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Neural and Neural Crest-Derived Stem Cell Distribution in the Adult Human Dental Pulp

Distribución de Células Madre Neurales y Derivadas de la Cresta Neural en la Pulpa Dental Humana Adulta

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SUMMARY: Despite comprehensive studies and reports about the properties of dental pulp stem cells (DPSCs) *in vitro*, we still need to confirm whether these *in vitro* characteristics coincide with the nature of DPSCs in situ. The anatomical location of DPSCs populations in the dental pulp has yet to be investigated. Moreover, the mesenchymal DPSCs have been much more studied than the neural crest-derived DPSCs. In this study, well-recognized neural/neural crest stem cell markers NCAM1, Nestin, SNAIL/SLUG, SOX9, and S100 are being investigated by immunohistochemistry to localize the precise location of these populations of DPSCs within the human adult dental pulp. All previously mentioned markers were expressed in the dental pulp, and their intensity and location of expression were reported.

KEY WORDS: NCAM1; Nestin; SNAIL/SLUG; SOX9; S100; Human dental pulp; Stem cells; Neural stem cells; Neural crest.

INTRODUCTION

The dental pulp is a loose connective tissue surrounded by specialized dentin-forming cells called odontoblasts. The dental pulp stem cells (DPSCs) originate from the cranial neural crest (Chai *et al*., 2000; Miletich & Sharpe, 2004) and the mesoderm, and both populations are heterogeneous (Gronthos *et al*., 2000, 2002; Liu *et al*., 2006; Honda *et al*., 2007; Jo *et al*., 2007; Sasaki *et al*., 2008; Paino *et al*., 2010; Spath *et al*., 2010; Janebodin *et al*., 2011; Abe *et al*., 2012; Ishizaka *et al*., 2013; Al-Zer *et al*., 2015; Al-Zer & Kalbouneh 2015). DPSCs from neural crest origin have phenotypic characteristics and differentiation abilities similar to those of neural crest (Stevens *et al*., 2008; Abe *et al*., 2012; Hamada K, Miura M 2012; Al-Zer *et al*., 2015). NCAM1, Nestin, SNAIL/SLUG, SOX9, and S100 are recognized neural/neural crest stem cell markers (Lendahl *et al*., 1990; Chou *et al*., 2003; Jessen & Mirsky, 2005; Hall, 2009a; Weledji & Assob, 2014). To our knowledge, no previous studies investigated the existence or the distribution of these neural/neural crest markers in the human adult dental pulp by immunohistochemistry. Accordingly, this study tries to illuminate the distribution and intensity of DPSCs that express these stem cell markers. The difference between these DPSCs populations' percentage *in situ* and *in vitro* could be further studied to elucidate the effect of culturing methods on the enrichment or extinction of these populations, as it was suggested earlier that culture conditions are responsible for such effects (Al-Zer *et al*., 2015; Alzer *et al*., 2021). Understanding the nature of neural/neural crest-derived stem cells *in situ* facilitates the isolation and enrichment of these populations *in vitro* for future recruiting in neural regenerative therapeutics.

MATERIAL AND METHOD

Sample collection. Wisdom teeth from healthy patients aged 17-19 years old were collected, and teeth were extracted for dental reasons.

Sample processing. Following extraction, the teeth were fixed in 10 % neutral buffered formalin and transformed to the laboratory. The teeth were immersed in the decalcification solution (1:1 volume of 8 % formic acid + 8 % HCL) and then incubated in a water bath at 50°C for 3h and left until they were soft enough to be sectioned. The teeth were fixed in formalin for 24h and then inserted into an auto processor where they were subjected to fixation in formal saline, dehydration with ascending concentrations of ethanol, clearing with xylene, and finally, infiltration and embedding in

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paraffin blocks. With the microtome, serial sections of 3-micrometer thickness were utilized and transferred onto immunohistochemistry slides.

Immunohistochemical staining. Slides were dried in the dryer for 15min and then incubated overnight at room temperature. Subsequently, they were deparaffinized with xylene for 15 min, rehydrated with graded ethanol (70 % - 100 %), then washed in distilled water. Heat-mediated antigen retrieval was performed using Tris/EDTA buffer pH9 or sodium citrate pH 6. Table I shows the data of the primary antibodies, dilutions, incubation conditions, and antigen retrieving methods utilized. The rest of the procedure was performed exactly as stated 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). The location and intensity of the signal were graded, as explained in the results in Table II.

