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Morphological, immunohistochemical and ultrastructural characteristics of human dental pulp stem cells after neuronal and glial cell differentiation

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List of Abbreviations

3G5	pericyte associated antigen
ALP	alkaline phosphatase
aMEM	alpha Minimal Essential Medium
BDNF	brain derived neurotrophic factor
BME	beta-mercaptoethanol
BM-MSC	bone marrow derived Mesenchymal Stem Cell
cAMP	cyclic adenosine monophosphate
CM	conditioned medium
CNPase	2', 3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
CNTF	ciliary neurotrophic factor
DAPI	4',6-diamidino-2-phenylindole
DFPC	dental follicle precursor cells
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DSPP	dentin sialophosphoprotein
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
ELISA's	enzyme-linked immunosorbent assays
ESC	embryonic stem cells
FABP	fatty acid binding protein
FACS	fluorescent activated cell sorting
FCS	fetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GABA	γ-amino butyric acid
GalC	galactocerebroside
GDNF	glial cell-derived growth factor
GFAP	glial fibrillary acidic protein
GusB	glucuronidase beta
HA/TCP	hydroxyapatite/tricalcium phosphate
hDPSC	human dental pulp stem cells
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IAN	inferior alveolar nerve
MAP2	Microtubule-associated protein 2

MBP	myelin basic protein
MEPE	matrix extracellular phosphoglycoprotein
mRNA	messenger ribonucleotide acid
MSC	Mesenchymal stem cells
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DRG	dorsal root ganglia
NCAM	Neural Cell Adhesion Molecule
NF-H	neurofilament heavy chain
NGF	nerve growth factor
NGFR	nerve growth factor receptor
NRG	Heregulin-β-1
NSE	neuron specific enolase
NT	neurotrophin
PBS	Phosphate Buffered Saline
PDGF R	platelet derived growth factor receptor
PDGFaa	platelet derived growth factor AA
PDLSC	periodontal ligament stem cells
PE	Phycoerythrin
PFA	paraformaldehyde
PNS	peripheral nervous system
RA	*all trans*-retinoic acid
RER	rough endoplasmatic reticulum
RT-PCR	reverse transcriptase polymerase chain reaction
SEM	standard error of the mean
SCAPs	stem cells from apical papilla
SCF	stem cell factor
SCF R	stem cell factor receptor
SC-hDPSC	Schwann-like cell differentiated human dental pulp stem cell
SHED	stem cells from human exfoliated deciduous teeth
TEA	tetraethyl ammonium
TEM	transmission electron microscopical
TGF	transforming growth factor
TTX	tetrodotoxin
VEGF	vascular endothelial growth factor

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General Introduction and Aims

1.1 Stem cells

Stem cells are defined as cells possessing the capacity of self-renewal and differentiation. This means that cells are able to give rise to two daughter cells with one being identical to the mother (self-renewal) and the other one being differentiated into a more specialized cell. Four types of stem cells have been established based on the ability and potency to differentiate into different cell types: (1) A fertilized egg can form an entire embryo including the extra-embryonic tissues like the supporting trophoblast which is required for the survival of the developing embryo. These cells are termed **totipotent** stem cells. (2) **Pluripotent** stem cells give rise to germ cells and cells of mesoderm, endoderm and ectoderm. These cells cannot form extra-embryonic tissues and therefore cannot develop into a complete organism. (3) Postnatal or adult stem cells are termed **multipotent** stem cells and are capable of multilineage differentiation in cells of only one germ layer. (4) Cells which can only differentiate into one defined cell type are called **unipotent** or progenitor stem cells. These cells possess a limited self-renewal capacity, defining them still as stem cells. Based on their origin, stem cells can be subdivided into embryonic and postnatal or adult stem cells [1].

1.1.1. Embryonic stem cells

At day 5 of embryonic development, the morula will develop into a blastocyst consisting of an inner cell mass and an outer epithelial layer called the trophoblast. From the inner cell mass of the blastocyst or from primordial germ cells of an early embryo, embryonic stem cells (ESC) can be isolated. ESC possess the capacity for unlimited self-renewal and are able to differentiate into germ cells and cells of all three germ layers: mesoderm, endoderm and ectoderm. Therefore, they are defined as pluripotent stem cells (Figure 1.1). ESC are widely investigated to elucidate the mechanisms and different molecular signaling pathways taking place during human development. Furthermore, they can be used to explore the origin of more differentiated / committed cells and the mechanism behind differentiation. Considering the fact that ESC can produce

almost all cells of the human body, they are ideal candidates for stem cell based or cell replacement studies. However, intensive studies have to be conducted for a better understanding of their properties and characteristics before they can be used in clinical applications. In addition, the major drawbacks of using ESC are the teratocarcinoma formation when applying ESC *in vivo* and high ethical considerations because human embryos early in their development, have to be manipulated and destroyed [2].

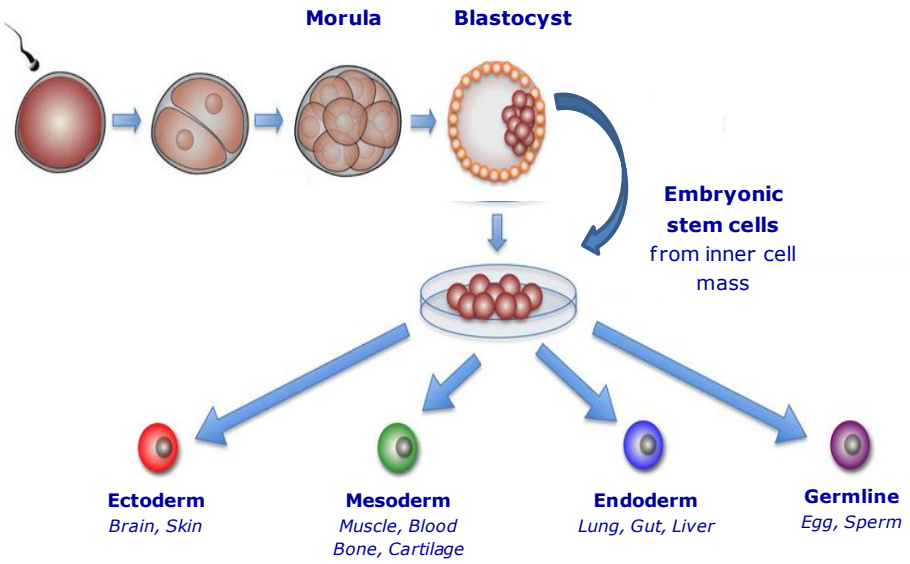


Figure 1.1: Blastocyst formation and embryonic stem cells residing within the inner cell mass. Adapted from Odessa Yabut *et al.* 2011 [3]

1.1.2. Adult stem cells

During the adult life, stem cells are still present within various tissues and organs. These stem cells are able to self-renew and differentiate into cells of one germ layer. In addition, they are able to repopulate the tissue after damage and contribute to a natural turn-over, meaning that they can provide a progeny even in the absence of injury [4]. Based on their differentiation capacity, adult stem cells are mostly referred to as postnatal or multipotent stem cells. Several adult stem cell types can be distinguished considering their presence within the body (hematopoietic stem cells, neural stem cells, mesenchymal stem cells,...).

Hematopoietic stem cells are present in bone marrow and can differentiate into all blood cells of the myeloid and lymphoid cell lineages. Neural stem cells can be isolated from the subventricular zone and from the subgranular zone of the hippocampal dentate gyrus and are able to differentiate into the three cell types of the brain (neurons, astrocytes and oligodendrocytes). Mesenchymal stem cells (MSC) can be found in a variety of tissues and organs like bone marrow, adipose tissue, umbilical cord blood and stroma, placenta, amniotic membrane, synovium, lung, dental pulp tissue... They are capable of differentiating into cells of the mesodermal lineage like adipocytes, chondrocytes and osteoblasts.

Mesenchymal stem cells

Friedenstein *et al.* were the first to identify a cell population in the stromal fraction of rat bone marrow, which adhered to a plastic surface *in vitro* and was able to undergo osteogenic differentiation. This cell population was initially called fibroblast colony-forming cells but is now referred to as marrow stromal cells or MSC [5]. Bone marrow derived MSC (BM-MSC) possess the self-renewal capacity to form colonies *in vitro* and are capable of differentiating into multiple mesenchymal cell lineage *in vitro* and *in vivo* [6, 7] (Figure 1.2). However, they are limited to a growth potential of 30 to approximately 50 population doublings following *ex vivo* expansion [8, 9]. BM-MSC consist of a heterogeneous cell population with cells displaying a fibroblast-like or spindle-shaped morphology. After expansion, a more homogeneous cell population with a spindle-shaped morphology is derived. The precise identity of MSC remains a challenge due to the lack of a single definitive marker. Therefore, a panel of different cell surface antigens is used to identify MSC in a cell population. The cells should express associated markers CD29 (integrin β1), CD44 (hyaluronan receptor), CD73, CD105 (endoglin), the early bone marrow progenitor marker CD90 (Thy-1) and the extracellular matrix proteins vimentin, laminin and fibronectin. MSC should lack marker expression of CD11b and CD14 (monocytes and macrophages), CD34 (primitive hematopoietic stem cells and endothelial cells), CD45 (leukocytes), CD79α or CD19 (B-cells), and HLA-DR [10, 11]. Recently it has been shown that MSC can undergo transdifferentiation towards cells of the neural lineage like astrocytes and neurons [12, 13]. Furthermore, after

transplantation of BM-MSC into regions of central nervous injury, an improved functional recovery was observed in injured rodent brain or spinal cord [14, 15]. Mesenchymal stem cells yield great potential for stem cell based regenerative therapies, based on their multilineage (trans)differentiation potential and their high proliferative capacity. The identification of the regenerative potential of mesenchymal stem cells has encouraged intense research during the last years. Recently, various clinical studies are conducted using mesenchymal stem cells as transplants for treatment or to improve functional outcomes. For instance, the cardiac function of ischaemic hearts was improved after intracardial transplantation of bone marrow MSC [16-18]. Furthermore, the infusion of allogenic whole bone marrow or bone marrow MSC in children, suffering from type III osteogenesis imperfecta, showed a promising phenotypic improvement with reduced fracture frequency and improved growth velocity [16-18]. Taken together, although clinical trials show promising results, there is still a long way to go before MSC can be used in the clinic as standard treatment.

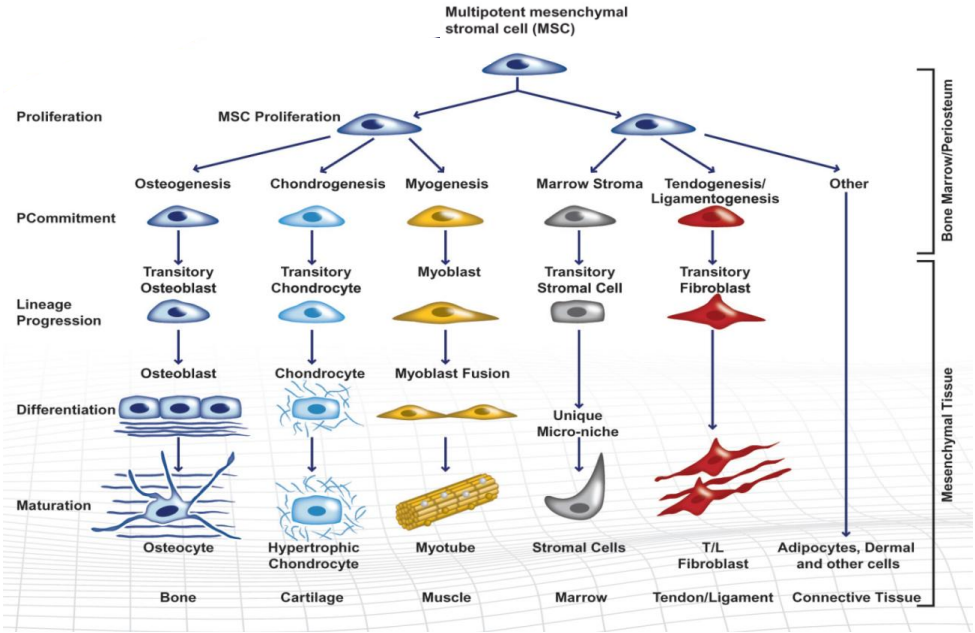


Figure 1.2: Differentiation potential of mesenchymal stem cells. Adapted from Caplan *et al.* 2009 [19]

1.2 Tooth development

Tooth development is regulated by sequential and reciprocal interactions between oral epithelium and the underlying ecto-mesenchymal cells, both originating from migrating neural crest cells. These interactions result in the formation of an outer layer of enamel formed by ameloblasts, derived from the oral epithelium, and an inner layer of mineralized dentin synthesized by odontoblasts, which are derived from the dental papilla. The central chamber of the tooth contains a soft mucoid connective tissue called the dental pulp, also derived from the dental papilla, and is infiltrated by a network of blood vessels and nerve bundles [20, 21]. By close interactions and signals exchanged between epithelial and mesenchymal cells, a tooth will develop through a series of different stages. This section will give a brief overview of the different stages during tooth development (Figure 1.3).

