenase (17018-029; Thermo Fisher Scientific, Boston, MA, USA) and 4 mg/mL dispase (17105-041; Thermo Fisher Scientific) followed by mechanical dissociation. DPSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % foetal bovine serum (FBS), 1 mM L-glutamine, 100 U/mL penicillin and 150 μg/mL streptomycin. DPSCs could be amplified and maintained in these conditions for a very long time (> 6 months). However, to avoid cell aging issues, only DPSCs that had been grown in culture for less than 3 months and had accumulated no more than 6 total passages were used. Comparative experiments between control and treatment conditions were always and without exception performed in parallel using DPSCs from the same donor.

### Notch and Wnt pathway pharmacological modulation

To block Notch signalling, 2.5 μM DAPT γ-secretase inhibitor (565784; Calbiochem, San Diego, CA, USA) was added to the culture medium 48 h prior to the assays, where DAPT-treated DPSCs were compared to DPSCs treated only with the control vehicle dimethyl sulfoxide (DMSO). To overactivate Wnt signalling, 2.5 μM 6-bromoindirubin-3′-oxime (BIO), a GSK3-β inhibitor (361550; Calbiochem), was used, which was added to the medium 48 h prior to the assays. BIO-treated cells were compared to DPSCs exposed to 2.5 μM inactive analogue methyl-6-bromoindirubin-3′-oxime (MBIO) (361556; Calbiochem). Wnt-3a recombinant protein (5036-

**Table 1a.** Primer pairs used. * transcript variants (*Pou5f1*, *Oct4a* and *Pou5f1p1*, *Oct4pg1*).

| Primers | | Sequence 5´-3´ | Annealing (°C) | Amplicon (bp) |
|---|---|---|---|---|
| **β-actin** | Upstream | GTTGTCGACGACGAGCG | 58.5 | 93 |
| | Downstream | GCACAGAGCCTCGCCTT | 59.7 | |
| **Gapdh** | Upstream | CTTTTGCGTCGCCAG | 60.3 | 131 |
| | Downstream | TTGATGGCAACAATATCCAC | 60.8 | |
| **Notch1** | Upstream | ATAGTCTGCCACGCC | 54 | 149 |
| | Downstream | AGTGTGAAGCGGCCA | 54.9 | |
| **Notch2** | Upstream | AAGCCCAGACATTCTTGCAGCTTG | 64.1 | 107 |
| | Downstream | TCCAGGGCATAATTCCCAACAGGA | 63.7 | |
| **Notch3** | Upstream | ACCCCCAAGAGGCAAGTGT | 61.1 | 125 |
| | Downstream | AGGATGAAAAAGACTAAAAGGAAGGAA | 59 | |
| **Notch4** | Upstream | GCGATAATGCGAGGAAGATACG | 59.4 | 118 |
| | Downstream | TCGGAATGTTGGAGGCAGAAC | 60.6 | |
| **Hes1** | Upstream | GGTACTTCCCCAGCACACTT | 59 | 138 |
| | Downstream | TGAAGAAAGATAGCTCGCGG | 57.7 | |
| **β-catenin** | Upstream | GAAGCTGGTGGAATGCAAGC | 60.1 | 279 |
| | Downstream | GACAGTACGCACAAGAGCCT | 60 | |
| **Nestin** | Upstream | GGTCCTAGGGAATTGCAGC | 57.9 | 144 |
| | Downstream | CTCAAGATGTCCCTCAGCCT | 58.8 | |
| **Jagged1** | Upstream | AGATCTCAATTACTGTGGGAC | 57.1 | 88 |
| | Downstream | GCAGGAACACTGATATTTGTC | 58.7 | |
| **Jagged2** | Upstream | TCTTGCAAAAACCTGATTGG | 62.6 | 86 |
| | Downstream | CAGTCGTTGACGTTGATATG | 59.2 | |
| **Lef1** | Upstream | TGCCAAATATGAATTAACGACCCA | 59 | 151 |
| | Downstream | GAGAAAAGTGCTCGTCACTGT | 58.5 | |
| **Oct4*** | Upstream | CGTGAAGCTGGAGAAGGAGA | 60.7 | 137 |
| | Downstream | CATCGGCCTGTGTATATCCC | 60.1 | |
| **c-Myc** | Upstream | GTCAAGAGGCGAACACACAAC | 60 | 162 |
| | Downstream | TTGGACGGACAGGATGTATGC | 60.1 | |
| **Sox2** | Upstream | ATAATAACAATCATCGGCGG | 61.1 | 90 |
| | Downstream | AAAAAGAGAGAGGCAAACTG | 57.8 | |
| **Klf4** | Upstream | TCTTGAGGAAGTGCTGAG | 56.5 | 147 |
| | Downstream | ATGAGCTCTTGGTAATGGAG | 58.3 | |

WN-010; R&D Systems, Minneapolis, MN, USA) was added to the DPSCs cultures to overactivate Wnt signalling at two concentrations: 2.5 μM and 5 μM.

**Osteogenic differentiation of DPSCs**

The following protocol was used to induce DPSC differentiation to mature osteoblasts: 6 μM β-glicerophosphate (G9422; Sigma-Aldrich, St. Louis, MA, USA), 10 nM dexamethasone (D4902; Sigma-Aldrich) and 52 nM ascorbic acid (127.0250; Merck, Darmstadt, Germany) were added to the cells cultured in DMEM + 10 % FBS for three weeks. The DPSCs had been previously subjected to preconditioning treatment with DMSO, DAPT, MBIO or BIO for 48 h, as described. Terminal osteoblast differentiation was assessed by detection of extracellular calcified bone matrix deposits by Alizarin Red S staining using 2 g/100 mL Alizarin Red S (400480250; Across Organics, Geel, Belgium) at pH 4.3. The DPSCs were fixed with 10 % formalin (F7503; Sigma-Aldrich) for 30 min. Then, cells were incubated with Alizarin Red S for 45 min before being washed four times with PBS to remove any background staining. The Alizarin Red S absorbance at 450 nm was quantified using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Also, alkaline phosphatase (ALP) staining was used to assess osteoblast differentiation, as this enzymatic activity is present in mature bone matrix-secreting cells. One SIGMA FAST™ BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablet (B5655-5ATB; Sigma-Aldrich) was dissolved in 10 mL milliQ water and added to the DPSCs fixed for 1 min with 10 % formalin. Next, cells were washed with PBS containing 0.05 % Tween 20 (STBB3609; Sigma-Aldrich). ALP activity was quantified by measuring the absorbance at 405 nm with a Synergy HT Multi-Mode Microplate Reader (BioTek).

**Adipogenic differentiation of DPSCs**

To induce adipogenic differentiation, DPSC cultures were treated with 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX) (I5879; Sigma-Aldrich),

**Table 1b.** Primer pairs used.

| Primers | | Sequence 5′-3′ | Annealing (°C) | Amplicon (bp) |
|---|---|---|---|---|
| **Nanog** | Upstream | GTCAAGAAACAGAAGACCAG | 56.4 | 184 |
| | Downstream | GCCACCTCTTAGATTTCATTC | 59.2 | |
| **Lin28** | Upstream | CTGGTGGAGTATTCTGTATTG | 56.2 | 81 |
| | Downstream | ACCTGTCTCCTTTTGATCTG | 58.3 | |
| **Rex1** | Upstream | TATCTCAACCTGTTCATCGAG | 59.3 | 130 |
| | Downstream | CCACATTCAGGTAGATGTTC | 56.9 | |
| **Ssea1/Fut4** | Upstream | ACAAAATCATCTGTTGGGAC | 58.9 | 85 |
| | Downstream | AGCAGATAAGCACTTTCAAC | 56.2 | |
| **Stella/Dppa3** | Upstream | GAGGAGTAAGAACATTGCTG | 56.5 | 133 |
| | Downstream | CTTGATTCCTTCTTAACTCCC | 58.3 | |
| **Snail/Snai1** | Upstream | AACAATGTCTGAAAAGGGAC | 58.1 | 94 |
| | Downstream | ATAGTTCTGGGAGACACATC | 55.4 | |
| **Slug/Snai2** | Upstream | AAACAACCTGAAGACTTGTG | 56.8 | 157 |
| | Downstream | TTCTTTGTACAGTGGTTTGG | 57.7 | |
| **Sox10** | Upstream | ACTTAGTGGAGTTCTCATCC | 54.7 | 106 |
| | Downstream | AAGAATGAGGTTATTGGCAC | 58.1 | |
| **Pax3** | Upstream | ATCAACTGATGGCTTTCAAC | 59.2 | 120 |
| | Downstream | CAGCTTGTGGAATAGATGTG | 58.3 | |
| **Pax7** | Upstream | AGGAGTACAAGAGGGAAAAC | 56.4 | 108 |
| | Downstream | TAATCGAACTCACTGAGGG | 57.8 | |
| **Neurogenin2** | Upstream | AGGGAAGAGGACGTGTTAGTGC | 61.9 | 225 |
| | Downstream | GCAATCGTGTACCAGACCCAG | 61 | |
| **Twist1** | Upstream | CTAGATGTCATTGTTTCCAGAG | 57.9 | 136 |
| | Downstream | CCCTGTTTCTTTGAATTTGG | 60.9 | |
| **Wnt3** | Upstream | CTGTGACTCGCATCATAAG | 56.8 | 186 |
| | Downstream | ATGTGGTCCAGGATAGTC | 54.3 | |
| **Wnt1** | Upstream | CTATTTATTGTGCTGGGTCC | 58.5 | 125 |
| | Downstream | AGAAACTGAGGAGAGAAGAG | 54.2 | |
| **Hnk1** | Upstream | TGTGAGTGCTGGTAATGAG | 57.2 | 169 |
| | Downstream | ACTGCCCTCATCCTTATG | 57.5 | |

1 μg/mL insulin (91077C; SAFC Biosciences, St. Louis, MA, USA) and 1 μM dexamethasone (D4902; Sigma-Aldrich) for four weeks after a preconditioning treatment with DMSO, DAPT, MBIO or BIO for 48 h. Subsequently, DPSCs were fixed with 10 % formalin for 10 min and then washed with PBS containing 60 % isopropanol. Lipid droplets in mature adipocytes were detected using Oil Red O staining solution, which contained 5.14 μM Oil Red O stock (O-0625; Sigma-Aldrich) diluted in milliQ water. Cells were stained for 10 min and the Oil Red O absorbance was measured at 490 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek).

**RNA extraction, conventional RT-PCR and quantitative Real-Time PCR (qPCR)**

Cell pellets were frozen and stored at -80 °C. Total RNA was extracted from the cells using the RNeasy kit (74104; Qiagen, Hilden, Germany) and purity was checked by calculating the 260/280 absorbance ratio using the Nanodrop Synergy HT (BioTek). cDNA (50 ng/μL) was obtained by reverse transcription of total extracted RNA using the iScript cDNA kit (1708890; BioRad, Hercules, CA, USA) with the following reagents: iScript Reverse Transcriptase (1 μL), 5× iScript Reaction Mix (4 μL) and Nuclease-free water (variable) to a final volume of 20 μL. Gene expression was analysed using 1 μL of cDNA (5 ng/μL) diluted in 4 μL of My TaqTM Red Mix (BIO-25043, Bioline, St. Petersburg, Russia), 1 μL of primers (0.625 μM) and Nuclease Free Water, for a total volume reaction of 10 μL for a conventional RT-PCR. Amplification products were separated by electrophoresis in a 2 % agarose gel. qPCR experiments were conducted in an iCyclerMyiQ™ Single-Color Real-Time PCR Detection System (BioRad) using 4.5 μL of Power SYBR® Green PCR Master Mix 2× (4367659; Applied Biosystems™ Applied Biosystems, Carlsbad, CA, USA), 0.5 μL of primers (0.3125 μM), 0.3 μL of cDNA (1.5 ng/μL) and Nuclease Free water for a total volume reaction of 10 μL. All primers were obtained from public databases and checked for optimal efficiency (> 90 %) under our qPCR experimental conditions. The relative expression of each gene was calculated using the standard $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and normalised to the average between *β-actin* and *Gapdh*, as internal controls. All reactions were

performed in triplicate. qPCR was run on an ABI PRISM® 7000 (Thermo Fisher Scientific). Data were processed by CFX Manager™ software (BioRad). We assessed that all qPCR reactions yielded only one amplification product by the melting curve method. Primer pairs used were obtained through the Primer-Blast method (Primer Bank) and they are listed in Table 1**a**,**b**.

