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Enrichment and Schwann Cell Differentiation of Neural Crest-derived Dental Pulp Stem Cells

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Abstract. *Background/Aim: As already described in previous studies, neural crest stem cells (NCSCs) can be found in adult human dental pulp. The present study investigated the methodology for enrichment and differentiation-induction of the above mentioned cells. Materials and Methods: Dental pulp was extracted from human wisdom teeth of four patients and subsequently cultured as explants on fibronectin-coated plates in neurobasal medium supplemented with B27, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin, l-glutamine and neuregulin-β1. The cells were then characterized by immunofluorescence, while their differentiation-potential was tested by the attempt to induce cells into different lineages, i.e. osteogenic, melanocytic and glial. Results: The enriched cell population expressed nestin, CD271 and SOX10, which are well-known markers for NCSCs. Consequently, the cells were successfully induced to differentiate into osteoblasts, melanocytes and Schwann cells, expressing the corresponding differentiation markers. Conclusion: Human adult dental pulp contains a population of stem cells with neural crest ontogeny, which can thus be recruited for multiple regenerative therapies.*

The neural crest (NC) was identified by the Swiss embryologist Wilhelm His in 1868 as a group of cells originating in the ectoderm of the vertebrate embryo, between the neural tube and the epidermis. The NC is a transient embryonic structure, containing cells that contribute to the formation of diverse tissues and organs, as for example the peripheral nervous system, smooth muscle cells of the cardiovascular system, skin pigment cells, as well as craniofacial bones, cartilage and connective tissue (1-3). Although neural crest cells (NCCs) undergo developmental restrictions with time, at least some NCCs maintain the capacity of self-renewal and show a certain developmental potential. Such neural crest-derived (NC-derived) stem cells are not only present in the embryonic NC, but have also been isolated from various other fetal and adult NC-derived tissues. These post-migratory neural crest stem cells (NCSCs) functionally resemble their embryonic counterparts in their ability to differentiate into a variety of cell types (1, 4-6). The cranial NC contribute to the formation of the majority of the cell types in the dental pulp (7). However, intrapulpal blood vessels are the source of some dental pulp mesenchymal cells (8). Mesenchymal dental pulp stem cells (DPSCs) have been described for the first time by Gronthos and Shi, and their heterogeneity, as well as their wide differentiation capacities, have already been reported in many studies (2, 9, 10). In the present study, the isolation of a population of DPSCs, which is closer to be NC-derived rather than the previously reported mesenchyme-derived stem cells, was aimed, *i.e.* a population able to differentiate into Schwann cells and melanocytes, which are NC derivatives but not mesenchyme derivatives.

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Materials and Methods

Sample collection. Human wisdom teeth from 26 patients aged between 16 and 26 were collected according to the corresponding Hamburg authority for privacy protection. The samples from 22 patients out of these were utilized in order to establish appropriate culturing and differentiation conditions. Experiments were performed on triplicates or tetrads on the left of four samples.

Tissue culture. Teeth were cleaned from gingival and periodontal tissues, immersed briefly in 80% alcohol before the pulp was extracted, cut-down into small pieces and then cultured as explants