1.2.1 Lamina stage

At approximately 5-6 weeks of human embryonic development, the first morphological sign of tooth development can be observed and is defined as the dental lamina. During the lamina stage, cells in the dental epithelium and underlying mesenchyme are dividing at different rates, the latter more rapidly, resulting in thickening of the oral epithelium, called dental (ectodermal) placodes. This takes place within the primary epithelial bands were tooth organs will develop. Dental placodes are responsible for the formation of each tooth family by dictating the fate of the underlying ecto-mesenchyme through sequential and reciprocal signaling [20, 21, 23].

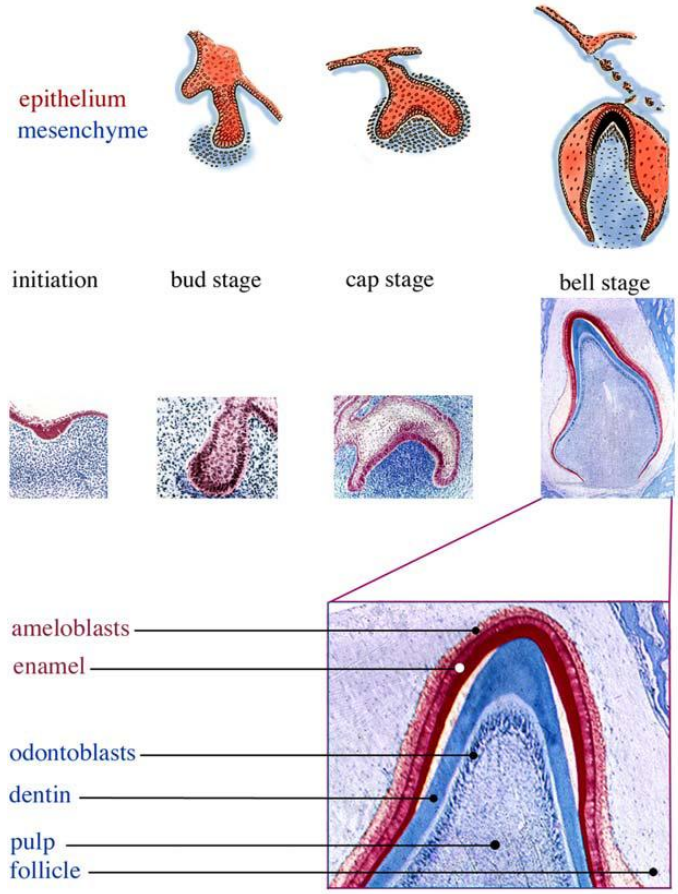


Figure 1.3: Different stages during tooth development. Adapted from Bluteau *et al.* 2008 [22]

1.2.2 Bud stage

During the bud stage the dental epithelium segregates into two histologically distinct cell lineages, (1) the peripheral basal cells contacting the basement membrane, and (2) centrally located loosely arranged cells, called the stellate reticulum, which are derived from the suprabasal cell layers of the surface ectoderm. These two tissue layers will form the epithelial components of the stem cell niche in the continuously growing teeth. The dental mesenchyme condenses around the bud and segregates into two cell lineages, the dental

papilla which later becomes surrounded by dental epithelium and gives rise to the tooth pulp and dentin producing odontoblasts, and the peripheral dental follicle giving rise to the cementoblasts and periodontal tissues [24]. The budding of the dental epithelium is accompanied by the formation of a condensate of dental mesenchymal cells around the bud, followed by the induction of a signaling center at the tip of the epithelial bud, the enamel knot. This signaling center is required for the transition of the epithelial bud to a cap-like structure, marking the onset of tooth crown development. This transition is very critical for tooth morphogenesis [25].

1.2.3 Cap stage

At this stage, the dental epithelium continues to proliferate into the ecto-mesenchyme extending around the periphery forming a cap-like structure. The epithelial outgrowth is referred to as the enamel organ, forming eventually the enamel of the tooth. Within the enamel organ, epithelial cells organize themselves into three distinct regions: the outer epithelium, the inner epithelium and the central stellate reticulum [26]. The ball of condensed ecto-mesenchymal cells, the dental papilla, will form the dentin and pulp tissue. The condensed ecto-mesenchyme lining the dental papilla and encapsulating the enamel organ, called the dental follicle, will give rise to the supporting tissues of the tooth [20]. Recent observations suggest that the shape of a tooth may be regulated by signaling centers present in the enamel knots. It has been suggested that the enamel knot plays a role in the regulation of cusp patterns of teeth. An interesting feature is that cells within the enamel knot can undergo apoptosis providing an important mechanism in the regulation of the tooth shape. The enamel knot will eventually disappear during the cap stage [21, 27].

1.2.4 Bell stage

During the early bell stage, continuous growing of the tooth germ gives the enamel organ a bell-like shape. The tooth crown assumes its final shape and the cells making the hard tissues of the tooth (ameloblasts and odontoblasts)

acquire their final phenotype. Cells lining at the periphery of the enamel organ will assume a cubical shape and form the outer enamel epithelium. The inner epithelium is formed by cells bordering the dental papilla and is connected to the outer enamel epithelium by a cervical loop. At this region, cells continue to divide until the tooth crown reaches its full size and will give rise to the epithelial component of root formation, the so-called Hertwig's epithelial root sheath. At the centre of the enamel organ, some cells will organize and secrete glycosaminoglycans into the extracellular compartment between the epithelial cells. Glycosaminoglycans will pull water and growth factors inside the enamel organ. Cells will obtain a star-like shape and together they will form the stellate reticulum. Cells of the stratum intermedium, formed between the inner enamel epithelium and the stellate reticulum, play a functional role in the enamel formation together with the cells of the inner enamel epithelium. Lining against the inner enamel epithelium, a layer of odontoblasts will develop, making contact with the ameloblasts layer [20, 28]. The dental lamina that connects the tooth organ to the oral epithelium gradually disintegrates at the late bell stage. Cells of the inner enamel epithelium continue to divide at different rates to determine the shape of the crown. At this time, the dental papilla is referred to as dental pulp tissue. Furthermore, odontoblasts begin to produce pre-dentin that finally becomes mineralized into dentin and stimulate the production of enamel by the ameloblasts. Teeth continue to develop postnatally. The outer covering of enamel gradually becomes harder and root formation which is essential for tooth function, only starts to occur as part of tooth eruption [29]. When the root of the teeth develop, the dental follicle gives rise to the cementoblasts depositing dental cementum as well as to the fibrous periodontal membrane connecting the roots of the teeth to the alveolar bone [30]. After crown morphogenesis, the roots of the teeth develop and subsequently the teeth erupt into the oral cavity.

1.3 Stem cells in dental tissues

Due to certain shortcomings of obtaining BM-MSC including pain, morbidity and low cell number upon harvest, alternative sources for MSC have been explored. During the last several years, various MSC-like cell types have been identified in dental tissues (see table 1.1). The first type of dental stem cells was isolated from pulp tissue and were termed dental pulp stem cells (DPSC) [31]. Subsequently, more dental stem cell populations were isolated and characterized: stem cells from human exfoliated deciduous teeth (SHED) [32], periodontal ligament stem cells (PDLSC) [33] and stem cells from apical papilla (SCAPs) [34, 35]. Recent studies have identified a fifth dental tissue derived stem cell population referred to as dental follicle precursor cells (DFPC) [36]. Dental tissues are specialized tissues that do not undergo continuous remodeling as shown in bony tissue: therefore, dental tissue derived stem/progenitor cells may be more committed or restricted in their differentiation potency in comparison to BM-MSC. Additionally, dental mesenchyme is termed ecto-mesenchyme due to its earlier interaction with the neural crest. From this perspective, ecto-mesenchyme-derived dental stem cells may possess different characteristics [37].

	DPSC	SCAP	SHED	PDLSC	DFPC
Location	Permanent tooth pulp	Apical papilla of developing root	Exfoliated deciduous tooth pulp	Periodontal ligament	Dental follicle of developing tooth
Differentiation Potential	Odontoblast, osteoblast, chondrocyte, myocytes, neurocyte, adipocyte, melanocyte, iPS	Odontoblast, osteoblast, neurocyte, adipocyte, iPS	Odontoblast, osteoblast, chondrocyte, myocyte, neurocyte, adipocyte, iPS	Odontoblast, osteoblast, chondrocyte, cementoblast, neurocyte	Odontoblast, osteoblast, neurocyte
Tissue repair	Bone regeneration, neuroregeneration, myogenic regeneration dentin-pulp regeneration	Bone regeneration, neuroregeneration, dentin-pulp regeneration, root formation	Bone regeneration, neuroregeneration, tubular dentin	Bone regeneration, root formation, periodontal regeneration	Bone regeneration, periodontal regeneration

Table 1.1: Overview Dental stem cells. Adapted from Estrela *et al.* 2011 [38]

1.3.1 Dental pulp stem cells

The possibility that dental pulp tissue might contain mesenchymal stem cells was first suggested by the observation that severe tooth damage penetrates both enamel and dentin and into the pulp which stimulated a limited natural repair process. Consequently, new odontoblasts were formed producing new dentin to repair the lesion [39-41]. DPSC were for the first time isolated and characterized from dental pulp tissue by Gronthos *et al.* in 2000. DPSC are described as a high proliferative cell population possessing the self-renewal ability and multilineage differentiation potential [31, 42]. Furthermore, they can be induced to differentiate into odontoblast-like cells, characterized by polarized cell bodies and accumulation of mineralized nodules [43]. DPSC display mesenchymal characteristics such as a fibroblast-like morphology, adherence to a plastic surface and the ability to form colonies when cultured *in vitro*. A similar expression pattern of mesenchymal and hematopoietic surface markers is found in both DPSC and BM-MSC: a positive expression of the mesenchymal markers CD29, CD44, CD59, CD90, CD106 and CD146 and a negative expression for hematopoietic makers CD34, CD45 and CD11b [37, 44, 45]. Alge *et al.* also compared DPSC and BM-MSC regarding their proliferation rate, colony formation, clonogenic potential and mineralization potential. They reported that DPSC have a higher proliferation rate, a greater clonogenic potential, a higher number of stem/progenitor cells in the population and may have increased mineralization potential compared to BM-MSC [44].

DPSC are ectodermal derived stem cells, originating from migrating neural crest cells and possess mesenchymal stem cell properties. They are multipotential cells being able to differentiate *in vitro* into cells of the odontogenic, adipogenic, osteogenic, chondrogenic, myogenic, melanogenic, and neurogenic lineages under the appropriate culture conditions [31, 46-52]. *In vivo* studies reported the formation of an ectopic pulp dentin-like tissue complex when transplanted with a hydroxyapatite/tricalcium phosphate (HA/TCP) carrier into immunocompromised mice. A vascularized pulp-like tissue was formed surrounded by a layer of odontoblast-like cells expressing dentin sialophosphoprotein (DSPP), which produced dentin containing dentinal tubules

similar to those in natural dentin. DPSC could also produce bone when implanted subcutaneously in immunocompromised mice with HA/TCP as carrier [31, 53]. Huang *et al.* showed that also a dentin-pulp-like complex with a good vascularity could be regenerated in an emptied root canal space by DPSC [54]. In addition, DPSC can express neural markers, produce and secrete neurotrophic factors, induce axon guidance and differentiate into functionally active neurons, suggesting their potential as cellular therapy for neuronal disorders [47, 48, 55-59]. This topic will be further discussed in section 1.4 'Dental pulp stem cells in neurological disorders'.