**Protein extraction**

Cells were washed several times with 0.9 % NaCl and the proteins were extracted with 100 µL of lysis buffer [50 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 % IGEPAL® CA-630 in $dH_2O$ and 1 : 100 Proteinase Inhibition Cocktail Set III (539134; Calbiochem)]. Protein quantification was performed before each Western Blot (WB) using the DC™ Protein Assay (5000112; Bio Rad).

**Western Blot (WB)**

Samples were diluted in NuPAGE sample buffer (NP0007; Novex, Life technologies, Carlsbad, CA, USA) and loaded onto a 4-12 % Invitrogen NuPAGE BisTris Gel (1 mm × 10 wells; NP032180X, Novex, Life Technologies). Subsequently, they were transferred onto 0.45 µm-pore nitrocellulose membranes (Inmmobilon® Transfer Membranes; Millipore, St. Charles, MO, USA) using a XCell Sure Lock Electrophoresis machine (NP0007; Novex, Life Technologies). For WB analyses, the following antibodies were used: 1 : 1000 rabbit anti β-actin (4967; Cell Signaling Technology (CST), Danvers, MA, USA), 1 : 10000 mouse anti-Gapdh (MAB374; Millipore), 1 : 2000 rabbit anti-PARP (9542S; CST), 1 : 500 rabbit anti-Sox2 (GTX101506; GeneTex, Irvine, CA, USA), 1 : 1000 rabbit anti-Nanog 1,2 (D73G4; CST, 1 : 1000 rabbit anti-Notch 1-receptor intracellular domain (N1ICD) (ab8925; Abcam, Cambridge, UK), 1 : 4000 rabbit anti-total β-catenin (ab6302; Abcam) and 1 : 500 mouse anti-active β-catenin (05-665; Millipore). The secondary antibodies (P0260; DAKO, Hovedstaden, Denmark; NA9340, GE Healthcare, Amersham, UK) were used at a 1 : 2000 dilution. The membranes were stripped using Red Blot reagent (M2504; Inmmobilon® EMD, Millipore).

**Immunofluorescence (IF)**

DPSCs cultured on glass coverslips were fixed with 4 % paraformaldehyde for 10 min and washed with PBS. Blocking was performed by 10 min incubation with Normal Goat Serum (501972; Thermo Fisher Scientific). Next, DPSCs were incubated overnight at 4 °C with primary antibodies diluted in PBS + 2 % BSA + 0.1 % Triton X-100. The rabbit anti-N1ICD (ab8925; Abcam), anti-Oct4 (ab19857; Abcam), anti-c-Myc (ab32[9E10]; Abcam), anti-Ki67 (ab15580; Abcam) and anti-total β-catenin (ab6302; Abcam) antibodies were used at 1 : 200, 1 : 100, 1 : 1000, 1 : 100 and 1 : 3000 dilutions, respectively. Goat anti-rabbit Alexa Fluor (488: A11012; 594: A11012; Invitrogen, Carlsbad, CA, USA) were employed as secondary antibodies at a 1 : 200 dilution, followed by DAPI, used to counterstain cell nuclei. Images were captured with an epifluorescence ZEISS Axioskop (ZEISS, Jena, Germany) operated with Nikon NIS-Elements and an Apotome Confocal Microscope (ZEISS) operated with Nikon DS-Qi1Mc software (Nikon, Tokyo, Japan). Samples fluorescence intensities were quantified by Fiji-ImageJ (Schindelin *et al.*, 2012) after background subtraction.

**Cell proliferation and death assays**

20 µg/mL propidium iodide (PI; Sigma-Aldrich) was used to detect cell death and 5 µM calcein-AM (Life Technologies) to detect cell viability. Both fluorescent dyes were incubated for 30 min at 37 °C in culture medium and subsequently cells were washed 3 times with PBS. Fluorescence quantification was calculated by microfluorimetry, measuring light emission at 495 nm (calcein-AM: green fluorescence) and 630 nm (PI: red fluorescence) in a Fluoroskan Ascent plate reader (Thermo Fisher Scientific).

**Statistical analyses**

Statistical analyses were performed with Excel, IBM SPSS Statistics v.9 (SPSS, Chicago, IL, USA) and GrapH Pad v.6 software (Graph Pad Inc., La Jolla, CA, USA). All data sets were subjected to a Kolmogorov-Smirnov normality test prior to analysis. For small sample sizes, non-parametric tests were chosen by default. Comparisons between only two groups were made using U-Mann Whitney test. Comparisons between multiple groups were made using Kruskal-Wallis followed by Dunn´s *post hoc* test. $p \leq 0.05$ was considered to be statistically significant.

## Results

**Notch activity was required for the expression of core pluripotency factors and self-renewal of DPSCs**

Control DPSCs showed detectable amounts of core pluripotency factors, such as *Oct4* (*Pou5f1* and *Pou5f1p1*), *Sox2*, *Nanog*, *Lin28*, *Rex1*, *Stella*, *Ssea1* and *c-Myc*, measured by RT-PCR amplicon bands. To assess whether DAPT treatment would affect the expression of core factors in DPSCs, we exposed the DPSC cultures to transient applications of 2.5 µM DAPT for 48 h. Interestingly, for many of these factors, the RT-PCR bands were significantly reduced or lost when the DPSCs were treated with DAPT (Fig. 1**a**). As an internal control to assess Notch activity, we examined the expression of the Notch target gene *Hes1*. A clear decrease in *Hes1* expression was consistently observed following DAPT treatment. Next, these changes were validated by qPCR, confirming that all core factors had significantly decreased levels of expression when DPSCs were exposed to DAPT. In some cases, such as that of *Rex1* or *Ssea1* expression, the decrease in expression

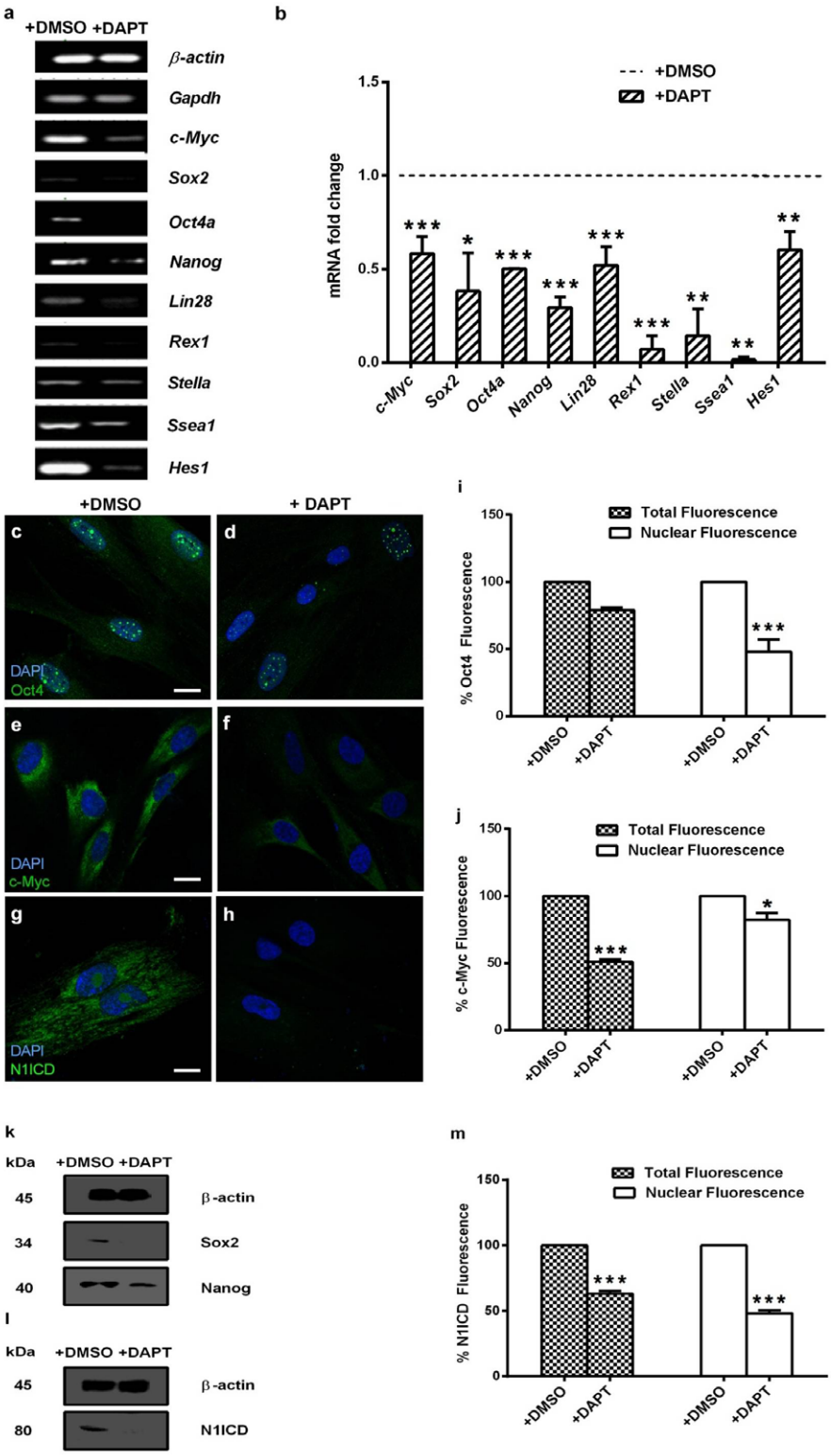


**Fig. 1.** Notch inhibition by DAPT reduced the expression of core pluripotency factors in DPSCs. (**a**) RT-PCR revealed differences in the expression of *c-Myc*, *Sox2*, *Nanog*, *Oct4a* and *Hes1* expression following DAPT exposure. (**b**) qPCR analysis confirmed a decrease in *c-Myc*, *Sox2*, *Nanog*, *Oct4a*, *Rex1*, *Stella*, *Ssea1* and *Hes1* transcripts between the control (DMSO) and DAPT conditions. Data were normalised to reference *β-actin* and *Gapdh* levels and presented as the mean + SEM ($n = 3$). The dashed line represented normalised gene expression in control conditions. (**c-h**) IF images of DPSCs grown for 48 h in presence or absence of DAPT and stained for (**c**,**d**) Oct4, (**e**,**f**) c-Myc and (**g**,**h**) N1ICD (in green). DAPI labelled cell nuclei (in blue). Scale bar = 20 μm. (**i**,**j**,**m**) Bar charts showing relative total and nuclear (**i**) Oct4, (**j**) c-Myc and (**m**) N1ICD fluorescence in control and DAPT-treated DPSCs. The data are presented as mean + SEM ($n = 3$). Representative WB showing (**k**) Sox2, Nanog and (**l**) active cleaved N1ICD. β-actin was used as protein loading control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. U-Mann Whitney test.

was more than 90 % with respect to control (Fig. 1**b**). Finally, these changes were also corroborated by IF and WB, where Oct4 (nuclear active form Oct4-a; Atlasi *et al.*, 2008; Ferro *et al.*, 2012; Liedtke *et al.*, 2007), Sox2, Nanog (canonical isoform 1; Saunders *et al.*, 2013; Wang *et al.*, 2008), c-Myc and Notch activity marker N1ICD, had also a consistently reduced expression at the protein level (Fig. 1**c-l**).