Table I. The primary antibodies utilized for Immunohistochemistry. Their clone, source, dilutions, incubation conditions, and antigen retrieval methods.

Antibody	Clone	Source	Dilution/ incubation conditions	Antigen retrieval method
NCAM1	Mouse monoclonal [RNL-1]	Abcam, UK	1:100 overnight 25°C	Tris/ EDTA pH 9
Nestin	Mouse monoclonal	Abcam, UK	1:500 overnight 4°C	Tris/ EDTA pH 9
SNAIL/ SLUG	Rabbit polyclonal	Abcam, UK	1:100 overnight 4°C	Tris/ EDTA pH 9
SOX9	Rabbit polyclonal [196908]	Abcam, UK	1:500 overnight 4°C	Tris/ EDTA pH 9
S100	Rabbit polyclonal	Dako, Denmark	1:500 overnight 4°C	Sodium citrate pH 6

Table II. The distribution and intensity of expression of NCAM1, Nestin, SNAIL/ SLUG, SOX9, and S100 in the adult human dental pulp.

Antibody	Locations of marker expression	Intensity
NCAM1	Cell clusters scattered in the dental pulp - large	++
	Cell clusters scattered in the dental pulp - small	++
	Cell sheets in the subodontoblastic zone	++
	Accessory canals	++
Nestin	Dental pulp core cells	+
	Cell clusters scattered in the dental pulp - small	+
SNAIL/SLUG	Cell clusters scattered in the dental pulp - small	+
	Cell clusters - subodontoblastic	+
SOX9	Dental pulp core cells	+
S100	Dental pulp nerve bundles	+++
	The area of Raschow plexus	+++
	Cell clusters scattered in the dental pulp - small	+++

The intensity of expression is defined in all the locations where the antibodies were detected as follows: +++intense, ++ strong, + moderate.

RESULTS

All five stem cell markers were identified in the dental pulp. Figures 1 to 3 and Table II summarize each marker's localization and intensity of expression.

NCAM1 was expressed strongly in rarely seen small and large cell clusters scattered in the dental pulp (Fig. 1A,B). Moreover, cell sheets of positive NCAM1 cells were observed in the subodontoblastic zone and entering the accessory canals (Fig. 1C,D). Nestin expression was detected in dental pulp core cells, and a few small cell clusters scattered in the dental

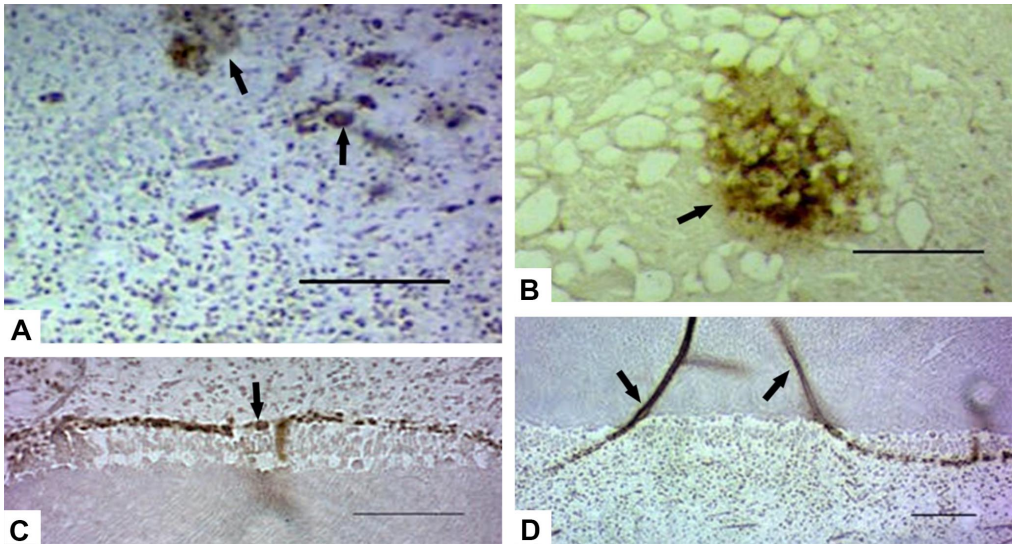

Fig. 1. The distribution of NCAM1 expression in the adult human dental pulp. A) and B) NCAM1 was expressed strongly in rarely seen small and large cell clusters scattered in the dental pulp (black arrows), both 10x. Cell sheets of positive NCAM1 cells were observed in the subodontoblastic zone C), 20x, and entering the accessory canals D), 10x. Scale bar 50 µm.