Several studies have demonstrated that DPSC retain their stem cell properties following cryopreservation. DPSC cultures can be established from extracted human third molars with a high efficiency, even after the whole tooth is cryopreserved [60-62]. Given that extracted human teeth are routinely discarded as medical waste, cryopreservation of DPSC, dental pulp tissue or even of a whole tooth, yield great advantages for tissue engineering and regenerative medicine. Therefore, DPSC should be used for cell banking.

1.3.2 Stem cells from human exfoliated deciduous teeth

Miura *et al.* were the first to isolate and characterize a multipotent stem cell population within dental pulp tissue derived from exfoliated deciduous teeth (SHED) [32]. Compared to DPSC, SHED possess a higher proliferation rate, increased cell-population doublings, sphere like cluster formation, osteo-inductive capacity *in vivo* and failure to reconstitute a complete dentin pulp-like complex. SHED were found to express the cell surface molecules Stro-1 and CD146, two early mesenchymal markers present in BM-MSC and DPSC. *In vitro* studies showed that SHED were able to differentiate into cells of osteogenic, adipogenic, neurogenic, myogenic and chondrogenic lineages [32, 63-65]. Furthermore, when SHED were subcutaneously transplanted in immunocompromised mice, they formed ectopic dentin-like tissue, but they were unable to regenerate a dentin-pulp-like complex. SHED did not differentiate into osteoblasts but induced new bone formation by forming an osteo-inductive template to recruit murine host osteogenic cells. These data suggest that deciduous teeth may not only provide guidance for the eruption of

permanents teeth, as generally assumed, but may also be involved in inducing bone formation during the eruption of permanent teeth [32]. A recent study of Sakai *et al.* used the combination of *in vitro* and *in vivo* approaches to investigate the potential of SHED to differentiate into functional odontoblasts and angiogenic endothelium. They showed that SHED were capable of differentiating into functional blood vessels that connected with the host vasculature. Furthermore, the mineralized tissue generated by SHED in the pulp chamber of the tooth slice/scaffolds had morphological features of dentin, including the presence of dentinal tubules and predentin, which distinguish it from osteoid tissue [66].

Not only the osteo/odontogenic differentiation capacity of SHED is investigated during the last several years, but also the neurogenic induction of SHED has gained much attention. Previous investigations into the neural potential of SHED have shown that under non-neuronal inductive conditions, these cells expressed the neural progenitor marker, nestin, and the glial marker, glial fibrillary acidic protein (GFAP), at both the mRNA and protein levels. *In vitro*, neural differentiation studies of SHED demonstrated that this cell population was able to differentiate into neural cells based on cellular morphology and the expression of early neuronal markers. Furthermore, it was shown that SHED can survive for more than 10 days when transplanted into the adult rodent brain and expressed neural markers such as neurofilament M [32]. The study of Wang *et al.* indicated that SHED were able form neural-like spheres in a medium optimized for neural stem cells *in vitro* and were able to further differentiate into a cell population that contained specific dopaminergic neurons. Moreover, SHED and SHED-derived spheres survived and differentiated into dopaminergic neurons after transplantation in a rat animal model of Parkinson's disease partially improving the behavioral impairment [65]. Taken together, SHED probably represent a cell population of more immature multipotent stem cells than DPSC.

1.3.3 Periodontal ligament stem cells

The periodontal ligament is a specialized soft connective tissue derived from the dental follicle and also originates from neural crest cells. The periodontal ligament connects the cementum (a thin layer of mineralized tissue covering the roots of the teeth) to the alveolar bone and functions primarily to sustain and help constrain teeth within the jaw. The periodontal ligament does not only have an important role in supporting teeth, but also contributes to tooth nutrition, homeostasis, and repair of damaged tissues [20]. It has been shown that the periodontal ligament contains a stem cell population, periodontal ligament stem cells (PDLSC), which have the potential to form periodontal structures such as cementum and periodontal ligament and were able to differentiate *in vitro* into osteoblasts, cementoblasts, adipocytes and chondrocytes [33, 67, 68]. PDLSC also showed a high expression of scleraxis, a specific transcription factor associated with tendon cells. Moreover, PDLSC were able to differentiate into spontaneously contracting myotubes, neurofilament positive neuron-like cells, GFAP positive astrocyte-like cells and CNPase positive oligodendrocyte-like cells [69]. When transplanted into immunocompromised rodents, PDLSC showed the capacity to form collagen fibres, similar to Sharpey's fibres, connecting to the cementum-like tissue generating a cementum/periodontal ligament-like structure. PDLSC contributed in this way to periodontal tissue repair, suggesting the potential to regenerate periodontal ligament attachment [33]. Upon implantation into the tooth socket of the mandible of a minipig, PDLSC transplanted with HA/TCP as a carrier formed an artificial bioroot encircled with periodontal ligament tissue [34, 70]. PDLSC have thus the potential for forming periodontal structures, including the cementum and periodontal ligament.

1.3.4 Dental follicle precursor cells

Dental follicle is an ecto-mesenchymal tissue that surrounds the enamel organ and the dental papilla of the developing tooth germ prior to eruption. This tissue contains progenitor cells that form the periodontium: cementum, alveolar bone and periodontal ligament. Dental follicle cells form the periodontal ligament by differentiating into periodontal ligament fibroblasts that secrete collagen and

interact with fibres on the surfaces of adjacent bone and cementum. Precursor cells (DFPC) have been isolated from human dental follicles of impacted third molars using enzymatic digestion of dental follicle tissue and express the stem cell markers Notch1, Stro-1 and nestin. DFPC have the ability to differentiate into osteoblasts/cementoblasts, chondrocytes, adipocytes and neuron-like cells when grown under the appropriate culture conditions *in vitro* [36, 71, 72]. In addition, immortalized DFPC were transplanted into immunocompromised mice and were able to recreate a new periodontal ligament-like structure after 4 weeks [73]. Human DFPC were transplanted in immunocompromised mice in combination with hydroxyapatite powder to investigate the developmental potential *in vivo*. Transplants generated a structure compromised of fibrous or rigid tissue which showed an increased human specific osteocalcin and bone sialoprotein expression and a decreased collagen type I expression indicating the osteogenic differentiation of DFPC. However, there was no dentin, cementum or bone formation observed in the transplants *in vivo* [36]. More *in vivo* studies are necessary to elucidate the differentiation potential of DFPC for hard tissue regeneration.

1.3.5 Stem cells from apical papilla

In developing teeth, root formation starts as the epithelial cells from the cervical loop proliferate apically, and influence the differentiation of odontoblasts from undifferentiated mesenchymal cells and cementoblasts from follicle mesenchyme. It has been known that the dental papilla contributes to tooth formation and eventually converts to pulp tissue. As the root continues to develop, the location of the dental papilla becomes apical to the pulp tissue [20, 74]. The group of Sonoyama *et al.* discovered a potentially new type of stem cells in the apical papilla of human immature permanent teeth [34, 35]. Stem cells from apical papilla (SCAPs) appear to be a different population of stem cells from DPSC. SCAPs possess a higher proliferation rate and are more committed to osteo/odontogenic differentiation. Furthermore, SCAPs express mesenchymal stem cells markers similar to DPSC and the additional cell surface marker CD24 which is down-regulated in response to osteogenic stimulation. CD24 expression

is found in SCAPs cultures but not in DPSC or BM-MSC cultures. Despite that SCAPs express many osteo/dentinogenic markers, they express lower levels of dentin sialophosphoprotein (DSPP), matrix extracellular phosphoglycoprotein (MEPE), transforming growth factor β receptor, fibroblast growth factor receptor and CD146 compared to DPSC [75]. Several studies also report the adipogenic and neurogenic differentiation capacity of SCAPs [34, 35, 76]. In addition, under standard culture conditions, SCAPs already express some neural markers (nestin, β-III-tubulin and GFAP) which decreased after neurogenic induction. After stimulation, additional neural markers were expressed, including NeuN, neurofilament M, neuron specific enolase and glial markers CNPase [35, 76]. *In vivo* studies indicate that SCAPs are also capable of forming a dentin-pulp-like complex after transplantation into immunocompromised mice in an HA/TCP carrier [34, 35]. Furthermore, SCAPs, seeded onto a synthetic scaffold and placed into a human root segment, were transplanted into immunocompromised mice to investigate the regeneration capacity in an emptied root canal space. The regeneration of vascularized pulp-like tissue and the formation of dentin-like mineral structures depositing onto the existing dentinal wall in the root canal space was observed [54]. The study of Sonoyama *et al.* showed that SCAPs could also be used in combination with PDLSC to form a bioroot. Using a minipig model, autologous SCAPs and PDLSC were loaded onto HA/TCP and gelfoam scaffolds, respectively, and implanted into tooth sockets of the lower jaw. After 3 months implantation, the bioroot was encircled with periodontal ligament tissue and appeared to have a natural relationship with the surrounding bone. Although newly formed bioroots showed a lower compressive strength than that of natural swine root dentin, they seemed capable of supporting porcelain crown and resulted in normal functions [34].

The discovery of SCAPs may also explain a clinical phenomenon, apexogenesis, that can occur in infected immature permanent teeth with apical periodontitis or abscess. It is likely that stem cells residing in the apical papilla survived the infection due to their proximity to the periapical tissues. This tissue may be benefited by its collateral circulation, which enables it to survive during the process of pulp necrosis. After endodontic disinfection, SCAPs might, under the influence of the survived Hertwig's epithelial root sheats, give rise to primary odontoblasts to complete root formation [35, 77, 78]. SCAPs are derived from a

developing tissue that may represent a population of early stem/progenitor cells which can be a superior cell source for tissue regeneration.

1.4 Neural characteristics of dental pulp stem cells

Several studies confirm the neural characteristics of DPSC, such as the expression of neural markers, the production and secretion of neurotrophic factors and the possible (trans)differentiation into functionally active neurons [47, 48, 55-58]. Since DPSC are a neural crest-derived stem cell population, they might express certain neural markers in a undifferentiated state. It has been reported that naïve DPSC express nestin, neurofilament, vimentin, S100 and β-III-tubulin [64, 79]. However, the expression of neural markers in undifferentiated DPSC is dependent on the serum levels used during culture time [80]. When growth media with low serum levels (2%) were used, the expression level of nestin was increased compared to culture conditions with high serum levels (10%). In addition, no expression of β-III-tubulin and S100 could be detected in undifferentiated DPSC when using low serum conditions.

The neuronal differentiation potential of DPSC is widely investigated during the last several years [31, 47, 48, 55, 64, 79-81]. Several protocols are described to induce neuronal differentiation such as (1) transplantation of DPSC into injured rodent brain, (2) chemical and cytokine induction by using a mixture of neuronal inducing agents or (3) the generation of neurospheres [47, 55, 56, 81-83]. To determine the success of neuronal differentiation, several criteria must be fulfilled: (1) a neuronal morphology, it is a polarized cell with a single axon and multiple dendrites - (2) the expression of neural markers such as NeuN, β-III-tubulin, neurofilament and MAP2 - (3) the coexistence of voltage-gated sodium and potassium channels and being able to generate action potentials - (4) the presence of synapses, neurotransmitters and neurotransmitter receptors to communicate between neurons [84]. The neuronal differentiation of DPSC include the addition of media for epigenetic reprogramming and neural induction followed by a neuronal maturation step. Different media are described in the literature for the induction of neural differentiation leading to different results: (1) achieving only a reversible differentiation followed by either dedifferentiation

or massive cell death, (2) obtaining an incomplete neuronal differentiation, (3) acquiring fully functional neuron-like cells with voltage-gated sodium and voltage-gated potassium channels [48, 55, 85]. The study of Király *et al.* is the first to describe DPSC that are fully differentiated into functional neuron-like cells expressing neuronal markers NeuN, NSE and neurofilament, and displaying voltage-gated sodium and potassium channels which are necessary to generate action potentials [48]. Furthermore, they injected neuronally predifferentiated DPSC into the brain cortex of an animal model of traumatic brain injury and demonstrated that engrafted DPSC-derived neuronally predifferentiated cells integrated into the host brain and showed neuronal properties as expressing neuron-specific markers and exhibiting voltage dependent sodium and potassium channels [47]. However, these studies did not describe the presence of synapses or the generation of action potentials.