The proliferation rates of DPSCs in the presence or absence of DAPT were compared, to verify if the decreased expression of core factors in DPSCs also reflected a decreased ability for self-renewal and proliferation. DPSCs were incubated with a mixture of calcein-AM/PI, after being cultivated for 48 h in the presence or absence of DAPT. No PI fluorescence was detectable in either condition, indicating that cell viability was not compromised by DAPT treatment. However, calcein fluorescence was reduced by approximately 40 % in the DAPT conditions compared to control (Fig. 2**a-c**). To corroborate this result, the number of proliferative cells positive for Ki67, a marker of cell proliferation present during the G1, S, G2 and M phases of the cell cycle, but absent from non-dividing cells (G0), was evaluated. Ki67 labelling was significantly lower in the DAPT-treated DPSCs with respect to controls, thus indicating a reduction in the amount of cycling proliferative cells (Fig. 2**e-g**). Finally, it was confirmed that, despite inducing a reduction in DPSC proliferation, DAPT treatment did not cause any genomic damage or apoptotic cell death. The cleaved poly-ADP ribose polymerase (PARP) levels were assessed by WB and resulted negative in all conditions (Fig. 2**d**). Taken together, these results suggested that Notch inhibition caused a decrease in the expression of core pluripotency factors in DPSCs, which resulted in decreased self-renewal and proliferation capacities, without affecting cell viability.

### Notch and Wnt/β-catenin signalling interacted and positively regulated each other in DPSCs

To assess whether Notch signalling interacted with Wnt/β-catenin signalling in DPSCs, the effect of DAPT treatment on the expression of Wnt signalling

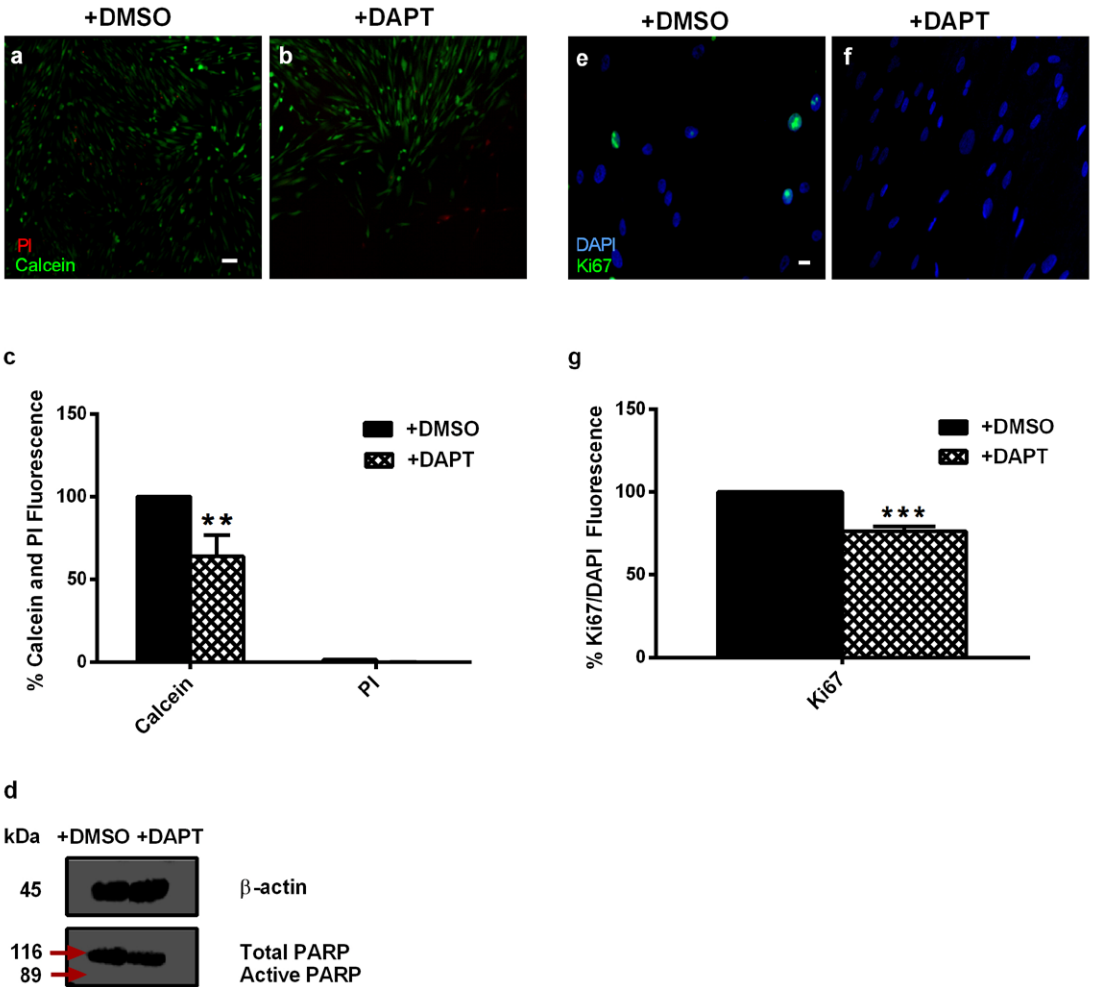


**Fig. 2.** Cell proliferation and cell death analysis in DPSCs exposed to DAPT. (**a**,**b**) Calcein-AM (green) and PI (red) staining of DPSCs grown in control and DAPT for 48 h. Scale bar = 200 μm. (**c**) Quantification of relative calcein-AM and PI fluorescence. Data are presented as mean + SEM ($n$ = 7). (**d**) WB showing absence of cleaved PARP protein (89 kDa; red arrow) in both DMSO and DAPT conditions in comparison with total inactive non-cleaved PARP (116 kDa), which was well detected. (**e**,**f**) IF for Ki67 in DMSO and DAPT conditions. Scale bar = 20 μm. (**g**) Quantification of Ki67 labelling in DAPT-treated and control DPSCs. Data are presented as mean + SEM ($n$ = 7). ** $p < 0.01$, *** $p < 0.001$. U-Mann Whitney test.

targets genes was studied by qPCR. Notch1-4 receptor expression was negatively affected by the treatment, whereas the expression of the Notch ligand *Jagged1* was unaffected (Fig. 3**a**,**b**). These results showed an overall downregulation of Notch signalling in DPSCs induced by DAPT treatment. Intriguingly, we also found out that DAPT treatment reduced the expression of the canonical Wnt mediators *Wnt-3a* and *β-catenin* and also that of the Wnt target gene *Lef1* by ~50-70 % compared to their respective control values (Fig. 3**a**,**b**). To confirm this result, β-catenin protein levels were assessed both by WB and IF. A significant decrease in the amount of both total and nuclear active β-catenin was found in DPSCs exposed to DAPT for 48 h (Fig. 3**c**-**f**). Therefore, Notch inhibition resulted in a parallel Wnt/β-catenin signalling inhibition in DPSCs.

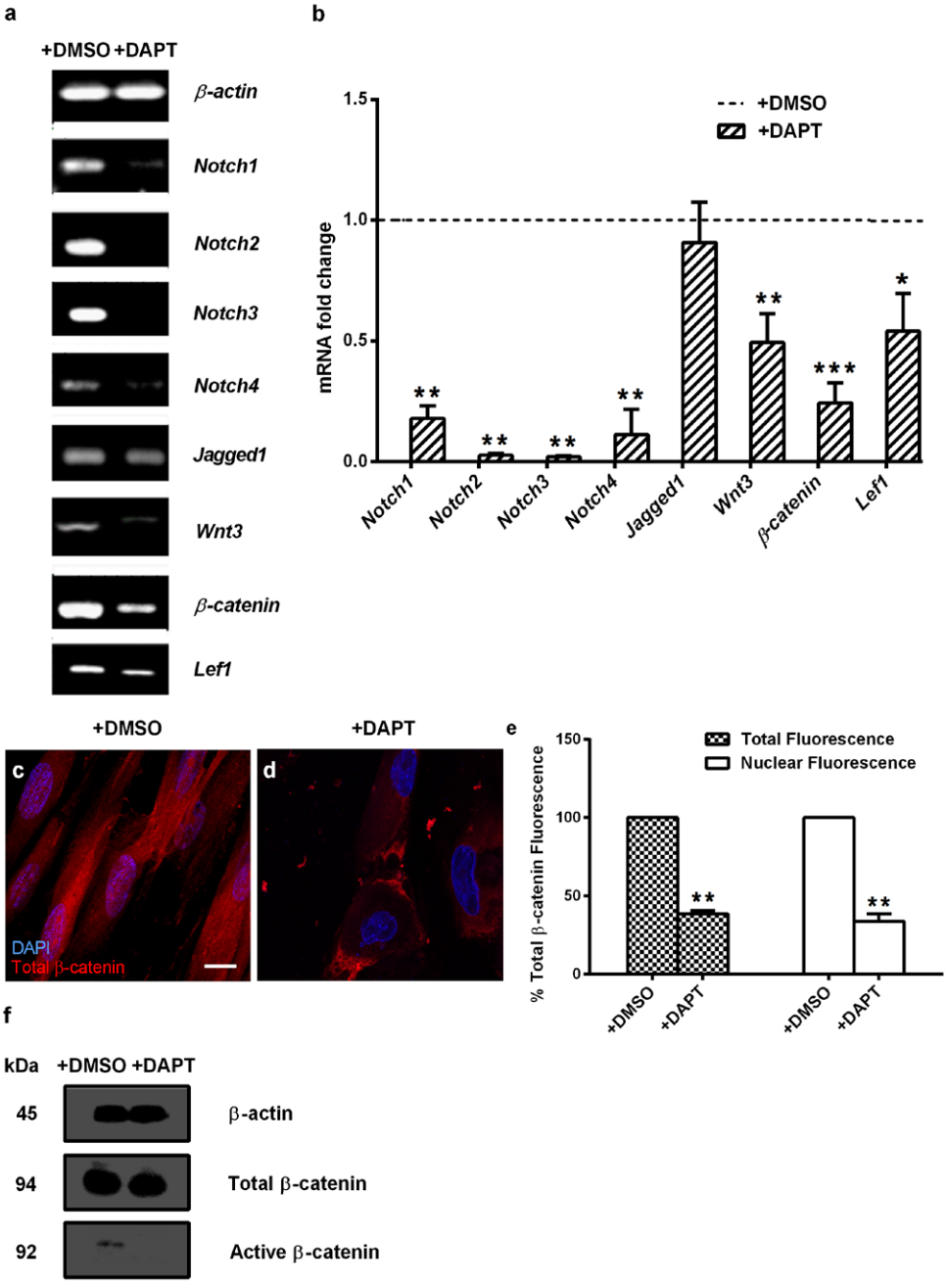


**Fig. 3.** Notch inhibition by DAPT negatively regulated Wnt/β-catenin signalling in DPSCs. (**a**) RT-PCR revealed differences in the expression of key Wnt signalling mediators and targets *Wnt-3a*, *β-catenin* and *Lef1* in DPSCs following DAPT exposure. (**b**) qPCR confirmed a decrease in both Notch receptors and Wnt factors between control (DMSO) and DAPT conditions. Data were normalised to *β-actin* and *Gapdh* levels and presented as the mean + SEM ($n = 4$). The dashed line represented normalised gene expression in control conditions. (**c**,**d**) IF images of total β-catenin in DAPT-treated DPSCs compared with controls. Scale bar = 20 μm. (**e**) Bar chart showing relative total and nuclear β-catenin fluorescence in control and DAPT conditions. (**f**) WB of total and nuclear active β-catenin in control and DAPT-treated DPSCs. β-actin was used as a protein loading control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. U-Mann Whitney test.