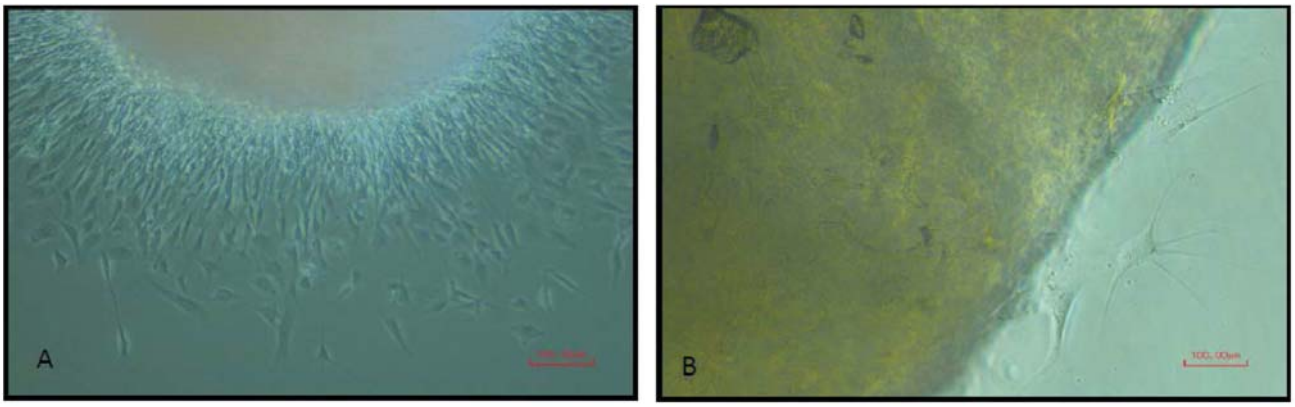

Figure 1. *Migration of NC-derived DPSCs. A) Migration of NC-derived DPSCs was observed only on fibronectin-coated wells starting, as early as 3 days after culturing, magnification 10×. B) Few cells were observed on a non-coated well, magnification 10×.*

on previously prepared 6-well plates coated with 2.5 μg/cm^2 human fibronectin (BD Biosciences, Bedford, MA, USA) in neurobasal medium supplemented with B27 supplement without vitamin A (Both are from Invitrogen, Karlsruhe, Germany), 20 ng/ml basic fibroblast growth factor (bFGF; Peprotech, Rocky Hill, NJ, USA), 20 ng/ml epidermal growth factor (EGF; R&D System, Minneapolis, MN, USA), 2.5 μM insulin (Sigma, Munich, Germany), 2 mM l-glutamine (Invitrogen) and 10 nM recombinant human neuregulin-β1 (kindly provided by Dr. S. Carroll, Division of Neuropathology, Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA) (4, 11-15). A non-coated well was kept as a control. Medium was changed 2-3 times a week. For appropriate passaging, the cells which reached confluence in any well of the 6-well plate were trypsinized then the cell number was halved and each half was cultured in a new well.

Fluorescent immunocytochemistry. Cells were fixed in 4% paraformaldehyde (PFA) for 10 min (minutes) and methanol for 5 min, before blocking them for 30 min in 10% goat serum (Dako, Hamburg, Germany). In order to permeate SOX10 nuclear protein, 0.3% triton X-100 (Sigma) was added to the blocking step. Cells were incubated with the primary antibodies overnight at 4˚C in the following dilutions: monoclonal anti-SOX10 1:2,000 (Sigma), monoclonal anti-nestin 1:250 (Millipore, Temecula, CA, USA), monoclonal anti-CD271 1:200 (BD Biosciences, Bedford, MA, USA) and polyclonal anti-S100β 1:500 (Dako). Bound antibody was then visualized using the Alexa Flour 1:1000 (Invitrogen).

Sphere formation. Sphere forming ability was tested by culturing the cells in ultra-low attachment plates (Corning, Corning, NY, USA) at a low density, clonally or by leaving the explant in the plate. Neurobasal medium was added, which was supplemented with B27 supplement without vitamin A, N2 supplement 1% (Invitrogen), 20 ng/ml EGF, 40 ng/ml bFGF, 32 IE/ml heparin (Ratiopharm, Ulm, Germany) and 2 mM l-glutamin (15, 16).

Osteogenic differentiation induction. Cells were incubated until confluence was achieved. After that, medium was changed to osteogenic differentiation induction medium: alpha-minimum essential medium (αMEM; Sigma) supplemented with 15% fetal calf serum (FCS; Biochrom, Berlin, Germany), 2 mM l-glutamine, 0.1 mM ascorbic acid (Sigma), 0.01 μM dexamethasone (Sigma) and 1.8 mM potassium phosphate monobasic (Sigma) (9, 17). Medium was changed twice-a-week for three weeks before performing osteoblast detection assays utilizing alizarin red and alkaline phosphatase. For alizarin red staining, cells were washed with PBS not containing Ca^{2+}/Mg^{2+}, subsequently fixed with 4% PFA for 30-45 min and then incubated with alizarin red S staining solution (Applichem, Darmstadt, Germany) at room temperature in a dark environment for 45 min. For alkaline phosphatase staining, cells were fixed with 4% PFA for 15 min, washed with previously mentioned PBS containing 0.05% tween 20 (Sigma) and then incubated in 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate solution (Sigma) at room temperature in a dark environment for 5-10 min.