Another approach for inducing neuronal differentiation is the generation of neurospheres wherein DPSC are cultured in specialized culture conditions such as the appropriate non adherence culture plates and culture medium supplemented with growth factors to induce neuronal differentiation. Also this method of neuronal differentiation led to the expression of neural markers nestin, β-III-tubulin, S100, MAP2 and CD81, a transmembrane protein present in neural progenitor cells [83, 86]. The electrophysiological properties and *in vivo* application of cells being differentiated via this approach remains to be elucidated.

DPSC are not only able to differentiate into functionally active neurons, but several studies have demonstrated that they are also capable of secreting many neurotrophic growth factors such as brain derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), fibroblast growth factor (FGF), glial cell-derived growth factor (GDNF), nerve growth factor (NGF) and vascular endothelial growth factor (VEGF), which were found to promote neuronal rescue and survival, neurite outgrowth and guidance *in vitro* and *in vivo* [55-58] and to stimulate neurogenesis after transplantation in the hippocampus [81].

The neuroprotective effect of DPSC in the central nervous system and central nervous disorders such as spinal cord injury, Alzheimer's and Parkinson's disease have been described in several studies. The production of BDNF, GDNF and NGF by DPSC have been found, both *in vitro* and *in vivo*, to promote the

survival of sensory and dopaminergic neurons, to favor the rescue of motorneurons in an animal model of spinal cord injury and to promote the survival and sprouting of neurons from trigeminal and sympathetic ganglia [57, 58, 87]. In addition, DPSC grafted into the hippocampus of immune-suppressed mice stimulated neural cell proliferation, shown by a significant increase in proliferating cells at the site of the graft and also caused the recruitment of endogenous neural cells to the site of engraftment. It was stated that the proliferation, differentiation, maturation and recruitment of endogenous neural cells was caused by altering the microenvironment in the brain through an increased production of neurotrophic factors CNTF, VEGF, NGF and b-FGF by the engrafted DPSC [81]. In a recent study, DPSC were transplanted into the cerebrospinal fluid of rats in which cortical lesion was induced. Those cells migrated as single cells into a variety of brain regions and were detected in the injured cortex expressing neuron specific markers. This showed that DPSC-derived cells integrated into the host brain may serve as useful sources of neuro- and gliogenesis *in vivo*, especially when the brain is injured [48]. The spontaneous differentiation potential of DPSC strongly suggests their possible applications in regenerative medicine. Taken together, DPSC yield great potential as cellular therapy for neurological disorders based on their neural crest origin and their neural characteristics *in vitro* and *in vivo*.

Although DPSC are extensively investigated for their role in central nervous disorders, no reports are available describing the potential role of DPSC in peripheral nerve injury.

1.5 Injury to the Inferior Alveolar Nerve

The inferior alveolar nerve (IAN) is a branch of the mandibular nerve that contains sensory fibres. It enters the mandibular foramen, runs in the mandibular canal, and supplies the mandibular teeth with sensory branches (Figure 1.4). The mandibular canal is located under the lower molars and runs in close relationship with the roots of teeth above. In some cases, this distance is very near and caution must be taken when performing extraction of the lower (third) molar. Parts of the root often extend around or in the canal so that the inner contents of the mandibular canal, the IAN, can be damaged after extraction of the tooth, leading to necrosis of the injured nerve (Figure 1.5).

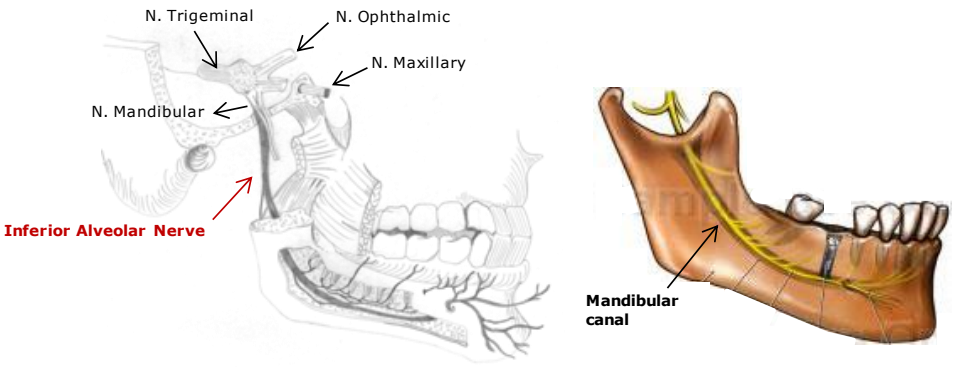


Figure 1.4: Anatomical image of Inferior Alveolar Nerve and its canal.

Extraction of the lower third molar is one of the common performed procedures in dental or oral and maxillofacial surgery. The majority of the patients show no post-extraction complications and fully recover. However, 0.5% to approximately 8% of the patients suffer from neurological problems including neuralgia or impaired sensibility not only in the area of the IAN but also in the entire area of the trigeminal nerve [88, 89]. Injury to the IAN can cause a variety of symptoms like altered sensation, pain, reduction in quality of life and associated psychological problems. Other causes for IAN injury are osteotomy, placement of dental implants, injection of local analgesics and facial traumas [90-92].

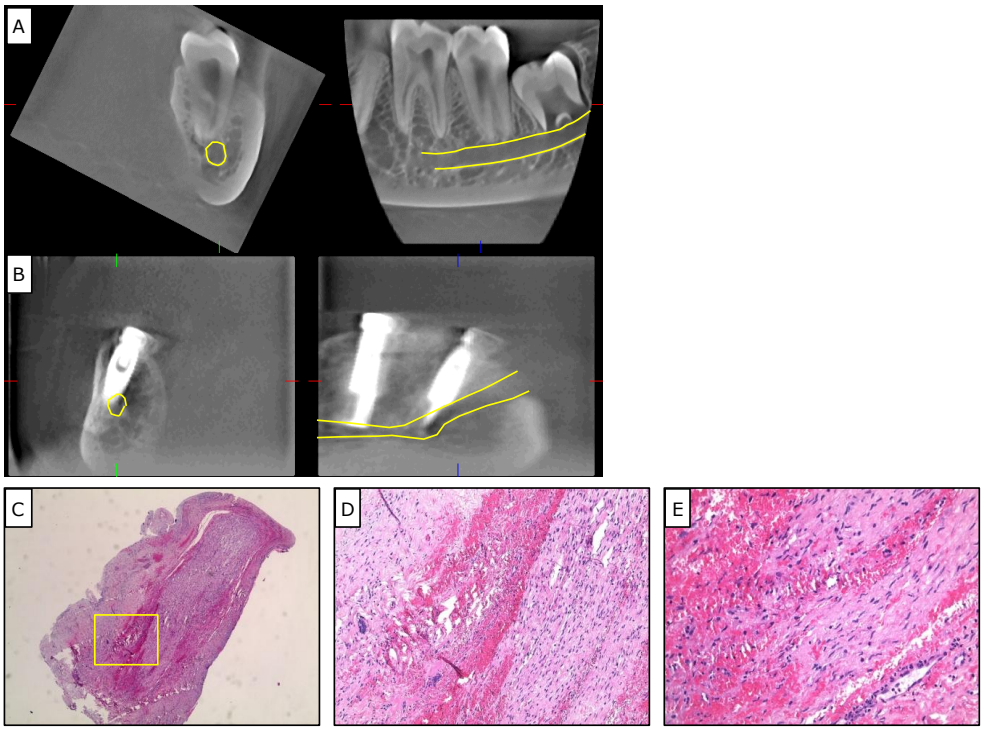


Figure 1.5: Mandibular canal and injured IAN. Cone Beam CT images of Mandibular Canal (A-B) and Histological image of inferior alveolar nerve injury. Necrosis of nerve fiber (C-E). (In courtesy of Prof dr R Jacobs and Prof dr C Politis)

Because the IAN lies within a the body of the mandible, repair of the nerve after injury is a complex and difficult procedure. Several surgical techniques have been developed during the last several years including anastomosis of the nerve ends with nerve or vein grafts, but the overall clinical results were not as good as expected [93]. To achieve an optimal peripheral nerve regeneration, the following conditions need to be provided: increase of the neural capability to induce axonal outgrowth, prevent loss of basal lamina and denervation changes in Schwann cells, and finally, impede inhibitory effects of extracellular matrix [94]. Cellular-based therapies might be a promising alternative as treatment for peripheral nerve injury since the current classical techniques have disappointing results. Schwann cells could be an interesting cell source considering that they play an important role during the regeneration of peripheral nerves. Denervated Schwann cells proliferate and promote the regeneration of axons following nerve injury [95]. However, the major drawback of using Schwann cells, is the loss

and sacrifice of another peripheral nerve for harvesting Schwann cells. In addition, Schwann cells have a slow and time consuming grow capacity *in vitro*, leading to the need of an alternative cell source with Schwann cell characteristics [96, 97]. Several *in vitro* and *in vivo* studies report the differentiation of mesenchymal stem cells to support peripheral nerve regeneration. MSC were shown to express Schwann cell markers and supported neurite outgrowth *in vitro*. In addition, several *in vivo* studies provided histological evidence of an improved axonal outgrowth but there are few studies examining functional outcomes and these studies had inconsistent findings [94, 96, 98-104]. Taken into account that DPSC are a neural crest-derived cell population and that they possess mesenchymal and neural characteristics, DPSC can be an optimal alternative cell source for regeneration studies of peripheral nerve injury.

1.6 Stem cells and dental materials

Over the past two decades, tissue engineering and regenerative medicine have become increasingly important areas of research. With a combination of biomaterials, competent (stem) cells and inductive growth and differentiation factors, the goal is to improve or fully restore the functions of the injured tissues or organs. Over the last few years, dentistry has begun to explore the potential application of stem cells and tissue engineering towards the repair and regeneration of dental structures. In tissue engineering, biodegradable and biocompatible scaffolds provide an environment for the adhesion of cells and their proliferation, migration and differentiation until these cells and the host cells begin to secrete and shape their own environment. Therefore, scaffolds are considered to be a critical component of tissue engineering [105, 106].

Biocompatibility of the scaffold is of utmost importance to prevent adverse tissue reactions. Since the host cells will interact with the scaffold, biodegradability should be tunable to facilitate constructive remodeling, which is characterized by scaffold degradation, cellular infiltration, vascularization, differentiation and spatial organization of the cells, and, eventually, replacement of the scaffold by the appropriate tissues [107]. Current research is exploring

the perfect formula for a reliable stem cell source, appropriate signaling molecules and a scaffold that will promote controlled cell growth and differentiation. Several dental stem cell populations, such as DPSC and SCAPs, have been shown to form functional tissues integrated with the scaffold *in vivo* (see chapter 1.3). Tissue engineering using dental stem cells, morphogens, and scaffolds may provide an innovative biologically-based approach for the generation of clinical materials and/or treatments for dental diseases or injuries [108].