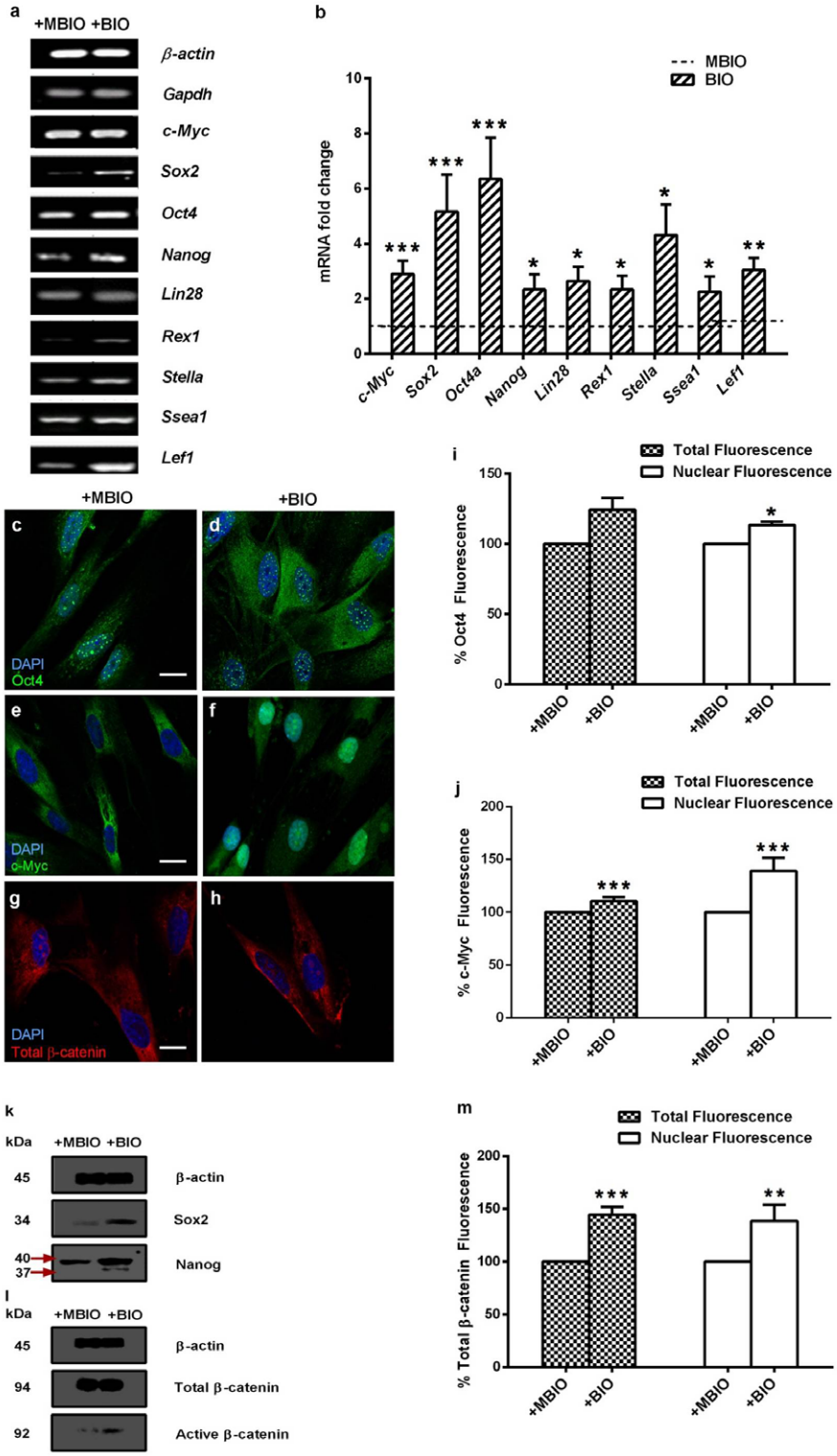


**Fig.4.** Wnt activation by BIO led to increased expression of core pluripotency factors in DPSCs. (**a**) RT-PCR revealed differences in the expression of *c-Myc*, *Sox2*, *Nanog*, *Oct4* and *Lef1* following BIO exposure. (**b**) qPCR analysis confirmed an increase in *c-Myc*, *Sox2*, *Nanog*, *Oct4a*, *Rex1*, *Stella*, *Ssea1* and *Lef1* expression between control (MBIO) and BIO conditions. Data were normalised to *β-actin* and *Gapdh* levels and presented as the mean + SEM ($n = 3$). The dashed line represented normalised gene expression in control conditions. (**c-h**) IF images of DPSCs grown in the presence of MBIO or BIO for 48 h and stained for (**c**,**d**) Oct4, (**e**,**f**) c-Myc and (**g**,**h**) total β-catenin. DAPI labelled nuclei in blue. Scale bar = 20 μm. (**i**,**j**,**m**) Bar charts showing relative total and nuclear (**i**) Oct4 (**j**) c-Myc and (**m**) total β-catenin fluorescence in MBIO and BIO-treated DPSCs. Data are presented as the mean + SEM ($n = 3$). (**k**,**l**) Representative WB showing an increase in (**k**) Sox2, native Nanog1 and Nanog2 isoforms, with slightly different molecular weights, and (**l**) in both total β-catenin and active β-catenin protein expression. β-actin was used as a protein loading control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. U-Mann Whitney test.

**BIO-induced Wnt activation enhanced the expression of pluripotency core factors in DPSCs**

To investigate whether Wnt/β-catenin activation would enhance the expression of pluripotency core factors in DPSCs, we used BIO treatment for 48 h to hyperactivate Wnt signalling by inhibiting β-catenin degradation. As a control for treatment efficacy, BIO-treated DPSCs showed increased levels of both total and active β-catenin protein, whereas other Wnt targets, such as *Lef1*, also demonstrated consistently increased expression (Fig. 4**a**,**b**,**g**,**h**,**k**-**l**). Therefore, BIO induced a strong hyperactivation of Wnt signalling in DPSCs. Then, the expression of pluripotency core factors in BIO-treated DPSCs by RT-PCR and qPCR was assessed. It was determined that BIO also induced overexpression of pluripotency factors at both the transcript (Fig. 4**a**,**b**) and protein levels (Fig. 4**c**-**k**).

In addition to boosting the expression of core pluripotency factors, BIO increased the proliferation ability of DPSCs. As shown by the calcein/PI assay, the number of viable cells in culture significantly increased after BIO treatment (Fig. 5**a**-**c**). Consistently, the Ki67/DAPI fluorescence ratio increased in response to BIO treatment, indicating a greater proportion of active dividing cells (Fig. 5**e**-**g**). Neither PI-positive cells nor an active PARP signal in BIO-treated cells were detected (Fig. 5**d**). Together, these experiments demonstrated that Wnt activation by BIO treatment increased the proliferation and self-renewal capacities of DPSCs without compromising their viability and genomic integrity.

**Wnt activation by BIO induced Notch upregulation in DPSCs**

Given that DAPT treatment affected Wnt signalling in DPSCs, the question whether Notch activity would also be affected in BIO-treated DPSCs needed addressing. It was found that in BIO-treated DPSCs, most Notch receptors did not undergo any change

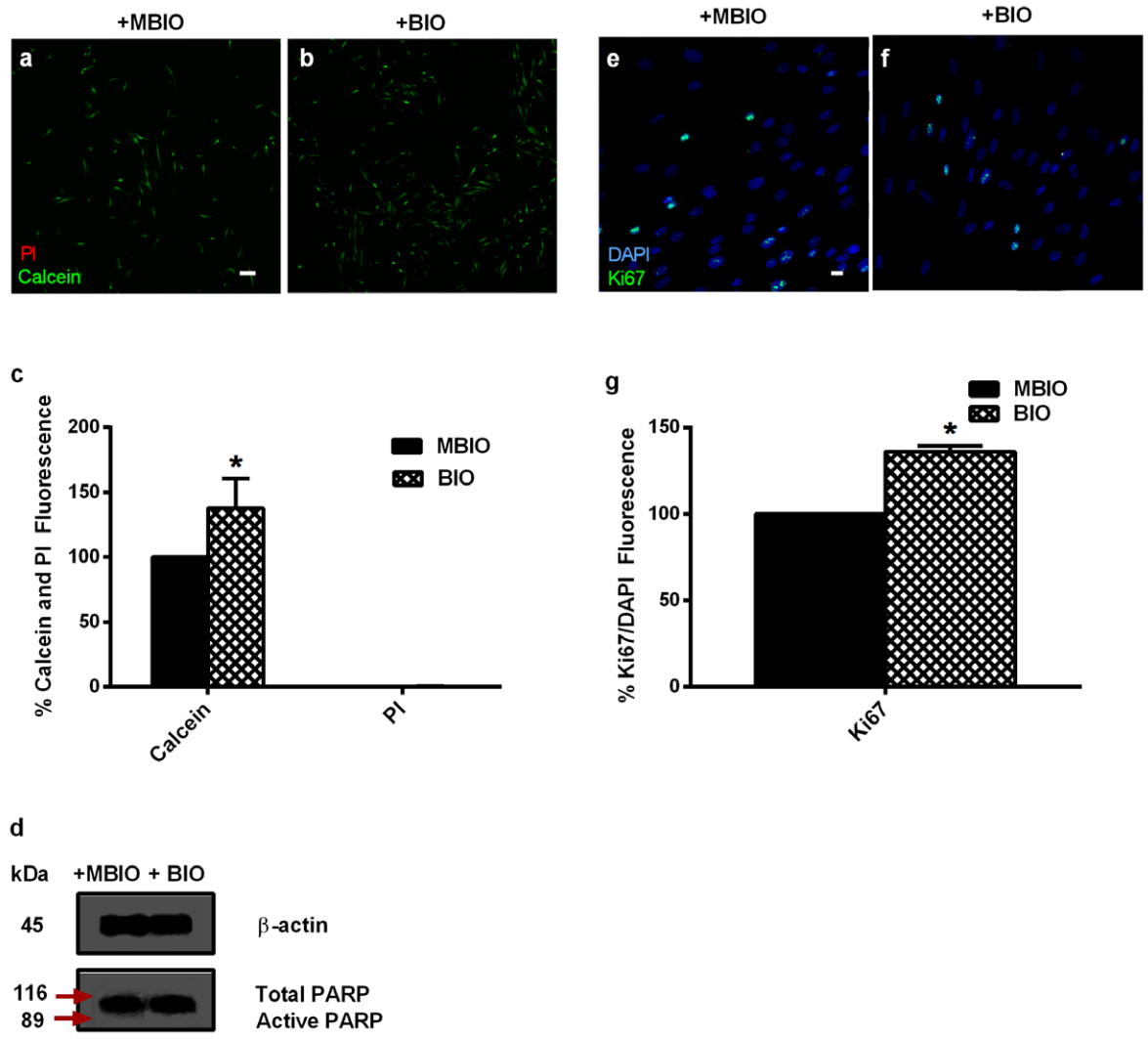


**Fig. 5.** Cell proliferation and cell death analysis in DPSCs exposed to BIO. (**a**,**b**) Calcein-AM (green) and PI (red) staining of DPSCs grown in MBIO and BIO conditions for 48 h. Scale bar = 200 μm. (**c**) Quantification of relative calcein-AM and PI fluorescence. Data are presented as mean + SEM ($n$ = 7). (**d**) WB showing absence of cleaved PARP protein (89 kDa; red arrow) in both MBIO and BIO conditions, in comparison with total inactive non-cleaved PARP (116 kDa), which was well detected. (**e**,**f**) IF for Ki67 in MBIO and BIO conditions. Scale bar = 20 μm. (**g**) Quantification of Ki67 labelling in MBIO- and BIO-treated DPSCs. Data are presented as mean + SEM ($n$ = 7). * $p < 0.05$. U-Mann Whitney test.

in expression, except the Notch2 receptor, which was found to be slightly upregulated at the transcript level (Fig. 6**a**,**b**). However, the Notch canonical ligand *Jagged1* underwent a much larger 8-fold increase in expression, when the DPSCs were exposed to BIO, followed by *Wnt-3a* and *β-catenin* with a ~5-fold increased expression (Fig. 6**b**). Moreover, *Hes1* was also prominently upregulated, which constituted solid evidence that Notch signalling was being hyperactivated by BIO treatment (Fig. 6**b**). Notch signalling upregulation at the protein level was also tested, using an antibody for active cleaved N1ICD. Increased levels of N1ICD were detected for BIO-treated DPSCs by both WB and IF (Fig. 6**c**-**f**).