pulp (Fig. 2). SNAIL/SLUG showed a moderate signal in rarely seen cell clusters scattered in the subodontoblastic zone (Fig. 3A). Some nerve fibers also moderately expressed it (Fig. 3 B). SOX9 was rarely detected as a moderate expression in the nuclei of a few pulp core cells (Fig. 3C). S100 expression was intense and uniform in dental pulp core nerve bundles, the area of Raschow plexus (Fig. 3D,E), and in small clusters scattered in the dental pulp (Fig. 3D).

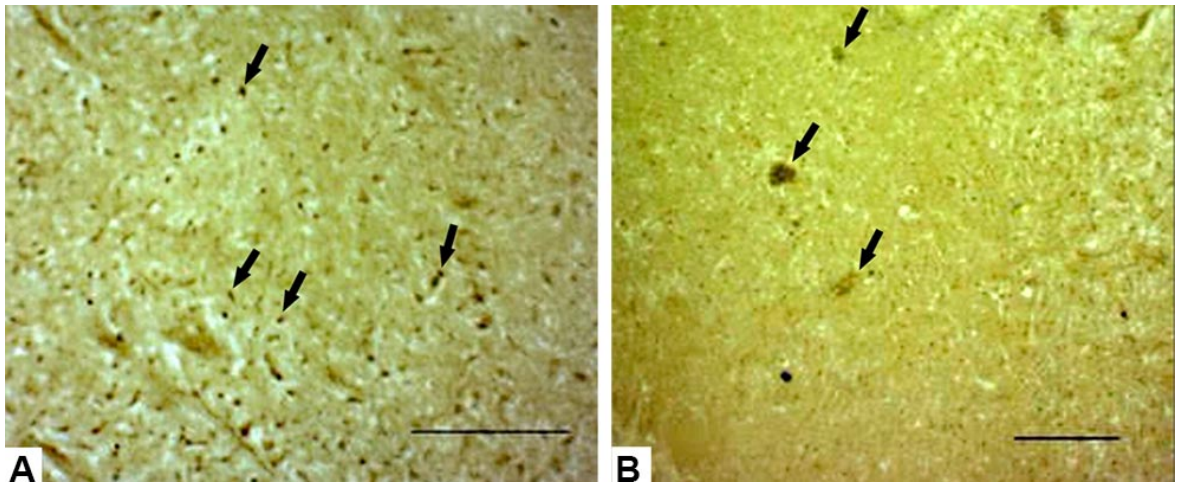

Fig. 2. The distribution of Nestin expression in the adult human dental pulp. Nestin expression was detected in dental pulp core cells A), 20x, and in a few small cell clusters scattered in the dental pulp, B), 10x. Scale bar 50 μm.