1.7 Aims of the study

Currently, no successful treatments are available for central or peripheral nervous injuries such as spinal cord injury and trigeminal neuralgia. Based on the high proliferative capacity, the neural differentiation potential and the ability to secrete neurotrophic factors, stem cells could be a promising alternative. Several stem cell populations are reported to be present within dental tissues. Among them are the DPSC, residing in dental pulp tissue, and stem cells from apical papilla. DPSC are described as a highly proliferative stem cell population with the ability of self-renewal and multipotential differentiation capacity. In addition, hDPSC can be easily isolated from orthodontic waste materials without any ethical concerns. Recently, it has been shown that DPSC are able to transdifferentiate into cells of the neural lineage. Therefore we investigated whether hDPSC possessed neuronal and glial characteristics and differentiation potential in *in vitro* models. Based on these results, *in vivo* models can be developed and eventually these stem cells could be applied clinically as treatment for peripheral/central nervous traumas.
In the first part of this thesis, the expression of neural and mesenchymal cell markers of DPSC in an undifferentiated state was elucidated via immunocytochemical analysis *in vitro* and *in situ*. In the next part, the immunocytochemical and ultrastructural characteristics present after neuronal and glial cell differentiation were analyzed. Furthermore, the secretion of neurotrophic factors by both naïve and glial cell differentiated hDPSC was investigated together with their influence on dorsal root ganglia cell cultures. In the last chapter, stem cells isolated from apical papilla were morphologically characterized and their multilineage differentiation capacity *in vitro* was elucidated.

Mesenchymal and neural marker expression in undifferentiated hDPSC (chapter 2)

DPSC possess mesenchymal characteristics such as the expression of typical mesenchymal surface makers and the potential to undergo multilineage differentiation into adipocytes, chondrocytes and osteoblasts. Recently, DPSC

are described to be able to differentiate into cells of the neural lineage. Before investigating the neural differentiation potential, knowledge about the expression of mesenchymal and neural markers of DPSC in an undifferentiated state is necessary. Therefore, the basal expression of these makers was determined via reverse transcriptase PCR and immunocytochemistry of *in vitro* cultures. Furthermore, the location of stem cells niches *in situ* was demonstrated by using the same cell-specific immunocytochemical markers.

Characterization of the neuronal differentiation potential of hDPSC (chapter 3)

In chapter 3, the neuronal differentiation potential of hDPSC was investigated by means of light and transmission electron microscopical analysis. Neuronal differentiation was induced via chemical induction and two different protocols were used: (1) neurosphere assay – (2) neural inductive media assay. The expression of neural markers upon differentiation was analyzed via immunocytochemistry. An ultrastructural analysis was performed to investigate the morphological changes after neuronal differentiation. In addition, whole cell patch clamp recordings were performed to analyze the presence of voltage-gated sodium and potassium channels after differentiation of hDPSC.

Characterization of the glial cell differentiation potential of hDPSC (chapter 4)

The fourth chapter of this thesis described the glial cell differentiation potential of hDPSC. As first, the pluripotency and neural crest-derived origin of undifferentiated cell cultures was studied via reverse transcriptase PCR. Next, glial cell differentiation was induced by adding a mixture of growth factors (PDGFaa, b-FGF, NRG) to the culture medium and the success of differentiation was determined via the expression of cell-specific markers and ultrastructural analysis. Furthermore, a human cytokine antibody array was performed to compare the production of neurotrophic factors between naïve and glial cell differentiated hDPSC. Finally, the effect of neurotrophic factors (secreted into

the culture medium by naïve and differentiated hDPSC) on DRG cell cultures was analyzed via different experiments: (1) the effect on the survival of DRG cells was addressed via MTT assay – (2) the length of neurite outgrowth of DRG cells was measured.

Characterization of stem cells from apical papilla (chapter 5)

In this last chapter, we investigated another dental stem cell population: stem cells isolated from apical papilla (SCAPs). The morphological and ultrastructural characteristics of this cell population was studied via light and transmission electron microscopy. Via immunocytochemistry, the expression of cell-specific markers was analyzed. In addition, the presence of pluripotency and neural crest markers on the mRNA level was determined using reverse transcriptase PCR. To evaluate the multipotency of SCAPs, the differentiation potential into cells of the adipogenic, chondrogenic and osteogenic lineages was investigated on the light microscopical and ultrastructural level.

2

Expression pattern of basal markers in human dental pulp stem cells and tissue

2.1 Abstract

In the adult organism, mesenchymal stem cell populations are present in most tissues and organs. Recently, dental pulp stem cells (DPSC), residing in dental pulp tissue, have been characterized as being a multipotent stem cell population. Besides their ability to differentiate into mesodermal cell lineages, DPSC have the potential to differentiate along a neural lineage. The *'de novo'* expression of neural markers after differentiation is mostly considered as a proof of differentiation. However, the basal level expression of neural markers is not well described. Therefore, in this study, an immunocytochemical analysis was performed to evaluate the basal expression level of mesenchymal and neural markers in *in vitro* cultures of naïve DPSC. Undifferentiated human DPSC uniformly expressed the mesenchymal markers CD29, CD44, CD105, CD117 and CD146 and the neural markers β-III-tubulin, S100 and synaptophysin. A subset of the population showed a positive immune-reactivity for Stro-1, GalC, neurofilament and NGFRp75.

In a second part of the study, the location of possible stem cell niches present in young dental pulp tissue was determined by means of immunohistochemistry. The results demonstrated the presence of a perivascular niche and a second stem cell niche present at the cementum-enamel border. In adult dental pulp, only a perivascular niche could be observed.

Based on the expression of neural markers in naïve DPSC, it has to be taken into account that not only the marker expression upon neural differentiation must be analyzed but in addition, an ultrastructural analysis of the morphological changes and functional studies must be included into the experiments to confirm a successful differentiation. Furthermore, we can conclude that depending on the developmental stage of the pulp tissue, several stem cell niches are present containing distinct multipotent stem cells.

2.2 Introduction

In the adult organism, most tissues and organs contain a small population of cells, adult (mesenchymal) stem cells, who maintain the stem cell characteristics of self-renewal and differentiation potential. By means of differentiation, more committed progenitor cells can be generated along multiple lineages [109]. Overall, adult stem cells remain quiescent within the adult tissue but they are able to respond to injury and play an essential role in tissue repair processes. The maintenance and regulation of a quiescent stem cell population is tightly controlled by the local microenvironment of the host tissue.

Recently, it has been confirmed that a stem cell population is present inside the dental pulp. Evidence can be found in a study from Téclès *et al.* in 2005 in which a migration of cells from perivascular regions was observed after pulp capping procedures. These migrating cells played a role in reparative dentinogenesis and dentin bridge formation. Migration of stem cells to the site of injury for differentiation into odontoblast-like cells will be an important event for cell recruitment during regeneration [41, 110]. Another attempt to identify a stem cell niche inside dental pulp tissue was performed via immunohistochemical analysis *in situ*. By using a combination of markers (stem cell marker Stro-1, the perivascular cell marker CD146, alpha smooth muscle actin and the pericyte associated antigen (3G5) it was suggested that a population of DPSC may reside in the perivascular niche within the adult pulp [110-113]. However, further research is necessary to determine if other stem cell niches could be present within dental pulp tissue.

Several stem cell populations residing in dental tissues have been identified during the last ten years. Gronthos *et al.* were the first to isolate and characterize the dental pulp stem cells (DPSC) from human dental pulp tissue [31]. Afterwards, other stem cell populations in the tooth and surrounding tissues have been characterized: periodontal ligament stem cells, stem cells from human exfoliated deciduous teeth, stem cells from apical papilla and dental follicle precursor cells [32-34, 36]. DPSC are described as being a highly clonogenic and proliferative adult stem cell type with the capability of forming a dentin-pulp-like complex *in vitro* and *in vivo* [31, 42]. They are an adherent fibroblast-like cell type, expressing commonly used mesenchymal stem cell

markers such as CD29, CD44, CD105, CD146 and Stro-1. After induction with the appropriate media, DPSC possess a multilineage differentiation potential towards adipogenic, chondrogenic and osteogenic lineages [52]. Furthermore, differentiation into odontogenic, myogenic, angiogenic and neurogenic lineages *in vitro* and *in vivo* have been described [31, 46, 49, 51, 55, 57, 78, 79]. A recent study shows that hDPSC are able to differentiate *in vitro* into odontoblast-like cells producing a mineralized extracellular matrix possessing the molecular and mineral characteristics of dentin *in vivo* [43]. Since DPSC can be easily isolated and expanded *in vitro* and they are able to undergo differentiation into several lineages, they are a very promising stem cell population for future regeneration studies. Furthermore, human third molars are frequently removed for orthodontic or therapeutic reasons, being an easily accessible source of adult stem cells.

Before investigating the multilineage differentiation potential of DPSC, an overview of marker expression in undifferentiated DPSC is indispensable. Most studies analyze the expression of markers to assess the success of differentiation. However, the basal expression levels of mesenchymal and neural markers in this cell population are not well described. Therefore, this study will focus on the expression of mesenchymal and neural markers in undifferentiated/naïve hDPSC both *in vitro* and *in situ*.

2.3 Materials & methods

2.3.1 Isolation and cell culture of stem cells from dental pulp tissue

Normal human third molars were collected from patients (18-24 years old) at the Ziekenhuis Oost Limburg, Genk, Belgium with the patient's informed consent. Teeth were mechanically fractioned and pulp tissue was isolated and collected in culture medium consisting of alpha Minimal Essential Medium (GIBCO Invitrogen Corp, Paisly, Scotland, UK) supplemented with 10% heat inactivated fetal calf serum (FCS) (Biochrom AG (Berlin, Germany), 2mM L-Glutamine, 100 U/ml Penicillin and 100µg/ml Streptomycin (GIBCO Invitrogen Corp, Paisly, Scotland, UK). Dental pulp was cut into pieces of $1mm^3$, transferred to a 6-well plate containing culture medium and cultured via the explant method. The explants were kept in culture for 14 days to allow cells to grow out of the explants. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 with medium change twice a week. After 14-21 days, confluency was reached and cells were subcultured.

2.3.2 Immunocytochemistry

To determine the expression profile of mesenchymal and neural markers in DPSC cultures, cells were seeded onto glass coverslips (5,000cells/cm²) and stained with the peroxidase-based EnVision System® (DakoCytomation, Glostrup, Denmark). When confluent, cells were fixed with paraformaldehyde (PFA) 4% for 20 minutes and washed with Phosphate Buffered Saline (PBS). When necessary, cells were permeabilized with Triton-X 0.05% for 30 minutes at 4°C and washed with PBS. To block non-specific binding sites, cells were incubated with 10% normal goat serum or normal donkey serum at room temperature for 20 minutes. After washing with PBS, cells were incubated with primary antibody (table 2.1) for 1h, followed by incubation with goat anti-mouse, goat anti-rabbit, or donkey anti-goat horseradish peroxidase-conjugated secondary antibodies for 30 minutes. To visualize the peroxidase, diaminobenzidine chromogenic substrate was used. Cells were counterstained

with Mayer's hematoxylin and mounted using an aqueous mounting medium (Aquatex, Merck, Darmstadt, Germany). The immune-reactivity of the cells was observed using a photomicroscope equipped with an automated camera (Nikon Eclipse 80i, Nikon Co., Japan).