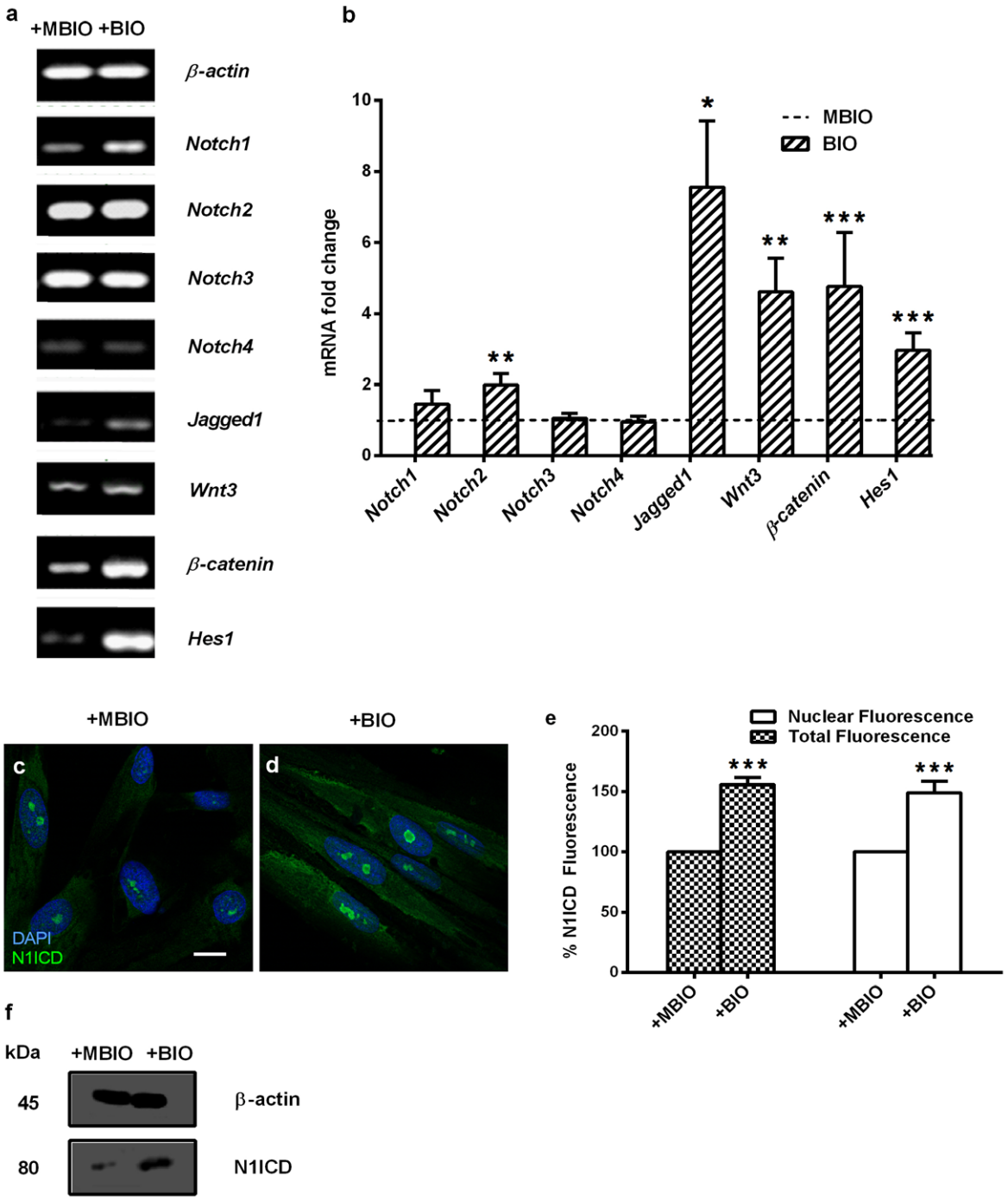


**Fig. 6**. Wnt activation by BIO positively regulated Notch signalling in DPSCs. (**a**) RT-PCR revealed differences in the expression of Notch mediators, ligands and receptors under BIO exposure. (**b**) qPCR analysis confirmed an increase in *Notch 2*, *Jagged1*, *Wnt3*, *β-catenin* and *Hes1* expression between control (MBIO) and BIO conditions. Data were normalised to *β-actin* and *Gapdh* levels and presented as mean + SEM ($n$ = 4). The dashed line represented normalised gene expression in control conditions. (**c**,**d**) IF images of active N1ICD, in cells treated with BIO and MBIO. Scale bar = 20 μm (**e**) Bar chart showing relative total and nuclear N1ICD fluorescence in MBIO- and BIO-treated cells. (**f**) WB showing an increase in N1ICD expression in BIO-treated DPSCs. β-actin was used as a protein loading control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. U-Mann Whitney test.

**Wnt/Notch activation enhanced expression of neural crest markers in DPSCs**

Notch/Wnt are crucial in NC induction and their activation enhanced core factor expression in DPSCs. Thus, it was important to test whether NC markers would also be affected by Notch/Wnt pharmacological modulation. It was found that both *Snai1* and *Snai2* were constitutively expressed in DPSCs, especially *Snail/Snai1* (Fig. 7**a**,**b**). Other NC gene markers, such as *Pax3, Neurogenin2, Twist1* and *Hnk1*, were also expressed in control DPSCs. However, when DSPCs were cultured with DAPT, the expression of all these markers was downregulated to less than 50 % of the basal levels as assessed by qPCR, with *Pax3* being downregulated to more than 90 % of its basal expression (Fig. 7**a**,**b**). Conversely, when cells were cultured with BIO, most markers showed a ~2-fold increased expression compared to control levels, with the notable exception of *Pax3*, which increased more than 10-fold (Fig. 7**c**,**d**). Some NC gene markers, which presented a very low expression in control DPSCs, such as *Sox10,* yielded consistent amplicon bands in conventional RT-PCR following BIO treatment. All these changes were corroborated by qPCR (Fig. 7**c**,**d**). Thus, Notch/Wnt activation upregulated the expression of both pluripotency and NC markers in DPSCs.

**Wnt/Notch pre-activation enhanced differentiation of DPSCs to osteoblasts and adipocytes**

Several *in vitro* protocols can be used to induce terminal differentiation of DPSCs to mature osteoblasts and adipocytes (Gronthos *et al.*, 2002; Langenbach and Handschel, 2013). The working hypothesis was that a short (48 h) pre-activation of the Notch/Wnt signalling pathway would render DPSCs more responsive to differentiation signals. Conversely, Notch/Wnt pre-inhibition would result in a reduced DPSC differentiation capacity. To test this hypothesis, DPSCs were exposed to BIO or DAPT for 48 h. Untreated DPSCs were used

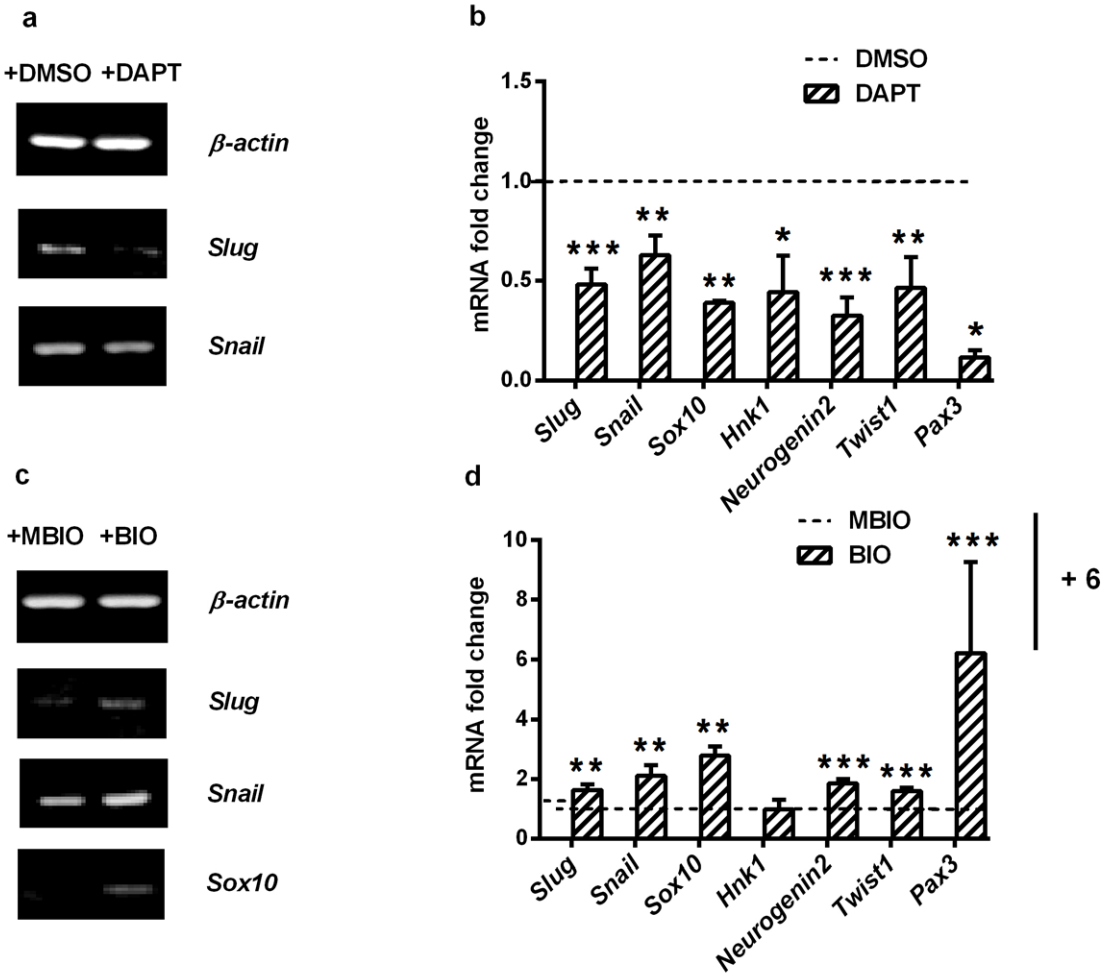


**Fig. 7.** BIO and DAPT antagonistically regulated the expression of NC markers *Snail/Snai1*, *Slug/Snai2*, *Sox10*, *Hnk1*, *Neurogenin2*, *Twis*t and *Pax3* in DPSCs. (**a**) RT-PCR showing NC marker gene expression in DMSO and DAPT-treated DPSCs. (**b**) qPCR showing relative differences on *Snai1*, *Snai2*, *Sox10, Hnk1, Neurogenin2, Twist* and *Pax3* expression. (**c**) RT-PCR showing NC markers gene expression in MBIO and BIO-treated DPSCs. (**d**) qPCR showing relative differences in *Snai1*, *Snai2*, *Sox10, Neurogenin2, Twist* and *Pax3* expression. Data were normalised to *β-actin* and *Gapdh* levels and represented as mean + SEM ($n = 3$). The dashed line represented normalised gene expression in control conditions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. U-Mann Whitney test.

