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Original Article

Odontoblasts or odontocytes, expression of stem cells markers and differentiation markers among human adult odontoblasts

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ABSTRACT

Objective: Despite that, the odontoblasts of the dental pulp are considered a terminally differentiated type of cell. We were interested in investigating if they express any embryonic, mesenchymal, or neural stem cell markers, along with other differentiation markers they were reported to express previously. **Methods**: An immunohistochemistry study was performed on wisdom teeth extracted from healthy donors aged between 17 and 19 for dental reasons. Nine markers were tested: c-Myc, SOX2, MCAM, CD73, NCAM1, STRO1, osteocalcin, S100, and Thy1. **Results**: Odontoblasts expressed the following markers: embryonic stem cell markers SOX2, c-Myc, mesenchymal stem cell marker MCAM, the neural differentiation marker S100, and the osteogenic differentiation marker osteocalcin. Odontoblasts did not express the following markers: mesenchymal stem cell markers CD73, STRO1, Thy1, and neural stem cell marker NCAM1. **Conclusion**: These findings suggest that odontoblasts' expression of these stem cell markers may enable them to dedifferentiate under certain conditions. Further investigation is needed into whether dental materials could induce such dedifferentiation for functional dentin regeneration.

1. Introduction

The dental pulp is a loose connective tissue surrounded by specialized dentin-forming cells called odontoblasts. While regenerative capacities of dental pulp stem cells and bone regeneration were previously negotiated (Patil et al., 2021; Sayed et al., 2021), the odontoblast potentials were not previously explored. Odontoblasts are considered terminally differentiated post-mitotic, neural crest-derived cells, some researchers argue they should be named odontocytes (Casasco et al., 1997; Chang et al., 2019; Klinz et al., 2013; Larmas, 2008; Pääkkönen et al., 2007; Palosaari et al., 2000; Ruch and Lesot, 1995), which implies that odontoblasts do not participate in dentin regeneration that happens when stem cells or pre-odontoblasts differentiate into odontoblasts in response to a stimulus. There is still some uncertainty around the origin and recruitment of pre-odontoblasts, although some studies suggest that they may come from a perivascular niche that migrates out of capillary walls in response to dentin matrix degradation (Shi and Gronthos, 2003). Attempts to regenerate dentin *in vitro* have not been entirely successful, as the resulting dentin lacks the organized tubular structure of natural dentin. This can have negative effects on the dentin's biological properties, such as sensation transduction and mechanical properties (Chang et al., 2019). It is essential to understand the exact nature and origin of odontoblasts, pre-odontoblasts, and stem cells that can differentiate into odontoblasts, as well as the mechanisms of odontoblast differentiation and polarization. This understanding will be crucial to develop functional dentin regeneration techniques. By characterizing odontoblasts and their markers, we can more easily isolate and enrich the stem cell populations responsible for replacing odontoblasts *in vitro*. Therefore, we investigated the odontoblast's expression of multiple embryonic, mesenchymal, and neural stem cell markers.

2. Materials and methods

2.1. Sample collection

Healthy patients aged between 17 and 19 years old had their wisdom teeth extracted for dental reasons. The study was approved by the Institutional Review Board and Ethics Committee in accordance with The Declaration of Helsinki and was given the number 2017/158. Informed consent was obtained from the patients to acquire their teeth

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for analysis, and data privacy was maintained.

2.2. Sample processing

After the teeth were extracted, they were fixed in 10 % neutral buffered formalin and taken to the laboratory. To prepare them for sectioning, they were placed in a decalcification solution consisting of a 1:1 vol of 8 % formic acid and 8 % HCL. They were then incubated in a water bath at 50 °C for 3 h until they became soft enough to be sectioned. Following this, the teeth were fixed in formalin for 24 h and then processed using an auto processor. The processing involved fixation in formal saline, dehydration with ascending concentrations of ethanol, clearing with xylene, and finally infiltration and embedding in paraffin blocks. Once this was done, the microtome was used to create serial sections of 3-μm thickness, which were then transferred onto immunohistochemistry slides.