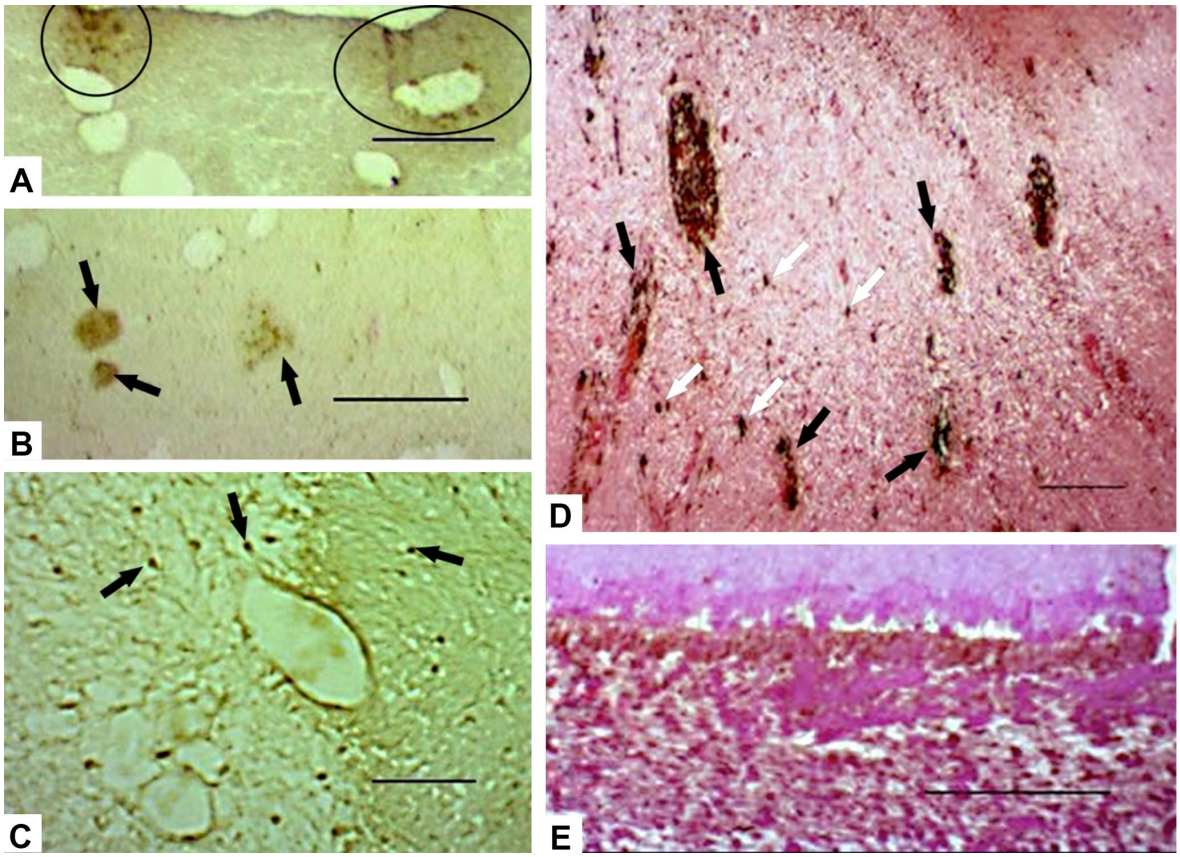

Fig. 3. The distribution of expression of SNAIL/SLUG, SOX9, and S100 in the adult human dental pulp. A) and B) SNAIL/SLUG showed a moderate signal in rarely seen cell clusters scattered in the subodontoblastic zone A). It was also moderately expressed in some nerve fibers B), both 10x, C) SOX9 was rarely detected as a moderate expression in the nuclei of few pulp core cells, 10x. D) S100 expression was intense and uniform in dental pulp core nerve bundles (black arrows) and small clusters (white arrows) scattered in the dental pulp, 10x, and in the area of Raschow plexus E), 20x. Scale bar 50 μm.

DISCUSSION

Immunohistochemical studies investigating the human adult dental pulp stem cell populations are limited. Moreover, the mixture of the investigated markers is different from those reported here. Accordingly, previous reports that examined the same markers of our choice are mentioned and compared to our study below.

Our results demonstrated the presence of a subodontoblastic neural stem cell niche, where SNAIL/SLUG, S100, and NCAM1 were observed. A second neural stem cell niche was observed in the core of the dental pulp, where all five markers NCAM1, Nestin, SNAIL/SLUG, SOX9, and S100 clustered.

Nestin is an embryonic intermediate filament expressed by the neuroepithelial stem cells of the neural tube and is downregulated when the neural stem cells differentiate. It is required for the survival and renewal of neural progenitor cells as it is involved in the organization and maintenance of the cell shape and the trafficking of intermediate filaments during mitosis of progenitor cells (Lendahl *et al*., 1990; Chou *et al*., 2003). Nestin expression was detected in dental pulp core cells and rarely in small cell clusters scattered in the dental pulp (Fig. 2), which coincided with earlier studies that performed immunohistochemistry on human wisdom teeth or human deciduous teeth and showed a wide distribution of expression of Nestin in the dental pulp tissue (Lizier *et al*., 2012; Martens *et al*., 2012).

S100 is a marker of neural crest and 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 & Mirsky, 2005) Here, we report that S100 expression was intense and uniform in dental pulp core nerve bundles and the area of Raschow plexus (Fig, 3, D, E), which is expected. Whether the expression demarcates neural crest stem cells or Schwann cells needs further investigation. Interestingly, S100 positive small clusters scattering in the dental pulp were detected (Fig. 3, D), which coincides with previous studies reporting a wide distribution of S100 positive cells in dental pulp cells *in situ* and in dental pulp *in vitro* cultured stem cells (Lombardi & Fiore-Donno, 1993). We presume the S100 positive small clusters to be niches of neural crest-derived stem cells, which need more investigations.