as controls. Next, control and treated cells were exposed to osteoblastic differentiation medium in the absence of BIO or DAPT. To measure terminal osteoblastic differentiation, ALP and Alizarin Red S assays were used. Interestingly, after 3 weeks of osteogenic treatment, Alizarin Red S staining and calcified matrix deposit formation, as assessed by light absorbance, were between two- and three-fold higher in the DPSCs pre-treated with BIO and significantly lower in DPSCs pre-treated with DAPT, when compared to controls (Fig. 8**a**-**d**,**m**). ALP enzymatic activity was also found to be significantly higher (*i.e.* osteoblastic differentiation more efficient) when DPSCs were pre-treated with BIO (Fig. 8**e**-**h**,**m**). To verify whether Notch/Wnt modulation could also affect DPSC differentiation into adipocytes, DPSCs were exposed to BIO or DAPT for 48 h. Control and treated cells were then exposed to adipogenic differentiation medium for 4 weeks. Oil Red O staining was performed to assess

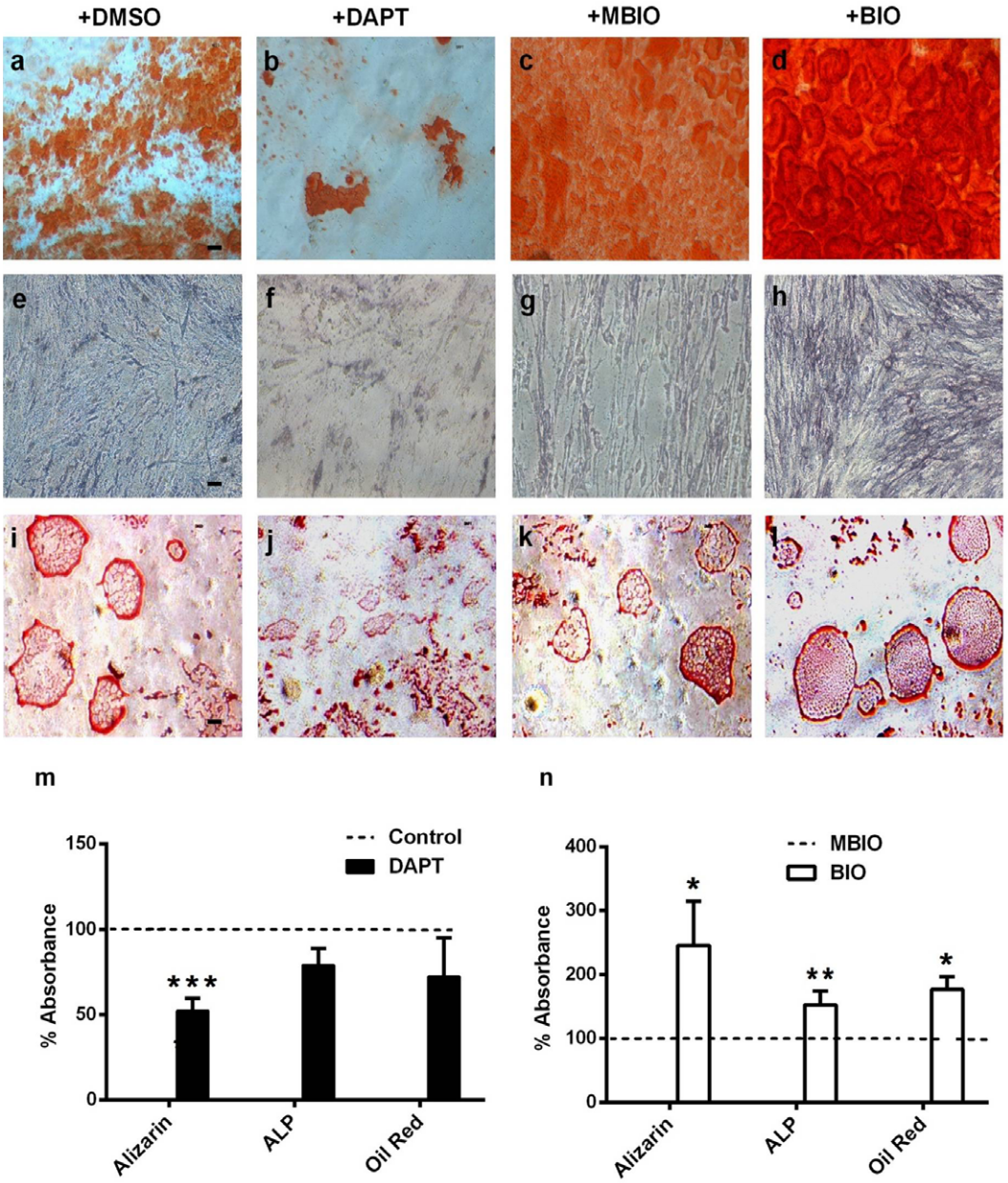


**Fig. 8**. A preconditioning exposure to BIO for 48 h enhanced DPSC differentiation efficiency into osteoblasts and adipocytes. (**a-d**) Alizarin Red S staining to assess calcified matrix formation and osteoblast differentiation in control (DMSO, MBIO), BIO and DAPT conditions. (**e-h**) ALP staining images at high magnification to detect osteoblastic commitment and differentiation in control (DMSO, MBIO), BIO and DAPT conditions. Scale bar = 100 μm. (**i-l**) Oil Red O staining to assess lipid droplet formation and adipocyte differentiation in control (DMSO, MBIO), BIO and DAPT conditions. Scale bar = 200 μm. (**m**) Relative absorbance quantification at 450 nm, 405 nm and 409 nm for Alizarin Red S, ALP and Oil Red O, respectively for DMSO- and DAPT-treated DPSCs. (**n**) Relative absorbance quantification at 450 nm, 405 nm and 409 nm for Alizarin Red S, ALP and Oil Red O, respectively for MBIO and BIO conditions. Data were normalised to DMSO and MBIO as internal controls and presented as the mean + SEM ($n = 6$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. U-Mann Whitney test.

adipocyte generation. Interestingly, it was found that BIO pre-treatment also enhanced DPSCs conversion to adipocytes as assessed by Oil Red O after the differentiation treatment period. Oil Red O staining was significantly higher in the BIO-pre-treated cells, near twice as much as controls (Fig. 8**i-l**,**n**).

**Wnt activation by exposure to human recombinant Wnt-3a protein for 48 h enhanced the expression of NC and pluripotency core factors in DPSCs**

Since BIO is known to be a general GSK3-β inhibitor (Meijer *et al.*, 2003), BIO treatment could affect other signalling pathways apart from Wnt. To prove that the observed effects on the expression of core and NC factors and stemness of DPSCs depended specifically on the canonical Wnt signalling pathway, Wnt-3a protein was used, a well-described prototypical canonical Wnt ligand (Famili *et al.*, 2015; Zhang *et al.*, 2009), to stimulate DPSCs for 48 h in similar conditions to BIO treatment. Two concentrations of human recombinant Wnt-3a were used: 2.5 μM and 5 μM, to verify any possible dose-dependent effects. Interestingly, pluripotency and NC factors showed increased expression when exposed to Wnt-3a, being the effect more pronounced at the concentration of

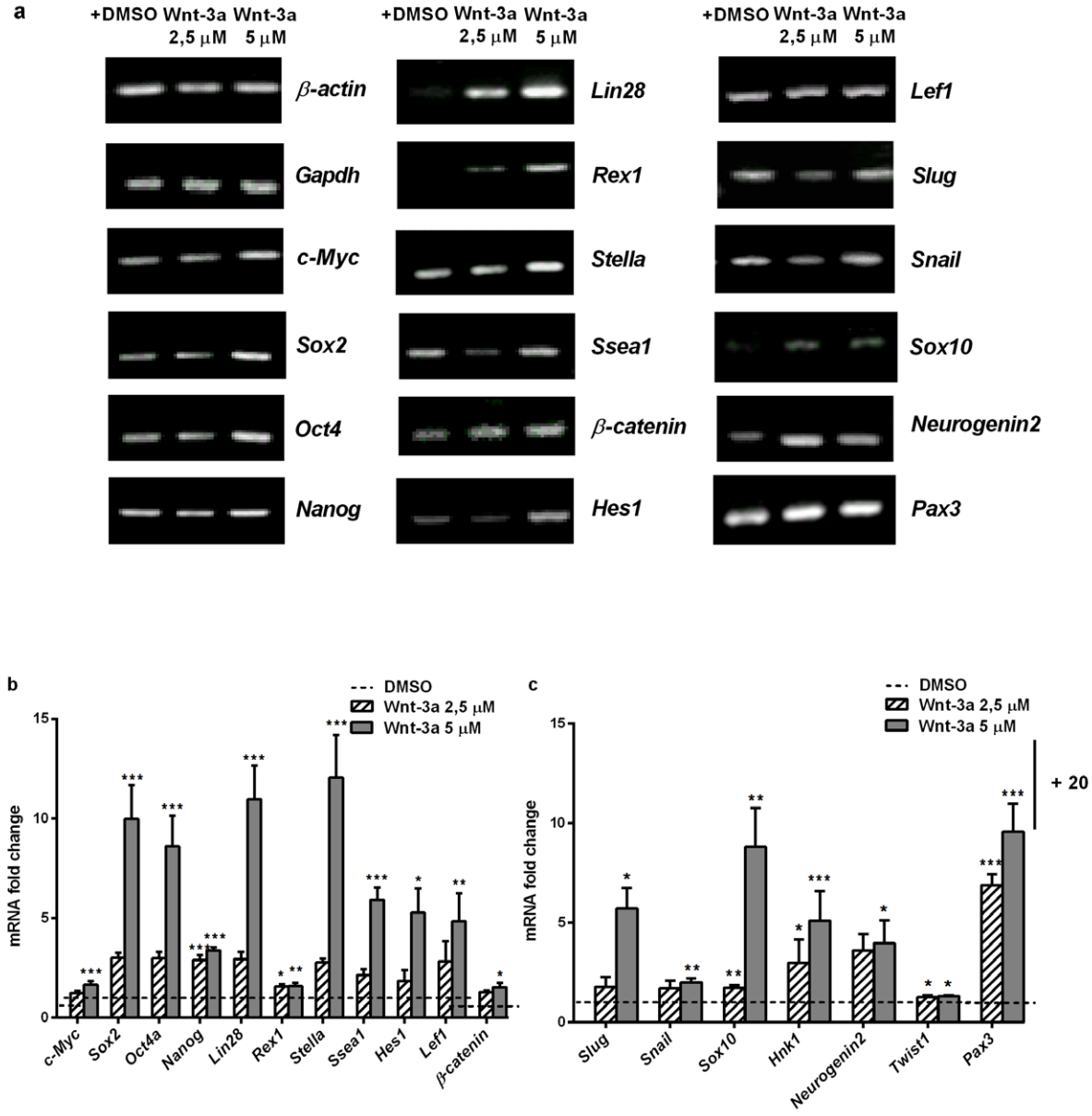


**Fig. 9.** Wnt activation by Wnt-3a increased the expression of pluripotency core factors, Notch markers and NC markers in DPSCs. (**a**) RT-PCR showing core factors (*c-Myc*, *Sox2*, *Nanog*, *Oct4*, *Rex1*, *Stella* and *Ssea1*), *Hes1* and NC markers (*Snai1*, *Snai2*, *Sox10*, *Hnk1*, *Neurogenin2*, *Twist* and *Pax3)* expression in DPSCs treated with DMSO and 2.5 μM and 5 μM Wnt-3a.(**b**,**c**) qPCR showing relative differences in the expression of (**b**) the core factors *c-Myc*, *Sox2*, *Nanog*, *Oct4*, *Rex1*, *Stella*, *Ssea1*, *Hes1*, *Lef1* and *β-catenin* and (**c**) the NC markers *Snai1*, *Snai2*, *Sox10*, *Hnk1*, *Neurogenin2*, *Twist* and *Pax3*. Data were normalised to *β-actin* and *Gapdh* levels and represented as the mean + SEM ($n = 3$). The dashed line represented normalised gene expression in control conditions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. Dunn´s Test, Kruskall Wallis H Test.