Glial differentiation induction. Cells were cultured on laminated wells for 24 h in αMEM containing 1 mM mercaptoethanol (Sigma) and without serum, for the next 3 days the medium was changed to 20% FCS αMEM containing 35 ng/ml retinoic acid (Sigma), then cells were incubated for the following 3 weeks in 20% FCS αMEM supplemented with 5 μM forscolin (Sigma), 10 ng/ml bFGF, 5 ng/ml platelet-derived growth factor (PDGF; Peprotech, London, UK) and 200 ng/ml recombinant human neuregulin-β1 (18, 19). Finally, the detection of the Schwann cell marker S100β was performed.

Melanocytic differentiation induction. Cells were cultured at 500-1,000 cell/well density in melanocyte supporting medium (Invitrogen), which contained 0.2% bovine pituitary extract, 0.5% fetal bovine serum, 5 μg/ml bovine insulin, 5 μg/ml bovine transferrin, 3 ng/ml bFGF, 0.18 μg/ml hydrocortisone, 3 μg/ml heparin, 10 ng/ml phorbol 12-myristate 13-acetate and 200 μM calcium chloride (20, 21). Then, tyrosinase activity detection by L-DOPA (the precursor to dopamine) staining was performed by incubating the cells in 10 mM L-DOPA (Sigma) at a pH of 6.8 and 37˚C in a dark environment for 18-24 h (19, 21).