2.3. Immunohistochemical staining

Slides were dried in the dryer for 15 min and then incubated overnight at room temperature. Subsequently, they were deparaffinized with xylene for 15 min, rehydrated with graded ethanol (70 % – 100 %), and then washed in distilled water. Heat-mediated antigen retrieval was performed using Tris/EDTA buffer pH9 or sodium citrate pH 6. Table 1 shows the data of the primary antibodies, dilutions, incubation conditions, and antigen-retrieving methods utilized. The remaining steps were carried out as per the instructions provided in the EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC Kit (ab80436) (Abcam/UK). Bound antibodies were visualized with a microscope (Nikon Eclipse Ts2).

3. Results

Odontoblasts were found to express the embryonic stem cell marker SOX2, the mesenchymal stem cell marker MCAM, and the osteogenic differentiation marker osteocalcin both in their nucleus and cytoplasm. Cytoplasmic expression of the neural differentiation marker S100 was observed, while the embryonic stem cell marker c-Myc was expressed exclusively in the nucleus. However, the odontoblasts did not express other mesenchymal stem cell markers like CD73, STRO1, Thy1, or the neural stem cell marker NCAM1. (Fig. 1 and Table 1).

4. Discussion

Understanding the nature of odontoblasts, their embryological origin, and the mechanisms of pre-odontoblasts and neural crest stem cells' differentiation into odontoblasts, followed by their polarization, is crucial for dentin regeneration therapies. Identifying odontoblast markers will facilitate *in vitro* isolation and enrichment of the stem cell population responsible for regenerating odontoblasts. Therefore, we investigated the odontoblasts' expression of multiple stem cells and differentiation markers. SOX2 and c-Myc are transcription factors expressed in human embryonic stem cells (ESC). They work synergistically with other transcription factors to determine pluripotency and self-renewal of human ESC and play a crucial role in early cell fate determination (Boyer et al., 2005; González et al., 2011; Karagiannis et al., 2018; Sumi et al., 2007; Yao and Wang, 2020), SOX2 and c-Myc have been induced in somatic cells to reprogram them into induced pluripotent stem cells (Takahashi et al., 2007; Velychko et al., 2019).

Moreover, SOX2 promotes the odontoblast differentiation of DPSCs (Yang et al., 2017). To our knowledge, no study has investigated the expression of these transcription factors in adult human odontoblasts by immunohistochemistry. Only one study investigated the role of SOX2 in odontogenesis in human fetuses by immunohistochemistry and reported that SOX2 is active during human tooth fetal morphogenesis and odontogenesis at varying stages (da Cunha et al., 2013). Previous studies investigated the expression of these transcription factors in *in vitro* cultures of dental pulp cells. They reported that low expression of c-Myc was observed in most dental pulp stem cell lines (Yang et al., 2017). Still, a rapid loss of these transcription factors' expression and the translocation of the signal from the nucleus to the cytoplasm follows (Liu et al., 2011). The translocation of transcription factor signal from the nucleus to the cytoplasm indicates losing stemness in favor of differentiation (van Schaijik et al., 2018). Here, we report that odontoblasts of human adult dental pulp showed a nuclear and cytoplasmic expression of SOX2 while c-Myc expression was nuclear (Figure 1, C and G).

MCAM or CD146 is a cell adhesion molecule and integral membrane glycoprotein that exhibits many functions in development, signaling transduction, cell migration, mesenchymal stem cells differentiation, angiogenesis, and immune response (Joshkon et al., 2020; Leroyer et al., 2019; Wang and Yan, 2013). MCAM is considered a stem cell and endothelial marker co-expressed with STRO1 on the walls of blood vessels and is reported to reside in the perivascular niche of the dental pulp (Shi and Gronthos, 2003). CD146 + DPSCs showed higher mineralization and adipogenic abilities than CD146- cells (Matsui et al., 2018).