Table 2.1: Primary antibodies for immunocyto- and immunohistochemistry

	Species	**Dilution**	**Company**
Mesenchymal stem cell markers			
CD29	Mouse	1:35	Abcam
CD44	Mouse	1:200	Abcam
CD105	Mouse	1:100	Abcam
CD117 (c-Kit)	Mouse	1:100	Santa Cruz
CD146	Rabbit	RTU	Abcam
Stro-1	Mouse	1:50	R&D systems
Neural markers			
β-III-tubulin	Mouse	1:2000	Sigma Aldrich
GalC	Mouse	1:20	Dr. V.W.Yong Calgary
GFAP	Mouse	1:200	NovoCastra
NGFRp75	Mouse	1:50	Dakocytomation
MBP	Mouse	1:200	Serotec
Neurofilament	Mouse	1:100	Dakocytomation
S100	Rabbit	1:400	Dakocytomation
Synaptophysin	Mouse	1:20	Dakocytomation
Other markers			
Cytokeratin	Mouse	RTU*	Dakocytomation
Nestin	Mouse	1:500	Chemicon
Vimentin	Mouse	1:500	Dakocytomation

* RTU : Ready to use

2.3.3 Immunohistochemistry (*in situ* expression)

Immunohistochemical analysis was performed on young dental pulp tissue using an indirect visualization system, based on a peroxidase-labelled polymer (Dako EnVision® System). Young dental pulp tissue was isolated from teeth showing less than 50% root formation. After fixation, paraffin embedded sections (7μm) were deparaffinized. Subsequently, sections were microwaved in 10 mM citrate

buffer pH 6.0 and endogenous peroxidase activity was quenched with 0.5% H_2O_2. Non-specific binding sites were blocked with 3% normal goat serum. Then, sections were stained with mouse or rabbit primary antibodies for 1 h (table 2.1) followed by incubation with a peroxidase-labelled polymer conjugated to a goat anti-mouse or goat anti-rabbit secondary antibody for 30 minutes and subsequently visualized using diaminobenzidine chromogenic substrate. Sections were counterstained with Mayer's hematoxylin, coverslipped with an aqueous mounting medium and examined using a photomicroscope equipped with an automated camera (Nikon Eclipse 80i, Nikon Co.).

2.3.4 RNA extraction – cDNA synthesis - Reverse Transcriptase PCR

Total RNA was isolated using the RNeasy Mini plus kit (Qiagen, Venlo, The Netherlands) following manufacturer's protocol. This kit ensures additional elimination of genomic DNA. The concentration and purity of total RNA was determined by measuring the optical density at 260nm and the 260/280nm ratio using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). 700ng of total RNA was reverse transcribed into cDNA according to the manufacturer's instructions of the Reverse Transcription System (Promega, Leiden, the Netherlands). Samples were stored at -80°C until further analysis. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed by means of Taq DNA Polymerase (1U/µl) (Roche Diagnostics, Vilvoorde, Belgium) (table 2.2). PCR reactions were performed by a BioRad Thermal cycler (Biorad, Hercules, CA, USA) under the following conditions: a denaturation step at 94°C for 5 minutes was followed by amplification over 35 cycles of denaturation (94°C for 1 minute), annealing (temperature dependent on the primers for 60 seconds) and elongation (72°C for 2 minutes). Sequences of the primers (Eurogentec S.A. Seraing, Belgium) can be found in table 2.3. The housekeeping genes β-actin, GusB and β2-microglobulin were used as a control for the PCR reaction. PCR products were separated on a 1.5% agarose gel (Invitrogen, Merelbeke, Belgium) and visualized with gel red.

Table 2.2: Composition of PCR reaction mix and applied PCR program

PCR mix per sample (µl)	
10X PCR buffer	2.5
Forward primer (25µM)	1
Reverse primer (25µM)	1
dNTPs (2mM)	0.25
Taq Polymerase (1U/µl)	0.75
MilliQ	19.1
cDNA	1

Table 2.3: Primer sequences for Reverse Transcriptase PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Tm (°C)	Product Length (bp)
Neural markers				
β-III-tubulin	**CAT-CCA-GAG-CAA-GAA-CAG-CA**	**GCC-TGG-AGC-TGC-AAT-AAG-AC**	56	566
GalC	**GCC-AAG-CGT-TAC-CAT-GAT-TT**	**GCA-GAG-ATG-GAC-TCC-CAG-AG**	58	161
GFAP	GCC-AAG-CCA-GAC-CTC-ACC-GC	GTG-TCC-AGG-CTG-GTT-TCT-CGA-ATC	60	509
MBP	**CCC-CGT-AGT-CCA-CTT-CTT-CA**	**TCC-CTT-GAA-TCC-CTT-GTG-AG**	56	178
NF-H	**AGG-AAC-CAG-ATG-ATG-CCA-AG**	**TGG-AGG-CTT-GCT-GTC-TTT-TT**	58	233
S100	**TGA-TCC-AGA-AGG-AGC-TCA-CC**	**CCC-TTG-AGG-GCT-TCA-TTG-TA**	56	160
Other markers				
Nestin	**AAC-AGC-GAC-GGA-GGT-CTC-TA**	**TTC-TCT-TGT-CCC-GCA-GAC-TT**	56	220
Vimentin	**GAG-AAC-TTT-GCC-GTT-GAA-GC**	**TCC-AGC-AGC-TTC-CTG-TAG-GT**	56	170

Housekeeping genes				
β actin	**AAA-TCT-GGC-ACC-ACA-CCT-TC**	**AGA-GGC-GTA-CAG-GGA-TAG-CA**	56	185
β2 microglobulin	**CTC-ACG-TCA-TCC-AGC-AGA-GA**	**CGG-CAG-GCA-TAC-TCA-TCT-TT**	56	213
Gus B	**AGC-CAG-TTC-CTC-ATC-AAT-GG**	**GGT-AGT-GGC-TGG-TAC-GGA-AA**	56	160

2.4 Results

2.4.1 Characterization of human dental pulp stem cells

Cultures of human DPSC (hDPSC) were isolated via the explant culture methods, in which cells were allowed to grow out of the explants (Figure 2.1 A). When confluency was reached, hDPSC were subcultured. *In vitro*, hDPSC were a heterogeneous cell population containing cells with a polygonal or spindle-shaped (fibroblast-like) morphology. Cells were adherent to a plastic surface and formed colonies *in vitro* (Figure 2.1 B-D). Furthermore, hDPSC were capable of differentiating into adipocytes, chondrocytes and osteoblasts when grown under the appropriate culture conditions (data not shown).

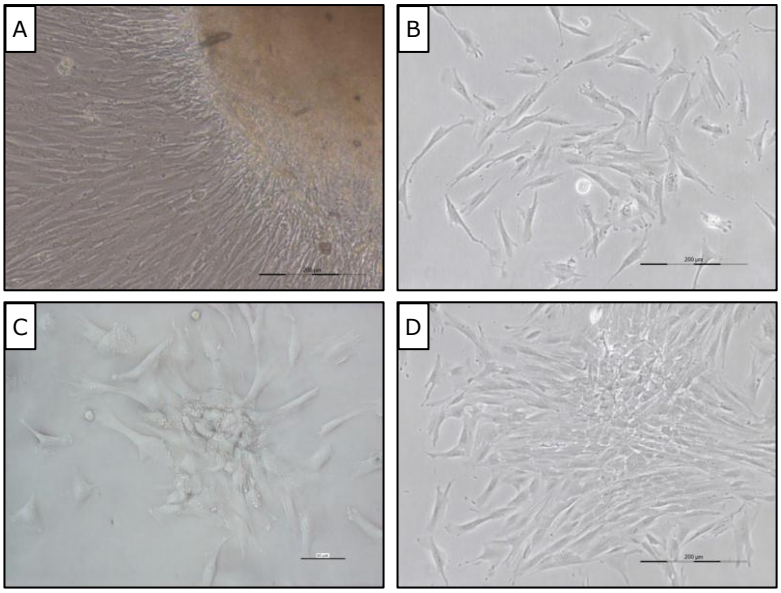


Figure 2.1: Phase contrast images of *in vitro* cultures of hDPSC. (A) hDPSC were cultured via the explant method. Cells grew out from pulp tissue explants on plastic substrates. (B-D) Isolated cells displayed a flattened polygonal morphology and were capable of forming colonies *in vitro*. Scale bar A-B-D = 200µm; Scale bar C = 50µm

To evaluate the expression pattern of classical mesenchymal surface markers *in vitro*, an immunocytochemical staining for CD29, CD44, CD105, CD117, CD146 and Stro-1 was performed. hDPSC showed uniformly a positive immune-reactivity for the mesenchymal markers mentioned. However, Stro-1 expression was only found in a subset of the cell population. After manual counting, 5.8% of the cell population was Stro-1 positive (Figure 2.2).

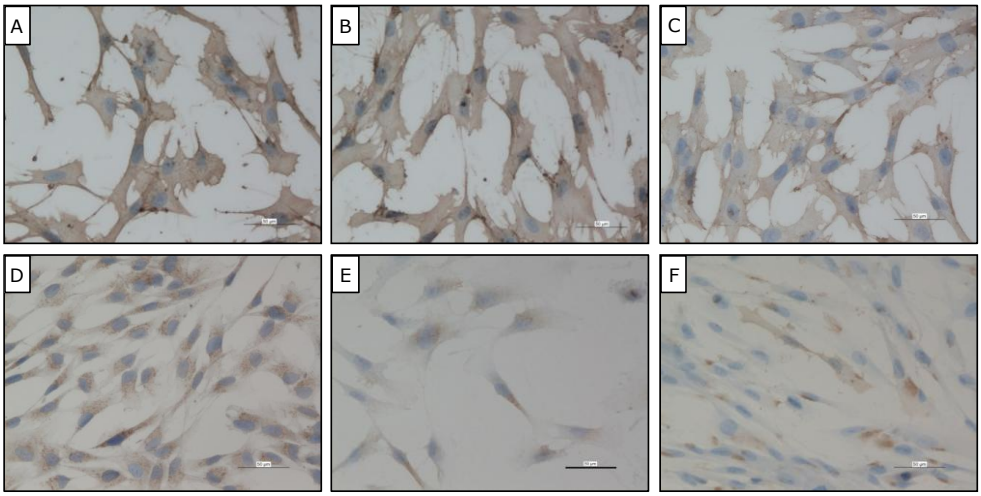


Figure 2.2: Immunocytochemical analysis of mesenchymal marker expression in undifferentiated hDPSC. (A-E) hDPSC stained uniformly for CD29, CD44, CD105, CD146 and c-Kit. (F) Stro-1 expression was only found in part of the population (5.8%; s.d. 2.2%). Scale bar = 50µm

2.4.2 Neural marker expression of undifferentiated hDPSC

RT-PCR was performed to determine the transcript levels of neural markers in undifferentiated hDPSC. hDPSC were shown to possess the neural genes β-III-tubulin, GalC, S100 and neurofilament at the mRNA level. No transcripts of GFAP and MBP were detected in undifferentiated hDPSC (data not shown). Furthermore, levels of nestin and vimentin, which are early neural markers, were found (Figure 2.3 B).

At the protein level, similar results were observed. Immunocytochemical analysis revealed that hDPSC stained uniformly for the neural markers β-III-tubulin, S100 and synaptophysin. Only a subpopulation of the cells showed a positive immune-reactivity for GalC, neurofilament heavy chain and Nerve Growth Factor Receptor p75 (NGFRp75) respectively 92.3%, 42.27 % and 4.07%. No GFAP and MBP expression was found (data not shown). In addition, the expression of early markers nestin and vimentin was also observed at the protein level in all undifferentiated hDPSC (Figure 2.3 A.G-H).

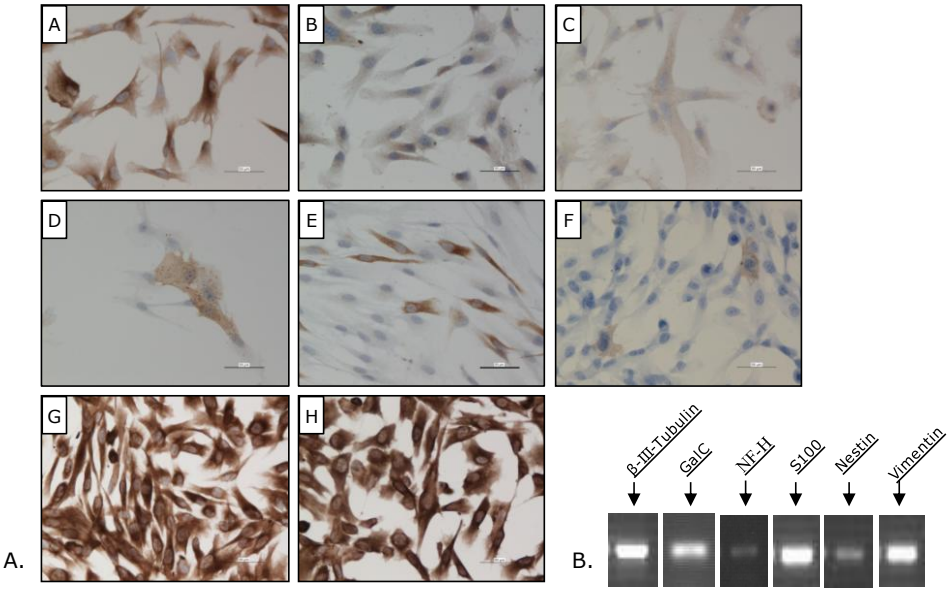


Figure 2.3: Analysis of neural marker expression in undifferentiated hDPSC A. Immunocytochemical staining of hDPSC. hDPSC stained uniformly for β-III-tubulin (A), S100 (B) and synaptophysin (C). Only a fraction of the cells expressed GalC (D), neurofilament (E) and NGFRp75 (F) respectively 92.3% 42.27% and 4.07%. Nestin (G) and vimentin (H) expression was observed in all hDPSC. Scale bar = 50μm B. Expression of neural genes at the mRNA levels in hDPSC. (NF-H = neurofilament heavy chain).