5 μM. Among the markers that were most strongly affected *Sox2*, *Oct4* (*Pou5f1* and *Pou5f1p1*), *Lin28*, *Stella*, *Ssea1*, *Slug*, *Hnk1* and *Sox10* were detected (all within a ~5-10-fold increase in expression) *Pax3* was also present (~30-fold increase with 5 μM Wnt-3a). As a control for Wnt activation, *Lef1* expression was solidly upregulated after Wnt-3a treatment. All expression changes were verified by RT-PCR and qPCR (Fig. 9**a**-**c**).

**Exposure to Wnt-3a upregulated Notch/Wnt crosstalk signalling in DPSCs**

Verification whether the Notch/Wnt interaction was also required, which was observed after DAPT/BIO treatments, and was also observed after treating cells with Wnt-3a. QPCR experiments had already showed a clear upregulation of *Hes1* expression in DPSCs exposed to Wnt-3a (Fig. 9**b**). This result was corroborated by IF and a significantly increased N1ICD nuclear fluorescence was found, especially at a concentration of 5 μM Wnt-3a (Fig. 10**a**-**c**,**k**). Similarly, nuclear β-catenin protein labelling was also found to be increased (Fig. 10**d**-**f**,**k**). Finally, similar results were observed for Ki67 labelling levels (Fig. 10**g**-**I**,**k**). Thus, 5 μM Wnt-3a induced a coordinated upregulation of Notch/Wnt cross-signalling and an increased proliferation capacity in DPSCs.

**A preconditioning treatment with human recombinant Wnt-3a enhanced the differentiation capacity of DPSCs to osteoblasts and adipocytes**

Finally, and in view of the previous results, verification was needed as to whether a preconditioning treatment with Wnt-3a would also render DPSCs

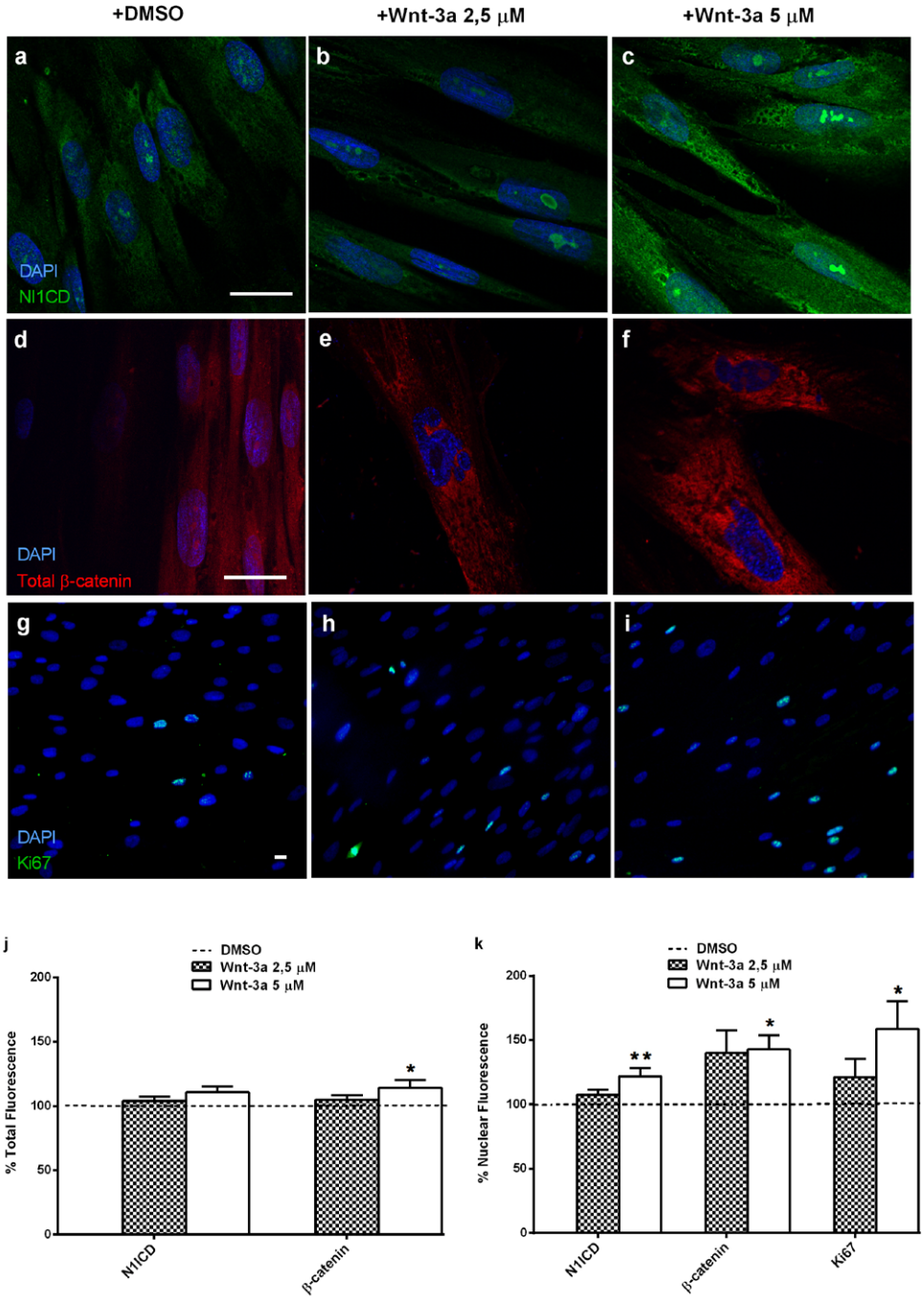


**Fig. 10.** Wnt activation by Wnt-3a upregulated Notch signalling in DPSCs. (**a-i**) IF images of DPSCs grown for 48 h in presence or absence of Wnt-3a at two different concentrations of 2.5 μM and 5 μM and immunolabeled for (**a-c**) N1ICD, (**d-f**) total β-catenin (**g-i**) and Ki67. DAPI labelled cell nuclei in blue. Scale bar = 20 μm. (**j**) Bar charts showing relative total N1ICD and total β-catenin fluorescence in control and Wnt-3a-treated DPSCs. (**k**) Bar charts showing relative nuclear N1ICD, β-catenin and Ki67 labelling fluorescence in control and Wnt-3a-treated DPSCs. Data are presented as mean + SEM ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$. Dunn´s Test, Kruskall Wallis H Test.

more responsive to osteogenic and adipogenic differentiation treatments. DPSCs were exposed to a preconditioning treatment with Wnt-3a for 48 h and subsequently cells were placed in osteogenic and adipogenic culture media, respectively – in similar conditions as shown before with DAPT/BIO. As predicted, pre-treatment with Wnt-3a enhanced the capacity of DPSCs to differentiate to osteoblasts and adipocytes (Fig. 11). The effect was more pronounced with 5 μM than with 2.5 μM Wnt-3a and was comparable to the one obtained with BIO (Fig. 8), with an almost 3-fold increase in Alizarin Red S staining and near to 2-fold increase in Oil Red O staining compared to control, non-pre-treated DPSCs cultures (Fig. 11**j**).

## Discussion

It was here reported for the first time that both Notch and Wnt signalling were required for the expression of NC and pluripotency core factors in DPSCs. Importantly, both pathways positively regulated each other in maintaining DPSC stemness and specific activation or inhibition of one pathway invariably affected the other in the same way. These findings shed light on the control mechanisms of DPSC self-renewal and maintenance and have important potential implications regarding the use of DPSCs for cell therapy.

There are several reasons for the interest in DPSCs and other dental stem cells. These cells are easily

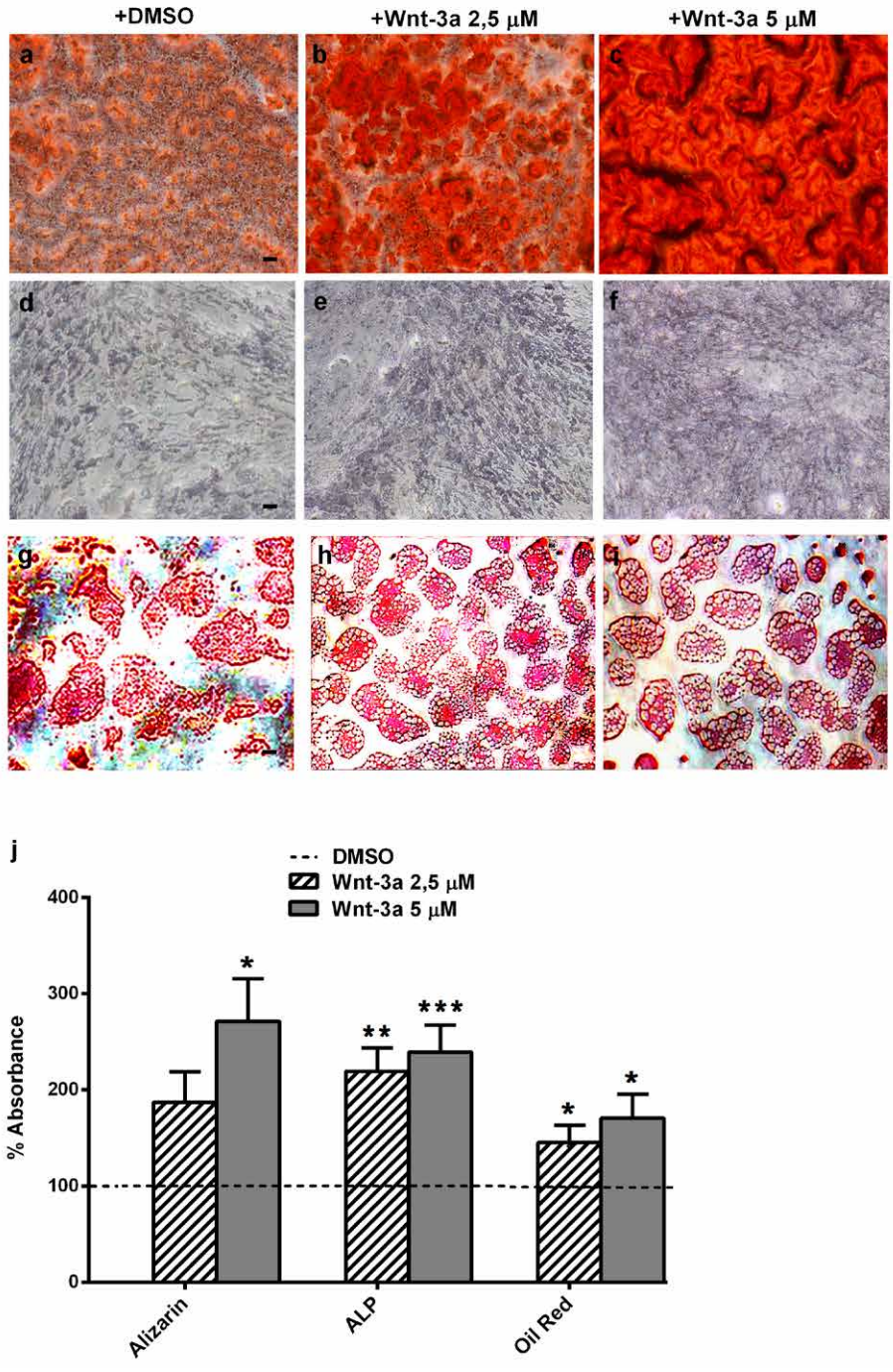


**Fig. 11.** A preconditioning treatment with Wnt-3a for 48 h enhanced DPSC differentiation efficiency into osteoblasts and adipocytes. (**a-c**) Alizarin Red S staining to assess calcified matrix formation and osteoblast differentiation in control (DMSO) and Wnt-3a (2.5 μM and 5 μM) conditions. (**d-f**) ALP staining images at high magnification to detect osteoblastic lineage commitment and differentiation in control (DMSO) and Wnt-3a (2.5 μM and 5 μM) conditions. (**g-i**) Oil Red O staining to assess lipid droplet formation and adipocyte differentiation in control (DMSO) and Wnt-3a (2.5 μM and 5 μM) treatment. Scale bar = 200 μm (**j**) Relative absorbance quantification at 450 nm, 405 nm and 409 nm for Alizarin Red S, ALP and Oil Red O, respectively. Data were normalised to DMSO as internal control and presented as mean + SEM ($n$ = 4).* $p < 0.05$. Dunn´s Test, Kruskall Wallis H Test.

accessible in both young and aged patients, possess a significant capacity for *in vitro* expansion and have non-tumourigenic phenotypes. Furthermore, evidence shows that DPSCs have a greater multi-lineage differentiation potential compared to other tissue-specific stem cells (Atari *et al.*, 2012; Kerkis *et al.*, 2006; Rosa *et al.*, 2016). It is illustrative of the growing acknowledgement of the stemness potential of DPSCs that some authors have referred to some subpopulations of these cells as dental pulp pluripotent stem cells or DPPSCs (Atari *et al.*, 2012).