In this study, odontoblasts showed cytoplasmic and nuclear expression of MCAM (Figure 1, H). Despite being a membrane glycoprotein, accumulated evidence considers the shuttling of adhesion transmembrane proteins between the plasma membrane and nucleus to be common, where adhesion transmembrane proteins interact with transcription factors to perform certain functions (Zheng and Jiang, 2022). The nuclear MCAM expression in odontoblasts, along with the fact that Sox2 and c-Myc are required for proper dedifferentiation of multiple

Table 1
The primary antibodies utilized for immunohistochemistry. Their clone, source, dilutions, incubation conditions, antigen retrieval methods, and the observed cellular location.

Antibody	Clone	Source	Dilution/ Incubation conditions	Antigen retrieval method	Cellular location
c-Myc	Mouse monoclonal [9E10]	Abcam, UK	1:500 1 h 25 °C	Tris/ EDTA pH 9	Nucleus
SOX2	Mouse monoclonal [20G5]	Abcam, UK	1:200 overnight 4 °C	Sodium citrate pH 6	Nucleus Cytoplasm
MCAM 1	Mouse monoclonal [P1H12]	Abcam, UK	1:500 overnight 4 °C	Tris/ EDTA pH 9	Nucleus Cytoplasm Membrane
CD73	Mouse monoclonal [7G2]	Abcam, UK	1:200 overnight 4 °C	Tris/ EDTA pH 9	Negative
NCAM1	Mouse monoclonal [RNL-1]	Abcam, UK	1:100 overnight 25 °C	Tris/ EDTA pH 9	Negative
STRO1	Mouse monoclonal [STRO-1]	Abcam, UK	1:200 1 h 25 °C	Sodium citrate pH 6	Negative
Osteocalcin	Rabbit polyclonal [AB10911]	Millipore, USA	1:500 overnight 4 °C	Sodium citrate pH 6	Nucleus Cytoplasm
S100	Rabbit polyclonal	Dako, Denmark	1:500 overnight 4 °C	Sodium citrate pH 6	Cytoplasm
Thy1	Rabbit Monoclonal [EPR3133]	Abcam, UK	1:500 overnight 4 °C	Sodium citrate pH 6	Negative