2.4.3 Expression of mesenchymal and neural markers in dental pulp tissue – Immunohistochemical analysis

Immunohistochemical stainings on human dental pulp tissue were performed to evaluate the expression pattern of mesenchymal and neural markers in young dental pulp tissue. Young dental pulp tissue was isolated from teeth showing less than 50% root formation. The mesenchymal markers CD29, CD44, CD117, CD146 and Stro-1 were expressed throughout the whole tissue with a more pronounced expression of CD29, CD44 and CD146 at the cementum-enamel border and CD29+ cells at the basis of the pulp tissue. Furthermore, an increase of CD29, CD44 and CD146 positively stained cells was observed at the cervical area towards the centre of the tissue and towards the apical region of the dental pulp tissue. The pattern was most obvious after staining with anti-CD44 (Figure 2.4 D). Upon staining with anti-CD105, a weak immune-reactivity was observed throughout the tissue (Figure 2.4). In addition, dental pulp tissue was homogenously stained with anti-nestin, anti-vimentin and anti-S100 (Figure 2.5 A-C). β-III-tubulin and NGFRp75 positive cells were mainly found at the subodontoblast zone. In the central part of the tissue, the expression of both markers was related to neural structures. Nerve fibers were also positively stained with anti-neurofilament, anti-MBP and anti-synaptophysin which was comparable with NGFRp75 staining (Figure 2.5 D-H). In contrast to the *in vitro* cultures of hDPSC, no GalC+ cells were observed in the dental pulp tissue. Furthermore, no GFAP+ cells could be observed in the tissue, which is consistent with no GFAP+ cells present in *in vitro* cultures. At the apical region of dental pulp tissue, fragments of epithelial cells were seen which stained positive for cytokeratin (data not shown).

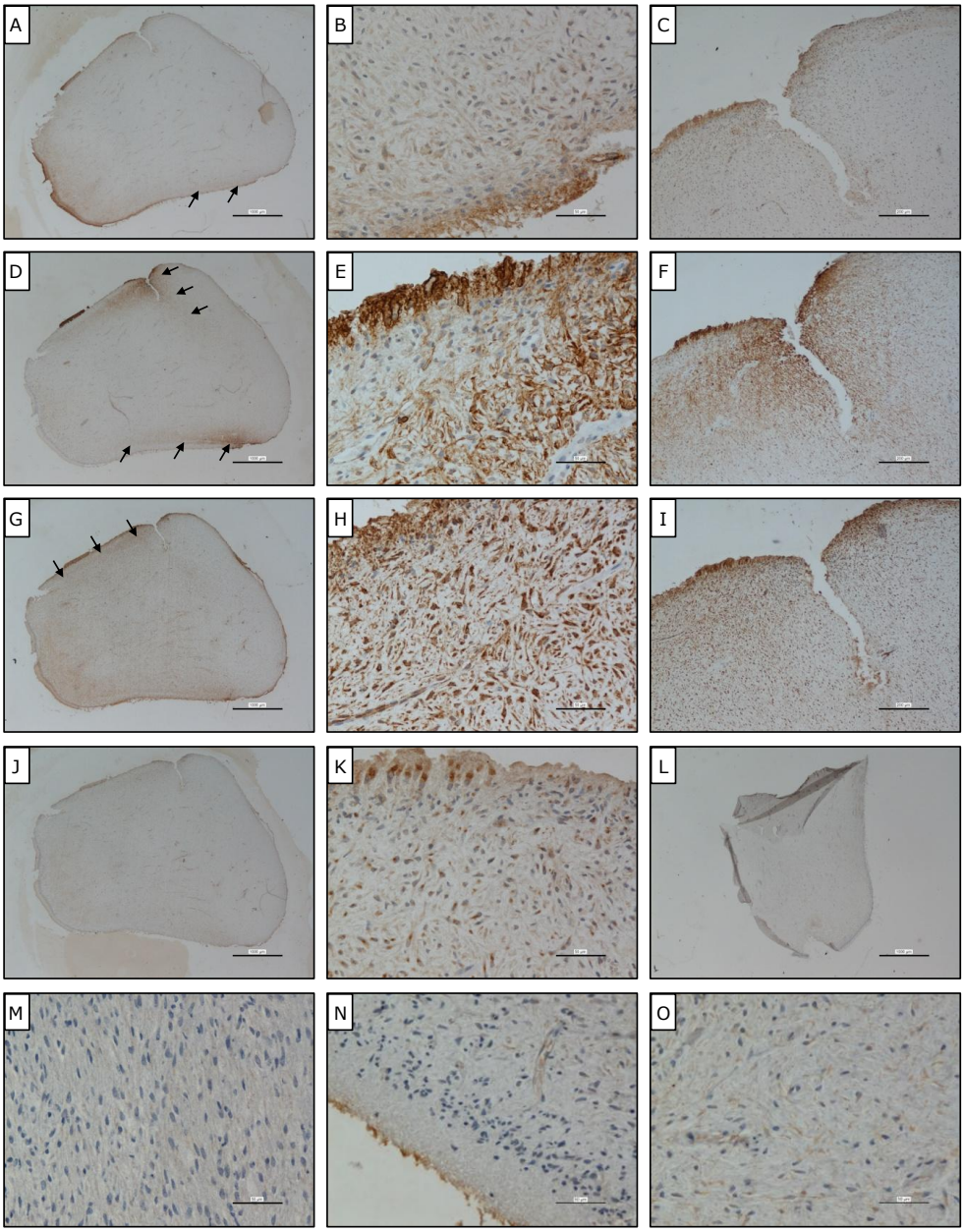


Figure 2.4 Immunohistochemical staining of mesenchymal markers in human dental pulp tissue. The expression of mesenchymal markers CD29 (A-C), CD44 (D-F), CD146 (G-I), CD117 (J-K), CD105 (L-M) and Stro-1 (N-O) was observed in dental pulp tissue. Arrows indicate a possible migration pattern (A,D,G). Scale bar A,D,G,J,L = 1000µm; Scale bar B,E,H,K,M,N,O = 50µm; Scale bar C,F,I = 200µm

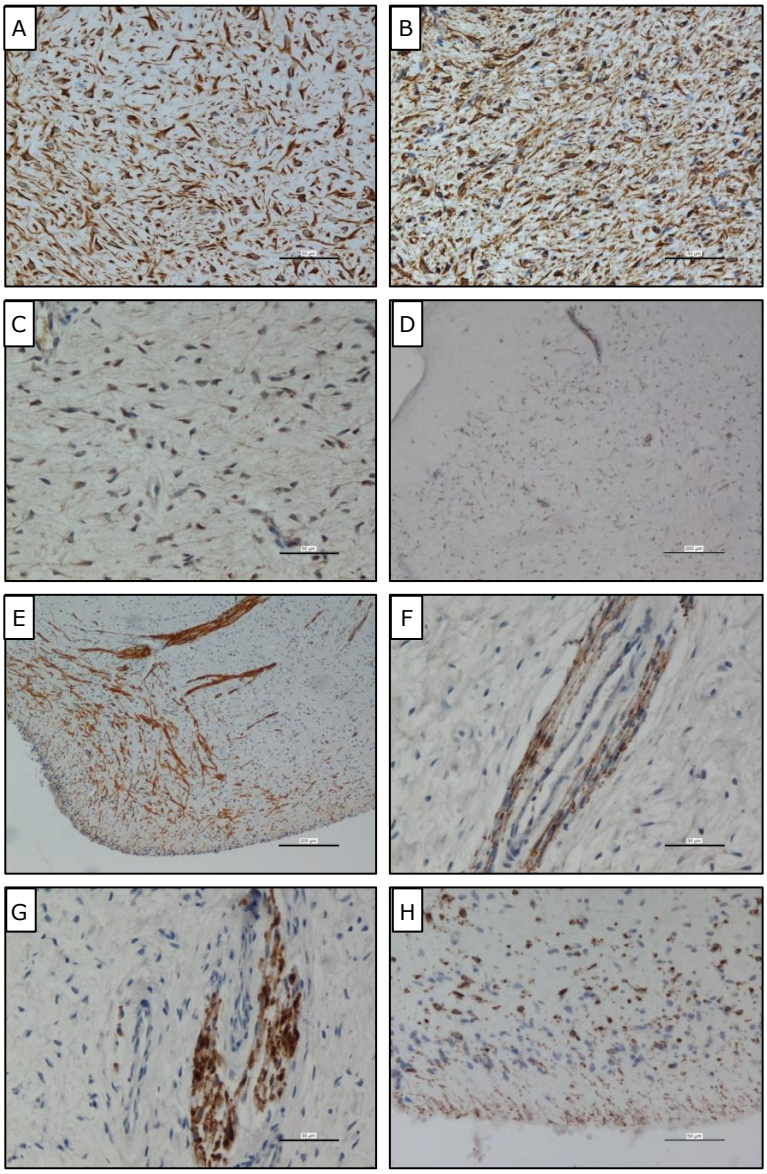


Figure 2.5: Immunohistochemical staining of neural markers in human dental pulp tissue. A positive expression of neural markers nestin (A), vimentin (B) and S100 (C) is found throughout the whole tissue. β-III-tubulin (D) and NGFRp75 (E) expression was observed at the subodontoblast zone. In the centre of the tissue, the expression of both markers was related to neural structures. Nerve fibers were also immune-reactive for neurofilament (F), MBP (G) and synaptophysin (H). Scale bar A,B,C,F,G,H = 50µm; Scale bar D-E = 200µm

2.5 Discussion and Conclusion

During the last 10 years, hDPSC have become a very promising alternative stem cell source of MSC for future regeneration studies based on their ease of isolation and expanding *in vitro,* and multilineage differentiation potential both *in vitro* and *in vivo*. The results of this study, concerning the isolation and morphological appearance of hDPSC *in vitro,* are comparable with a previous study of our group [52]. hDPSC cultured via the explant method were described as being an adherent colony-forming cell type with a fibroblast-like morphology. The expression of the mesenchymal surface markers CD29, CD44, CD105, CD146 and Stro-1 was found in undifferentiated hDPSC. Furthermore, hDPSC possessed the ability to differentiate into cells of adipogenic, chondrogenic and osteogenic lineages as previously described in our laboratory [52].