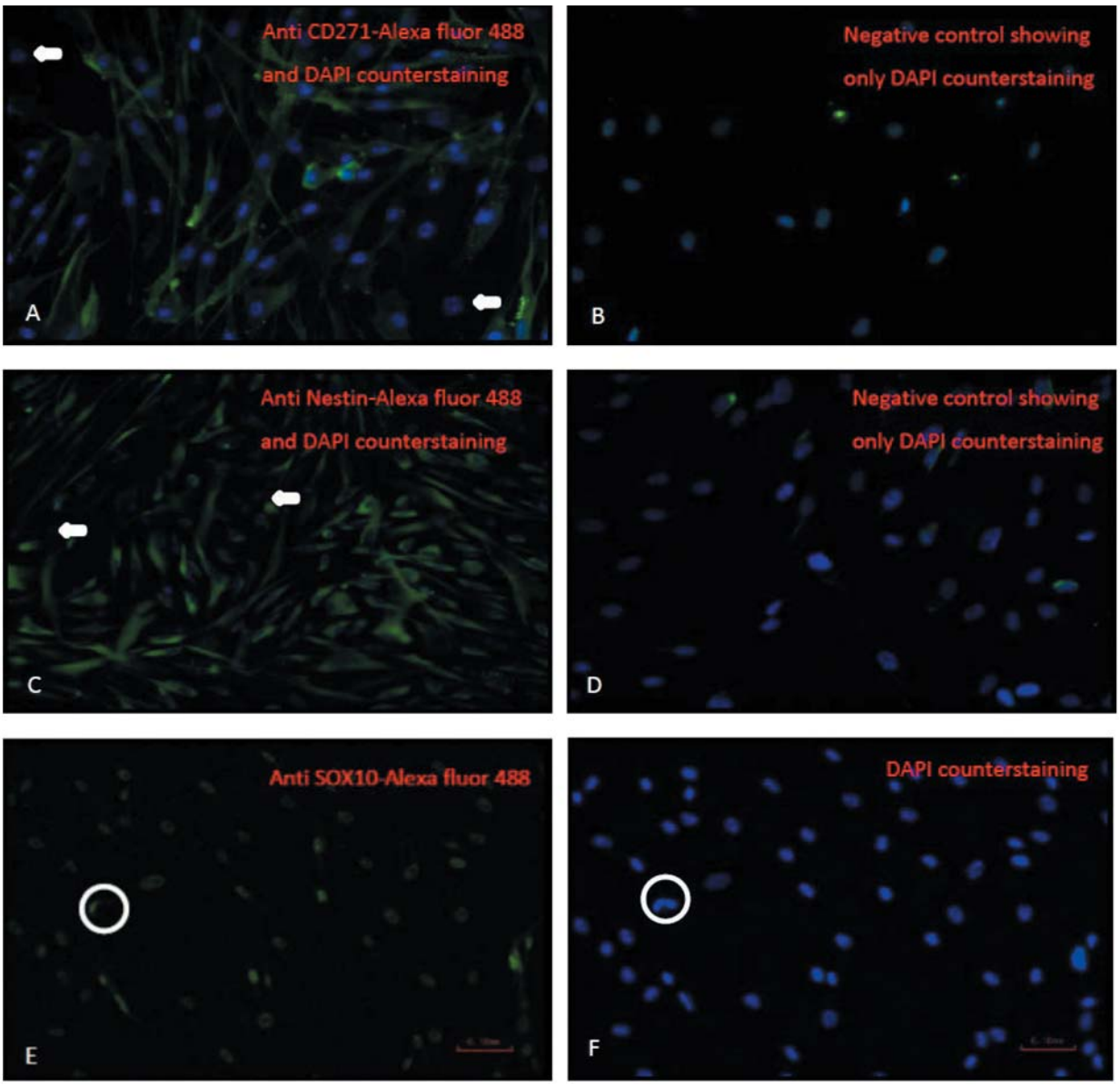

Figure 2. *Expression of neural crest stem cell markers in NC-derived DPSC culture. Cells were cultured in an 8-well chamber for one week before immunofluorescent staining. A) CD271-positive cells show green fluorescence, some CD271-negative cells are marked by arrows, magnification 20×. B) Negative control for CD271 shows only DAPI counterstaining, magnification 20×. C) Nestin-positive cells showing green fluorescence, some nestin-negative cells are marked by arrows, magnification 10×. D) Negative control for nestin shows only DAPI counterstaining, magnification 10×. E) Positive SOX10 cells show nuclei of cells expressing the nuclear protein visible in fluorescent green, magnification 20×. F) DAPI staining of the last figure E, some SOX10-negative cells are marked with a circle in F; as can be observed, they are not visible in E, magnification 20×.*

Results

Tissue culture. NC-derived DPSCs began to migrate exclusively on fibronectin-coated surfaces between day 3-10 (Figure 1). Overall, cells migrated faster from smaller explants. A concentration of fibronectin lower or higher than 2.5 μg/cm^2 prevented cell migration. When the cell cultures reached confluence after 4-8 weeks, they were passaged by splitting the cell number (1:2). At a lower density, cells gradually ceased to proliferate and eventually died. The passaged cells had a duplication rate of 4-5 days and were cryopreserved after 6 passages.

Fluorescent immunocytochemistry. The majority of the stem cells expressed the NCSCs markers CD271, SOX10 and nestin (Figure 2).

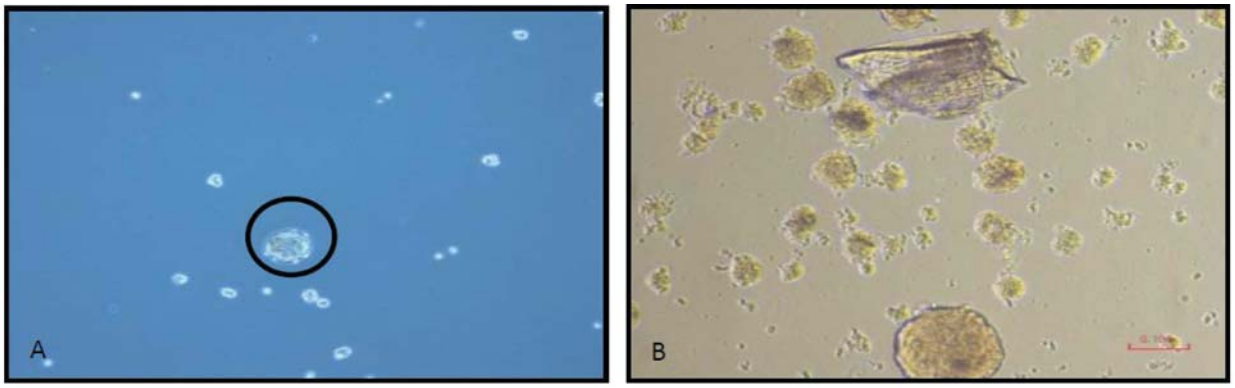

Figure 3. *Sphere formation ability. Sphere formation ability was tested each time the cells were passaged; cell cultures from four patients were tested and each was tested 5-6 times. A) Sphere measuring 1 mm in diameter (inside the circle) was observed within one week after cells were seeded in low density in ultra-low attachment plates with the medium described in (materials and methods - sphere formation), magnification 10×. B) Multiple spheres were observed when the explant was left in the low attachment plate, magnification 10×. All resulting spheres yielded secondary spheres only when cells were passaged in low density; they could not be cloned.*