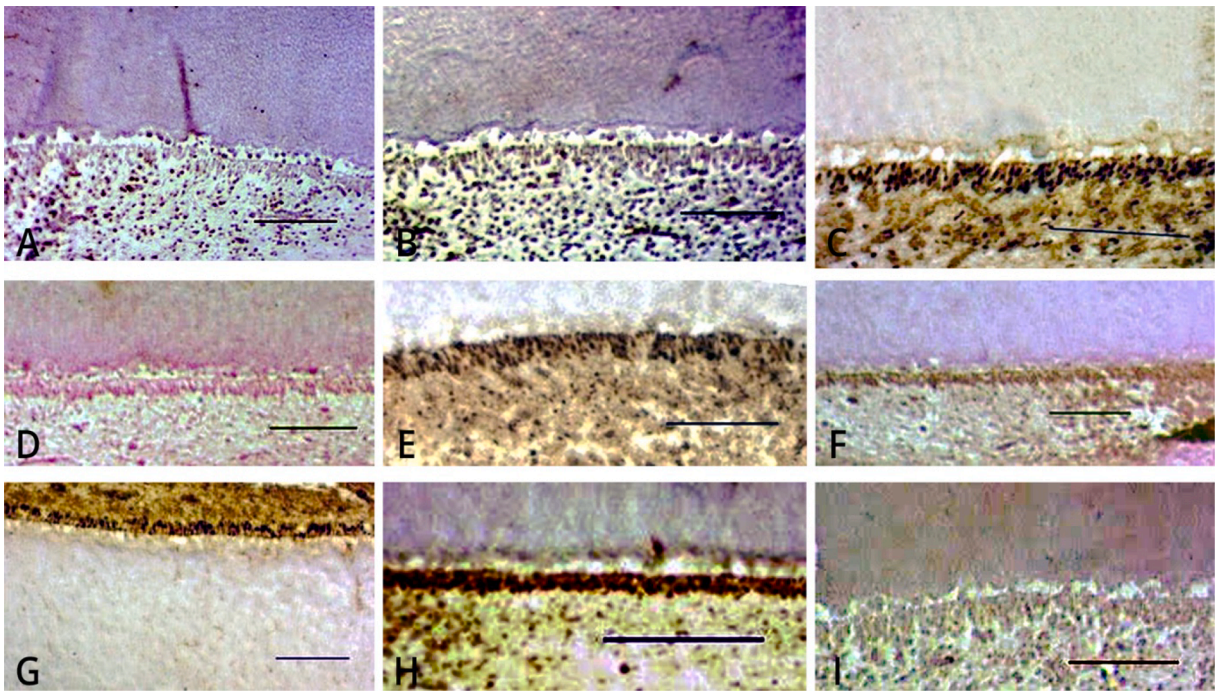

Fig. 1. Expression of stem cell markers and differentiation markers in the odontoblasts of the human adult dental pulp. A) Odontoblasts showed no expression of neural stem cells marker NCAM1, 10x. B) Odontoblasts showed no expression of the mesenchymal stem cells marker Thy1, 10x. C) Odontoblasts showed a nuclear and cytoplasmic expression of the embryonic stem cell markers SOX2, 20x. D) Odontoblasts showed no expression of CD73, 10x. E) Odontoblasts showed a nuclear and cytoplasmic expression of differentiation osteogenic markers osteocalcin, 10x. F) Odontoblasts showed a cytoplasmic expression of the neural differentiation marker S100, 10x. G) Odontoblasts showed a nuclear expression of the embryonic stem cell marker c-Myc, 10x. H) Odontoblasts showed a nuclear and cytoplasmic expression of the mesenchymal stem cells marker MCAM, 20x. I) Odontoblasts showed no expression of the mesenchymal stem cells marker STRO1, 10x. Scale bar 50 um.

types of cells during regeneration of injured tissues or in cancer (Herreros-Villanueva et al., 2013; Iovanna et al., 1992; Mashanov et al., 2015), leads us to wonder whether the expression of the transcription factors SOX2 and c-Myc and the mesenchymal stem cell marker MCAM in odontoblasts of the human adult dental pulp is related to differentiation, self-renewal or most essentially what we suggest here, dedifferentiation abilities.

Cell dedifferentiation is the process by which cells grow reversely from a partially or terminally differentiated stage to a less differentiated stage within their lineage, this mandates a change in the shape, gene expression pattern, protein expression pattern, and function. Dedifferentiation can be targeted as a new therapeutic approach for regenerative therapy (Yao and Wang, 2020). Physiological or pathological dedifferentiation of multiple cell types like cardiomyocytes, neurons, chondrocytes, epidermal cells, chondrocytes, endothelial cells, and vascular smooth muscle cells has been studied extensively (Yao and Wang, 2020), while no studies performed on the pathways, genes, biomolecules, culture conditions, niche, extrinsic stimuli, or inhibitory factors responsible for physiological or pathological dedifferentiation and redifferentiation of odontoblasts. On the other hand, the differentiation and polarization of odontoblasts were studied and documented previously (Chang et al., 2019; Ruch and Lesot, 1995). Induction of dedifferentiation and then redifferentiation of multiple types of terminally differentiated cells or stem cells are previously reported; the dedifferentiation process reverted cells into more primitive stem cells with improved differentiation potential, cell survival, migratory capacity, colony-forming ability, and increased expression of Oct4, SOX2, and Nanog (Paduano et al., 2021; Rui et al., 2015). In one study, DPSCs were differentiated into the osteogenic lineage and dedifferentiated into DPSCs; dedifferentiated DPSCs showed a more primitive phenotype and a significantly higher expression of SOX2, Klf4, and Nanog. Dedifferentiated DPSCs showed a substantially higher osteogenic differentiation quality when redifferentiated into osteogenic lineage again (Rui et al., 2015). The suggestion that odontoblasts still acquire this dedifferentiation ability needs further investigation, and noticing that extrinsic signals can play an essential role in dedifferentiation (Yao and Wang, 2020) can be experimented with for dentin regeneration using properly investigated dental materials to induce dedifferentiation and redifferentiation of odontoblasts.