To our knowledge, no previous reports have investigated SOX9, SNAIL/SLUG, and NCAM1 existence and location in the human dental pulp by immunohistochemistry. Accordingly, we will only discuss our results.

The transcription factor SOX9 is a member of the high-mobility group gene family. It regulates neural crest cell induction and survival (Hall, 2009a,b,c). SOX9 was rarely detected as a moderate expression in the nuclei of a few pulp core cells (Fig. 3, C). SNAIL and SLUG (sometimes called SNAIL 2) are genes expressed by the neural crest and required for neural crest specifications and migration. Both are critical regulators of epithelial-mesenchymal transition in the neural crest and are used as a marker for pre- and post-migratory neural crest cells (Aybar *et al*., 2003; Hall, 2009a; Tang & Weiss, 2017). Both were expressed in sphere-forming cells derived from dental apical papilla cells of immature open apex human dental pulp (Abe *et al*., 2012). SNAIL/SLUG showed a moderate signal in a few rarely detected cell clusters scattered in the subodontoblastic zone (Fig. 3 A). They were expressed moderately in some nerve fibers (Fig. 3 B). Both SOX9 and SNAIL/SLUG are expected to be rarely detected in the dental pulp as most neural crest cells commit differentiation. Interestingly SOX9 and SNAIL/SLUG, both neural crest cell markers, are presented in various locations in the dental pulp, which suggests that neural crest stem cells settled into diverse populations with distinctive characteristics.

The neural cell adhesion molecule (NCAM1) is an immunoglobulin-like neuronal surface glycoprotein that binds to other cell adhesion proteins to mediate adhesion, guidance, and differentiation during neuronal growth. It is expressed in migrating neural crest cells and some mesenchymal and neural stem cell populations (Hall, 2009a,c; Weledji & Assob, 2014) NCAM1 was expressed strongly in rarely seen small and large cell clusters scattered in the dental pulp (Fig. 1, A, B). Moreover, cell sheets of positive NCAM1 cells were observed in the subodontoblastic zone and entering the accessory canals (Fig. 1, C, D). DPSCs expressing SOX9, SNAIL/SLUG, and NCAM1 would provide an excellent source of neural crest-derived stem cells if their enrichment *in vitro* is optimized.

CONCLUSION

Information concerning DPSCs proliferation and differentiation is essential to understanding tooth response to regenerative stimuli. Since there is little data concerning DPSCs *in vivo*, we have investigated the localization of neural or neural crest-derived DPSCs populations in third molars of young adult humans using immunohistochemistry. The results demonstrated the presence of two neural DPSCs niches observed in the subodontoblastic dental pulp cell-rich zone and the pulp core. The observations that multiple DPSCs niches exist may have further implications for their recruitment *in situ* for multiple regenerative therapies. However, these stem cells' exact location, developmental

potential, and ontogeny are still largely unknown. In the present study, identifying neural and neural crest-derived DPSCs niches may help elucidate the fundamental conditions necessary to selectively maintain and expand these DPSCs populations *in vitro* and direct their potentials *in vivo*.

Ethics approval and consent to participate. The Institutional Review Board and Ethics Committee approved the study in accordance with The Declaration of Helsinki. Informed consent was obtained to acquire the teeth for analysis, and data privacy was preserved.

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RESUMEN: A pesar de estudios e informes exhaustivos sobre las propiedades de las células madre de la pulpa dental (DPSC) *in vitro*, todavía necesitamos confirmar si estas características *in vitro* coinciden con la naturaleza de las DPSC *in situ*. La ubicación anatómica de las poblaciones de DPSC en la pulpa dental aún no se ha investigado. Además, las DPSC mesenquimales han sido mucho más estudiadas que las DPSC derivadas de la cresta neural. En este estudio, se están investigando mediante inmunohisto química marcadores de células madre de la cresta neural/ neural NCAM1, Nestin, SNAIL/SLUG, SOX9 y S100 para localizar la ubicación precisa de estas poblaciones de DPSC dentro de la pulpa dental humana adulta. Todos los marcadores mencionados anteriormente se expresaron en la pulpa dental y se informó su intensidad y ubicación de expresión.

PALABRAS CLAVE: NCAM1, Nestin; SLUG DE CARACO; SOX9; S100; Pulpa dental humana; Células madre; Células madre neurales; Cresta neural.

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