Figure 4. *Alkaline phosphatase and alizarin red staining of stem cells induced into osteoblasts. Staining of stem cell derived osteoblasts in an 8-well chamber; positive alizarin red results in yellow-orange color (left) and positive alkaline phosphatase results in dark violet color (right). Each assay was performed three times on three different patient's cell cultures as shown. The two chambers at the bottom are non-induced cells used as controls.*

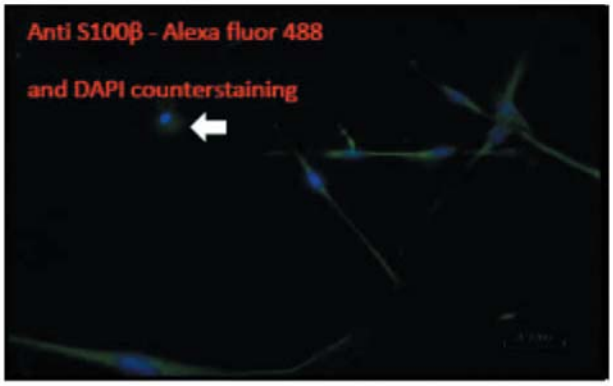

Figure 5. *Immunofluorescence staining for Schwann cell marker S100β. After glial induction of cells for four weeks, immunofluorescence staining for Schwann cell marker S100β was performed. Induced cells show positive S100β green fluorescence and a bipolar shape. A S100β negative cell is marked by an arrow, magnification 20×.*

Sphere forming ability. Cells were cultured in ultra-low attachment plates at a low density, clonally or by leaving the explant in the plate. Within one week, spheres could only be observed in the wells where cells were cultured in low density or when the explants were left in the culture (Figure 3). Clonally-passaged spheres never yielded secondary spheres. These were only observed after passaging the spheres in low densities (Figure 3).

Differentiation potential. After culturing the stem cells in osteogenic differentiation induction medium for three weeks, high activity of alkaline phosphatase and also calcium depositions were detectable with alkaline phosphatase and

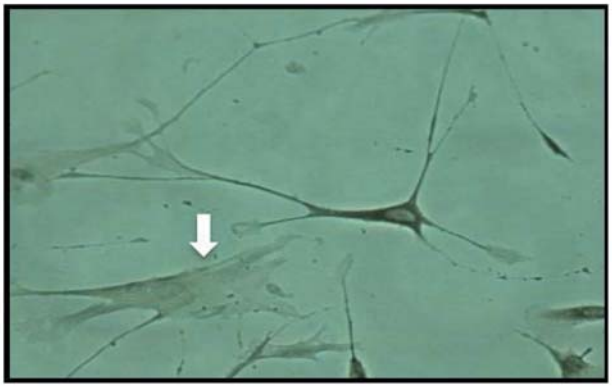

Figure 6. *Melanocytic differentiation induction. Differentiated melanocytes show positive brown L-DOPA staining and a dendritic morphology. An L-DOPA negative cell is marked by an arrow, magnification 20×.*