Arguably, induced pluripotent stem cells (iPSCs), derived from autologous somatic cells, could be regarded as the ideal source of stem cells for *in vivo* cell therapy. These cells are truly pluripotent (*i.e.* have no restriction to differentiate in any type of adult cell), can be extracted from any donor's tissue and have the donor's same genetic background. However, traditional cell reprogramming procedures rely on permanent gene transfection with Yamanaka or related core pluripotency factors (Takahashi *et al.*, 2007) and, for safety reasons, this is not yet acceptable. Hence, while these cell reprogramming technologies are not mature enough for clinical use, the interest in searching for alternative sources of autologous pluripotent-like cells, including DPSCs, is growing very fast. The possibility to boost the natural pluripotency-like features of DPSCs by methods that do not involve permanent chromatin modification or gene transfection is highly desirable for cell therapy.

Canonical Notch and Wnt signalling pathways are described as pivotal regulators of stemness and pluripotency (Clevers *et al.*, 2014; Dravid *et al.*, 2005; Fox *et al.*, 2008; Li and Chen, 2012; Lluis *et al.*, 2008; Lowell *et al.*, 2006; Park *et al.*, 2008; Simandi *et al.*, 2016; Yan *et al.*, 2010) and their pharmacological manipulation has already been tested as a strategy to enhance either cell differentiation (Kitajima *et al.*, 2016) or cell reprogramming (Ichida *et al.*, 2014). Notch inhibition by γ-secretase blockers is associated with an increased efficiency of keratinocyte reprogramming (Ichida *et al.*, 2014). However, a very different effect was found in the studied model system with DCPSs: Notch inhibition by DAPT decreased the expression of core factors in DPSCs and diminished their capacity to generate fully differentiated osteoblasts and adipocytes. In this regard, it was worthy to note that Notch effects have long been known to be extremely context-dependent: the same Notch signal can have very different outcomes depending on the cell type, physiological state and extracellular environment. Regarding Wnt signalling, its pharmacological activation by GSK3-β inhibitors in mouse iPSCs is associated with increased cell differentiation (Kitajima *et al.*, 2016). However, in the DPSC model, BIO application significantly increased the expression of core factors and enhanced DPSC differentiation potential towards adult osteoblasts and adipocytes. Again, these differences were attributed to the different cell culture systems that were tested in this and other studies.

One important difference between the current study and the aforementioned studies was the presence/absence of serum in the culture medium. The use of medium containing FBS is well established as the gold standard for manipulation and *in vitro* expansion of DPSCs (Gronthos *et al.*, 2002; Gronthos *et al.*, 2000). *In vitro* expansion of DPSCs is often inevitable due to the small amounts of tissue material and total cell numbers that can be collected from the human dental pulp, as compared to other stem cell sources. In this regard, despite ongoing progress in culture media formulations that do not contain foetal serum for maintenance of DPSCs (Bonnamain *et al.*, 2013; Eubanks *et al.*, 2014; Jung *et al.*, 2016; Xiao and Tsutsui, 2013), currently the addition of FBS or related agents to the culture media permits easily overcoming the issue of initial cell expansion. However, it is becoming increasingly apparent that FBS-containing media also induce differentiation of DPSCs into a default osteo/odontogenic pathway (Pisciotta *et al.*, 2012; Yu *et al.*, 2010) and this may not be the best choice to generate certain cell lineages, particularly neural cells, from DPSCs (Jung *et al.*, 2016). Moreover, upon continual expansion induced by the presence of 10 % FBS, DPSC cultures also tend to generate populations of committed/differentiated cells (Mokry *et al.*, 2010). Therefore, progressive stem cell exhaustion was also a concern in this study. We confirmed that the proliferation rates and core factor expression decreased steadily in DPSCs upon serial passaging (data not shown). This correlation between sustained stem cell aging and loss of multipotency is also reported elsewhere (Bose and Shenoy, 2016).

One important finding of this study was that Notch and Wnt pathways operated coordinately as part of a common network to maintain DPSC stemness. We found that the expression of core factors in DPSCs was completely dependent upon Notch/Wnt signalling. Notch inhibition by DAPT applications abolished the expression of core factors and decreased DPSC stemness. These results were accompanied by parallel Wnt inhibition. Conversely, Wnt activation by BIO or Wnt-3a significantly increased the expression of core factors and DPSC stemness. In view of this evidence, the question became what connection do these pathways have at the molecular level? Many positive and negative interactions are reported between both pathways in a myriad of model systems (Borggrefe *et al.*, 2016; Fukunaga-Kalabis *et al.*, 2015; Kwon *et al.*, 2011; Nicolas *et al.*, 2003; Shi *et al.*, 2015). In colorectal cancer cells, Wnt signalling affects Notch through β-catenin-mediated transcriptional activation of the gene *Jagged1* (Rodilla *et al.*, 2009). Jagged1 is a Notch ligand, which can activate Notch receptors and, consequently, Notch signalling. Consistently, BIO-induced Wnt activation in DPSCs elicited a sharp increase in the transcriptional levels of *Jagged1*. It is likely that such an increased *Jagged1* transcription would eventually activate Notch receptors in DPSCs cultures. In addition, β-catenin physically binds to and interacts with reprogramming factors, in

particular Oct4a (Simandi *et al.*, 2016), whereas *Oct4a* transcription levels are also positively regulated by Wnt signalling in pluripotent stem cells (Li and Chen, 2012; Simandi *et al.*, 2016). Finally, Wnt/β-catenin also increases *c-Myc* transcription, which promotes cell proliferation (Barker *et al.*, 2000). Consistently, DPSCs treated with BIO or Wnt-3a showed an increased transcription of *c-Myc*, which was related to an increased cell proliferation.

Together with enhanced core factor expression, activation of the Notch/Wnt signalling pathways also increased the expression of NC markers in DPSCs. Both Notch and Wnt signals are associated with NC induction (Gazarian and Ramirez-Garcia, 2017; Hari *et al.*, 2012; Leung *et al.*, 2016; Rogers *et al.*, 2012; Stuhlmiller and Garcia-Castro, 2012). Indeed, NC cells also express relatively high levels of core factors, which possibly relates to their ample capacity to generate very diverse cell lineages (Thomas *et al.*, 2008). It was found that the NC markers *Pax3*, *Hnk1*, *Twist*, *Neurogenin2*, *Snail/Snai1*, *Slug/Snai2* and *Sox10* had increased expression levels when the DPSCs were exposed to Wnt-3a or BIO and that their expression decreased in the presence of DAPT, thus showing a similar relationship to the one found with pluripotency core factors.

The results demonstrated that Wnt/Notch activation increased DPSC stemness. This could have important implications for optimising the clinical use of these cells. A short preconditioning treatment with Wnt-3a or BIO for 48 h was enough to improve the potential of DPSC to differentiate into at least two different adult cell lineages, osteoblasts and adipocytes, which are both of mesenchymal origin. This very important finding proved that DPSC exhaustion by continuous exposure to FBS could be at least partially prevented by transiently boosting the activity of Notch/Wnt pathways, by means of small molecules and/or recombinant proteins, which would enhance stemness traits of DPSC cultures just 48 h before use for differentiation or cell transplant. It would also be very interesting to verify whether such preconditioning would also be effective for other non-mesenchymal cell lineages of interest, such as Schwann cells (Martens *et al.*, 2014), neuron-like cells (Gervois *et al.*, 2015) and hepatocyte-like cells (Atari *et al.*, 2012). Finally, the adoption of this preconditioning strategy could also be applicable to differentiation protocols that do not rely on FBS (Eubanks *et al.*, 2014; Xiao and Tsutsui, 2013), allowing for a faster translation to clinical therapy.

## Conclusions

Our data showed that Notch and Wnt signalling were required for the maintenance of NC and core pluripotency factor expression in DPSCs. Furthermore, both pathways operated coordinately as part of the same signalling network to maintain DPSC stemness. Thus, a 48 h Notch/Wnt signalling activation preconditioning step by BIO or Wnt-3a significantly enhanced the capacity of DPSCs to respond to standard *in vitro* protocols for osteoblastic and adipogenic differentiation. Conversely, Notch/Wnt inhibition decreased DPSC stemness. These preconditioning changes involved levels of expression of core pluripotency and NC factors, which correlated with an enhanced differentiation potential of DPSCs. This strategy could be used alone or in conjunction with other cell reprogramming methods that do not involve permanent gene transduction or modifications at genome level to generate DPSCs and other tissue-specific adult stem cells with increased self-renewal and cell differentiation potential for cell therapy.

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### Discussion with Reviewers

**Benjamin Fournier**: Do you think that the supplements you used in this study (especially BIO) may become part of the serum replacement strategy, when growing DPSC? Did you experiment (or did you plan to experiment) the use of these factors on other stem cells?

**Authors**: The main advantage of the use of FBS is that it permits a very rapid amplification of the collected cells for various uses (*e.g.* cell therapy). This is particularly important for the dental pulp where the starting collected material is usually very small, compared to other stem cell sources. However, the downside is that upon continuous amplification, stem cell populations tend to exhaust (*i.e.* lose proliferation and multilineage differentiation capacity). One important implication of this work was that DPSC exhaustion by continuous exposure to FBS could be at least partially prevented by transiently boosting, just 48 h before use, the activity of Notch/Wnt pathways – by means of small molecules and/or recombinant proteins – which would enhance stemness traits of DPSCs cultures for *in vitro* differentiation. We really do not think that these treatments alone could work as a serum replacement strategy, but rather as a complementary mechanism to prevent stem cell exhaustion after several rounds of culture passaging. We do not plan to extend these experiments to other stem cell sources yet, but we think it would be a very interesting idea.

**Marco Tatullo**: Authors have clearly reported how the potency of DPSCs can be enhanced by short preconditioning treatments with Wnt activators. Is it reasonable to consider such treatments as part of a functionalised scaffold to be used in a specific site to aid regeneration?
**Authors**: We definitely think this is a very interesting idea, especially for Wnt-3a, which is an extracellular water-soluble protein and could be easily incorporated into a functionalised scaffold by a team expert in biomaterials research.

**Editor note**: The scientific editor for this paper was Christine Hartmann.