Osteocalcin is the main non-collagenous hydroxyapatite-binding protein synthesized by osteoblasts, odontoblasts, and hypertrophic chondrocytes. It regulates mineralization and is considered a marker of bone cell metabolism (L Ramos et al., 2016). S100 is a marker of neural crest-derived cells. S100 has a low expression in neural crest cells, and the expression increases with the propagation of these cells toward differentiation, especially in Schwann cells (Jessen and Mirsky, 2015). Therefore, the strong expression of these two differentiation markers by odontoblasts of human adult dental pulp was expected. This expression was observed as a nuclear and cytoplasmic expression of the osteogenic differentiation marker osteocalcin and a cytoplasmic expression of the neural differentiation marker S100 (Figure 1, E and F).

The neural cell adhesion molecule (NCAM) is an immunoglobulin-like neuronal surface glycoprotein that binds to various other cell adhesion proteins to mediate adhesion, guidance, and differentiation during neuronal growth. It is also expressed in neural crest cells and some populations of Mesenchymal stem cells (MSCs) and neural stem cells. (Weledji and Assob, 2014). CD73 is a cell surface protein that hydrolyzes extracellular nucleotides into permeable membrane nucleosides and is expressed in MSCs (L Ramos et al., 2016; Ranjbar et al., 2019; Tan et al., 2019). STRO1 is a cell surface antigen, like MCAM, is expressed by MSCs in the bone marrow and dental pulp blood vessels and is considered a marker of pre-osteogenic populations and an early marker of different MSCs populations to be found in the perivascular niche (Shi et al., 2001; Shi and Gronthos, 2003). Thy1, or CD90, is expressed on fibroblasts, neurons, endothelial cells, and thymocytes. It has been identified as an MSC marker and has a crucial role in MSC fate decisions (Saalbach, 2019). Thy1, STRO1, CD73, and NCAM1 are

markers of MSCs. The negative expression of these markers among odontoblasts (Figure 1, A, B, D, and I) suggests that MSCs do not contribute to odontoblasts' regeneration.

5. Conclusion

Understanding dentin regeneration requires investigating pre-odontoblast proliferation and differentiation in dental pulp. However, there is limited data available about odontoblasts' origin and regeneration. To shed light on the nature of these cells, we investigated using immunohistochemistry the odontoblasts' expression of multiple stem cell markers in the pulp tissue of young adult human males and females' third molars. Our findings show that odontoblasts express embryonic transcription factors c-Myc and SOX2 and the mesenchymal stem cell marker MCAM. This raises the question of whether these cells are terminally differentiated or retain the potential to dedifferentiate, and the underlying mechanisms need to be revisited. We also suggest that isolating and enriching DPSCs populations that are SOX2, c-Myc, and MCAM positive and studying their dentin functional regeneration *in vitro* would be worth investigating. This could facilitate controlling odontoblast cell fate under specific physiological or pathological circumstances for dentin regenerative applications.

6. Disclaimer statement

The authors have no conflict of interest to announce.

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Credit authorship contribution statement

Both authors participated in conceptualization, acquisition and curation of data, writing and revising the manuscript, investigation, validation, visualisation and analysis, also in methodology.

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