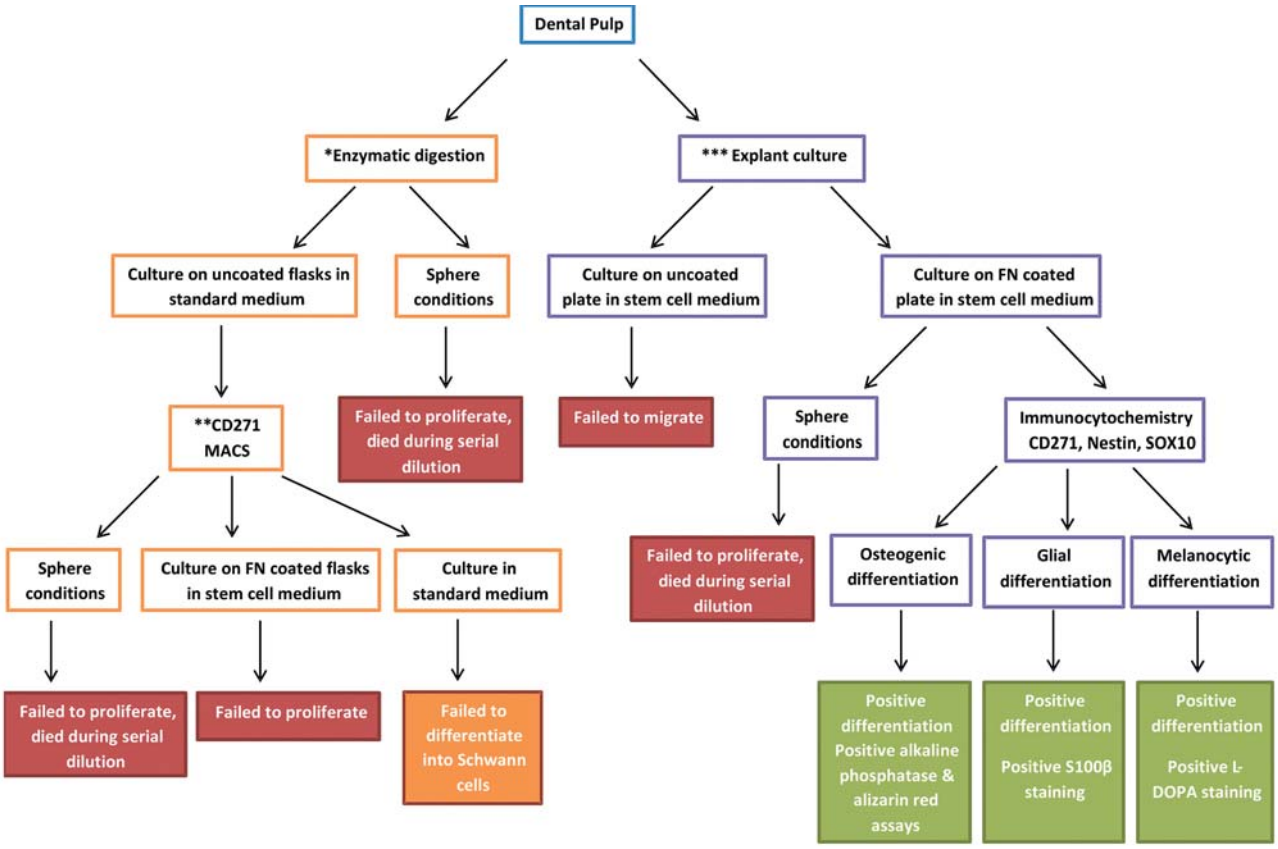

Figure 7. *Flow chart showing the different digestion/culturing methods employed in our laboratory to enrich NC-derived DPSCs. The Explant method is the only method discussed in this article. Two culturing methods were tested in order to enrich NC-derived DPSCs; the enzymatic digestion method and the explant method. *The enzymatic digestion method was described by Gronthos (2). Briefly, the pulp tissue was digested in a solution of 3 mg/ml collagenase type I (Sigma) and 4 mg/ml dispase (Gibco) for 1 h at 37˚C and then the cells were cultured and expanded in 20% FCS α MEM.**CD271 positive cells were isolated by magnetic cell sorting and cultured in 20% FCS α MEM. After that, they were induced into Schwann cells by the protocol described in (materials and methods – glial differentiation induction).These cells could not be differentiated into Schwann cells as the result in the solid orange box shows. ***The explant method is discussed in this article.*

alizarin red staining (Figure 4). After four weeks of glial differentiation induction of the cells, their morphology changed into a bipolar spindle-like phenotype and strong S100β expression was detectable (Figure 5). Finally, the cells induced to differentiate into the melanocytic phenotype by culturing in melanocyte-supporting medium, showed a morphology change into dendritic cells and expressed a strong L-DOPA staining (Figure 6). All differentiation induction experiments were performed in tetrads and on three patient's samples.

Discussion

Searching for NCSCs in the human dental pulp started when mesenchymal DPSCs demonstrated neurogenic/adipogenic differentiation potential *in vitro* (24), label-retaining ability and neurosphere forming ability (28, 29), as these are considered as characteristics of neural crest ontogeny.

When these former protocols (20-24), which always used serum in the composition of the DPSCs culture medium, were experimented, the isolated stem cell population was not able to differentiate into the glial lineage (Figure 7). Since different isolation methods of DPSCs are proven to be able to retrieve different stem cell populations (25), the enrichment of NC-derived DPSCs in this study was based on these methods of NCSCs isolation from other embryological and adult tissues. In our analysis, conventional cell culture methods of DPSCs did not succeed in preserving the stemness of NCSCs (26), possibly due to reasons like serum existence in the medium (27). On the other hand, dental pulp is a small-sized tissue and expanding the low cell-numbered primary cell-culture without serum is a great challenge. The established culture method in the study is able to overcome these hindrances by supplying factors supporting NCSCs survival and proliferation, as for example. extracellular

matrix (ECM), as fibronectin (28, 29), neuregulin-β1 (28, 30) and bFGF, EGF (27, 28), while eliminating factors that cause spontaneous differentiation or death of NCSCs, as for example serum and enzymatic digestion (26, 27). Neural crest cells are ECM-dependent; they need ECM to survive and migrate (28, 29). Fibronectin is described to be of high importance for them (29). Cells were allowed to migrate out of the explants, them being free of enzymatic digestion stress, which disturbs the pulp ECM needed for survival and migration of NCSCs. Medium formula was prepared in order to support NCSCs without using serum. Neurobasal medium and its supplement B27 were formulated to culture neural stem cells. EGF and FGF preserve stem cell (27, 28), moreover; neuregulin-β1 has been reported to support NCSCs in presence of ECM (28, 30). Passaging of cells in low cell density or without coating caused these cells to proliferate in a decreasing manner and eventually die resembling NCSCs' crucial need of ECM to survive (29). The cellular doubling time was approximately 4-5 days, which is similar to previously characterized human NCSCs cell lines (4).

Three NCSCs markers were chosen to be tested based on former work with NCSCs characterization: CD271 (20, 23, 30-32), SOX10 (33, 34) and nestin (11, 13, 35). The majority of cells express these markers.

Despite the fact that sphere-forming ability is considered a feature of neural stem cells, many reports disagree and argue whether this ability is a proof for stemness (12, 14, 16, 20, 27, 36-43). Clonality of these spheres was not always conditioned (5, 14). Clonal culturing of cranial NCSCs on low-attachment surfaces as spheres has not been reported before; Some investigators report sphere-like cell clusters that do not self-renew if cloned, others report a sphere formation on attachable surfaces (11, 12, 16, 20). Even clonal culture of neural crest embryonic cells as spheres continued only for 4 passages (27). The results accomplished here are in good agreement with former reports as, in this study, sphere forming ability was diminished. Possible reasons could be that NCSCs are cellular ECM-dependent, ecto-mesenchymal transition of NCCs before they leave the NC, non-optimal sphere forming conditions or, simply, the inability of NC-derived DPSCs to generate spheres.

To test the differentiation potential of the enriched cells, two lineages were chosen: ectodermal (Schwann cells, melanocytes) and mesodermal, (osteoblasts) as these are the lineages NCCs differentiate into naturally (29). NC-derived DPSCs differentiated successfully into osteoblasts after 3 weeks of induction utilizing previously reported osteogenic induction protocols (2, 44, 45). Alizarin red and alkaline phosphatase assays, both osteoblast detection assays (46, 47), delivered strong positive results compared to the negative results seen in non-induced NCSCs. NC-derived DPSCs successfully differentiated into melanocytes, which exclusively originate from NCSCs (48), after induction by medium containing factors that reported to support differentiation and survival of melanocytes (20, 21, 49-51). A change in morphology from fibroblastic shape into dendritic shape and a positive L-DOPA staining, both characteristics of normal melanocytes, (48, 52) confirm the achieved differentiation. Glial lineage differentiation in terms of Schwann cells from human NC-derived DPSCs is a very complex procedure, as a number of critical points determine whether the differentiation occurs or not. For instance, stem cells have to be cultured longer in FGF and EGF before induction, which has been observed previously in NCSCs (27). A differentiation induction period of at least four weeks should be considered, utilizing the components reported to induce NCCs differentiation into Schwann cells (41, 53-58). The differentiated Schwann cells were thin, elongated cells, with 2-3 main processes and an oval, blunt-ended nucleus oriented longitudinally in relation to the main axis of the cell, resembling a normal Schwann cell (59). These cells survived and multiplied for three passages showing an increased S100β expression over time (results not shown).

Conclusion

In the present study, a population of DPSCs, which resemble NCSCs, was enriched by culturing DPSCs under conditions favoring the survival of NCSCs. The enriched NC-derived DPSCs showed a great resemblance to the former isolated NCSCs. They are migratory cells, which depend on ECM to survive and to preserve stemness. They seem to be highly sensitive to serum and growth factor existence in the medium, as both contribute to their rapid differentiation. NC-derived DPSCs expressed nestin, CD271 and SOX10, three of the most known NCSCs markers. Most importantly, these cells could be induced to differentiate into NC derivatives, namely osteoblasts, melanocytes and Schwann cells.

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