When investigating the neural differentiation potential of MSC, the success of differentiation is often based on the expression of certain neural markers like nestin, β-III-tubulin, neurofilament... Nevertheless, the basal levels of neural marker expression in undifferentiated cells must be taken into account. Depending on the composition of the culture media, certain markers could already be expressed by the naïve stem cells. The study of Blondheim *et al.* in 2006, reported the expression of neural genes in undifferentiated bone marrow MSC (BM-MSC). In the culture media used, a high percentage of FCS combined with horse serum and epidermal growth factor supplement was present, possibly leading to the expression of nestin and neurofilament in naïve BM-MSC [114]. In the present study, undifferentiated hDPSC expressed uniformly β-III-tubulin, S100, synaptophysin, nestin and vimentin. Furthermore, a high percentage of the cell culture showed a positive immune-reactivity for GalC and neurofilament. Besides the expression of neural markers by hDPSC, several studies report the production and secretion of neurotrophic factors BDNF, GDNF and NGF [55, 57, 58, 87]. It must be taken into account that dental pulp is derived from neural crest tissue, giving a predisposition of differentiation towards neural lineages. Nevertheless, by using low-serum containing media, the expression of certain neural markers was lost in naïve DPSC [80]. Depending on the differentiation potential investigated, a good consideration of medium composition is

obligatory. By using high serum levels and/or growth factor supplements in basal culture media, this could lead to an early expression of neural markers in naïve mesenchymal stem cells. In this study, the dental pulp tissue stained negative for GalC in contrast to hDPSC *in vitro*. During cell culture, growth media containing high levels of serum (10% FCS) was used which could lead to the expression of GalC in hDPSC *in vitro*. Most likely, growth factors present in the serum could induce the expression of GalC in certain cells. This leads to the conclusion that indeed careful consideration of medium composition or cell culture conditions is needed for differentiation studies as certain cell culture conditions may interfere with the differentiation potential of hDPSC. As this study clearly demonstrated that undifferentiated hDPSC expressed neural markers such as β-III-tubulin, nestin, S100, synaptophysin, nestin and vimentin, when cultured in 10% FCS, these markers can never be used to confirm neural or glial differentiation of hDPSC. Therefore, besides the evaluation of marker expression before and after differentiation studies, an ultrastructural analysis of the morphological changes and/or functional studies must be included into the experiments to confirm a real successful differentiation.

Several studies report the presence of a perivascular niche of stem cells residing in the adult dental pulp [41, 110, 113]. By means of immunohistochemical analysis, the expression of various mesenchymal and neural markers was evaluated in young dental pulp tissue. Young dental pulp tissue was derived from teeth in a less than 50% root formation stage. Since a young dental pulp tissue, still being an immature tissue, is used for the immunohistochemical analysis, an overall expression of nestin and vimentin was found. The results further demonstrated the presence of a perivascular stem cell niche and a second niche at the cementum-enamel border. An increased amount of positively stained cells were observed at the cementum-enamel border towards the centre of the pulp tissue and towards the apex. Neural markers expression was mainly related to neural structures within the tissue. However, some cells residing in the subodontoblast zone stained positive for β-III-tubulin and NGFRp75. However, in adult pulp tissue, only a perivascular niche is present (data not shown). Leading to the conclusion that the niche at the cementum-

enamel border is lost during maturation of the tissue. A possible explanation can be that this niche contains cells from the dental papilla which will migrate apical forming the apical papilla in a later stage. It is general known that during tooth development, the dental papilla will contribute to tooth formation and converts to the pulp tissue inside the pulp chamber. As the root continues to develop, the location of the dental papilla becomes apical to the pulp tissue [20]. A second explanation could be that being an immature tissue, progenitor/stem cells are essential in the developmental processes of the tooth resulting in the presence of multiple niches including a stem cell niche at the cementum-enamel border. Once the tooth is fully matured, progenitor/stem cells are only required after injury to play a role in tissue repair processes leading to less stem cell niches being necessary. The presence of a perivascular niche in adult dental pulp tissue was confirmed by previous studies [41, 110, 113]. Moreover, by using a combination of specific markers, Karbanová *et al.* suggested the idea of multiple stem cell niches present within the dental pulp containing distinct multipotent DPSC [80]. Further research is necessary to identify the exact location of the niches which could be helpful in understanding if the isolated multipotent stem cells are derived from one highly proliferative multipotent stem cell population or from committed progenitors belonging to distinct lineages.

3

Neural differentiation of human dental pulp stem cells *in vitro*

3.1 Abstract

During the last several years, neuronal differentiation of mesenchymal stem cells (MSC) has been widely investigated in order to find new stem cell-based therapies in central and/or peripheral nervous disorders. The neuronal differentiation potential of dental pulp stem cells, resembling MSC, is in this study investigated by means of immunocytochemistry, ultrastructural analysis and whole cell patch clamp recordings. Neuronal differentiation is induced by two different approaches, the neurosphere assay and neural inductive media assay, resulting in differentiated cells being in different phases of neuronal differentiation. The neurosphere assay resulted in cells expressing the mature neuronal marker NeuN and displaying a polar distribution of synaptophysin in part of the population. The ultrastructural analysis confirmed the more mature differentiation due to the presence of multiple cell-cell contacts, vesicles containing neurotransmitters and presynaptic membrane formations. After neuronal differentiation by the second approach, a decreased immune-reactivity for Stro-1 and an increased expression of neurofilament was observed. However, ultrastructurally, no signs of mature neurons could be detected. Electrophysiological measurements of differentiated cells demonstrated the presence of voltage-gated sodium and potassium channels, but the cells were not able to fire action potentials. Our results demonstrated that hDPSC are capable of undergoing neuronal differentiation and that the two different approaches used, can result in different phases of neuronal differentiation. This makes them good candidates for stem cell-based therapies in central or peripheral nervous disorders.

3.2 Introduction

The adult nervous system has a limited capacity in the regeneration of neurons and glial cells after injury. Stem cell-based therapies may overcome this via neural cell replacement and/or via molecular mechanisms, whereby secreted factors induce changes in the host tissue [56]. Within the central nervous system, neural stem cells are present which can be expanded and differentiated into neurons, astrocytes and oligodendrocytes *in vitro* [115]. In addition, *in vivo* studies show that these neural stem cells, transplanted into the adult rodent brain, can survive and differentiate into neurons and glial cells [116-121]. Although neural stem cells yield great potentials, tumour formation has been reported after the transplantation of neural stem cells into an injured peripheral nervous system [122]. Furthermore, the inaccessibility of neural stem cells is one of the major limitations for clinical use, leading to the search for alternative (stem) cell types that are capable of neural differentiation.

The transdifferentiation potential of mesenchymal stem cells to differentiate into cells of neural lineages, has been widely investigated in order to find new strategies for the treatment of central nervous diseases such as Alzheimer's and Parkinson's disease. Woodbury *et al.* were the first to report the potential transdifferentiation of mesenchymal stem cells (MSC) into neurons *in vitro* [123]. During the following years, other groups confirmed the differentiation of MSC into cells expressing neuronal and glial markers *in vitro* and *in vivo* [123-128]. In addition, it has been demonstrated that transplanted MSC were able to improve the functionality of the central nervous system in rodents with focal cerebral ischemia, Parkinson's disease or other neurodegenerative disorders [128-130].

DPSC, resembling MSC, were first described by Gronthos *et al.* in 2000. DPSC are a clonogenic highly proliferative adult stem cell type with the capability of regenerating a dentin-pulp-like complex *in vitro* and *in vivo* [31]. Furthermore, they express mesenchymal surface markers such as CD29, CD44, CD105, CD146 and Stro-1 [52] and are able to differentiate *in vitro* and *in vivo* into cells of the mesodermal lineages (adipocytes, chondrocytes and osteoblasts) and into odontoblasts, myoblasts, endothelial cells and neural cells [31, 37, 46, 55, 131].

During tooth development, epithelial-mesenchymal interactions take place between pharyngeal epithelium and neural crest-derived mesenchymal cells. In a later phase, the mesenchyme will form the dental papilla which gives rise to the tooth pulp and odontoblasts [20, 21, 132]. Therefore, dental pulp tissue is thought to contain a neural progenitor cell type among it heterogeneous cell population. Considering their neural-crest derived mesenchymal origin, DPSC might possess a greater predisposition to differentiate into neuron-like cells compared with classical BM-MSC. Various studies already describe the neural differentiation potential of DPSC. When DPSC are cultured under the appropriate conditions, they can differentiate into neuron-like cells with a neuronal morphology, neuron specific markers and voltage-gated potassium and sodium channels [48, 55]. Furthermore, DPSC can survive for several months in the central nervous system, when grafted into the caudate nucleus [57]. When DPSC were injected into the hippocampus of immune-suppressed mice, they stimulated the proliferation, differentiation, maturation and recruitment of endogenous neural cells [81]. Taken together, DPSC might be appropriate candidates for future studies to investigate their regenerative potential and possible therapeutic role in central and peripheral nervous disease or injury. In the present study, we investigated whether hDPSC were able to undergo neuronal differentiation by using two different neural induction assays. The success of differentiation was determined by the expression of neural markers and the presence of voltage-dependent sodium and potassium channels after differentiation. Furthermore, an ultrastructural analysis was performed to evaluate the morphological changes upon differentiation. To our knowledge, no ultrastructural analysis has been previously performed on neuronally differentiated DPSC.

3.3 Materials & methods

3.3.1 Isolation and neural differentiation of human dental pulp stem cells

Human DPSC were isolated and cultured as previously described in chapter 2 (2.3.1). Neural differentiation was induced at passage 2 using two different approaches: the neurosphere assay and neural inductive media assay.

Neurosphere assay

Cells were removed from the culture disk by means of Trypsin/EDTA treatment and seeded at a density of 10,000 cells/well in ultimate low cell binding surface plates (Hydrocell, Cellseed, Japan). Cells were cultured in DMEM/F12 medium supplemented with 2% B27 (Invitrogen, Belgium), 100 U/ml Penicillin and 100µg/ml Streptomycin, 20ng/ml basic fibroblast growth factor (b-FGF) and 20ng/ml epidermal growth factor (EGF) (Immunotools, Friesoythe, Germany). Fresh culture medium including EGF and b-FGF was added to the cultures every 3-4 days. Neurospheres were cultured for 7 days and subsequently plated on pre-coated coverslips in culture medium containing EGF and b-FGF. Coverslips were incubated overnight at 37° in a solution of poly-L-ornithine (15mg/ml; Sigma-Aldrich, Bornem, Belgium). The next day, coverslips were rinsed with PBS and coated with fibronectin (500µg/ml) for at least 6 hours. Cells migrated out of the plated neurospheres to form a monolayer. Medium was changed every 3 days and after 7 days in culture, cells were fixed with 4% PFA or 2% glutaraldehyde and further processed for immunocytochemical staining and transmission electron microscopical (TEM) analysis respectively.

Neural inductive media assay

hDPSC at passage 2 were induced to undergo neural differentiation by changing culture medium to DMEM/F12 supplemented with 2% B27, 100 U/ml Penicillin, 100µg/ml Streptomycin, 20ng/ml b-FGF and 20ng/ml EGF. Cells were kept in culture for 10-14 days with medium change every 3 days. Cells were fixed with 4% PFA or 2% glutaraldehyde for light and TEM examination or cells were used for whole-cell patch clamp recordings.

3.3.2 Immunocytochemistry

To determine the expression of neural markers (table 3.1) in differentiated hDPSC, cells were fixed with 4% PFA for 20 minutes and stained with the peroxidase-based EnVision System® (DakoCytomation, Glostrup, Denmark) as previously described in chapter 2 (2.3.4). The immune-reactivity of the cells was observed using a photomicroscope equipped with an automated camera (Nikon Eclipse 80i, Nikon Co., Japan).

A double staining for anti-GFAP and anti-β-III-tubulin was performed in control and differentiated hDPSC. Donkey-anti mouse Alexa488 (1:500) and donkey anti-rabbit Alexa555 (1:500) were used as secondary antibodies (Molecular probes). Slides were mounted using DAPI with Prolong Gold Antifade (Invitrogen/Molecular probes, Merelbeke, Belgium). Immune-reactivity was visualized using a fluorescence microscope (Nikon Eclipse 80i, Kingston, England).

Table 3.1: Primary antibodies for immunocytochemistry

	Species	**Dilution**	**Company**
β-III tubulin	Mouse	1:2000	Sigma Aldrich
GalC	Mouse	1:20	Biomed
GFAP	Mouse	1:200	NovoCastra
GFAP	Rabbit	1:200	Neomarkers
MBP	Mouse	1:200	Serotec
NeuN	Mouse	1:200	Millipore
Neurofilament	Mouse	1:100	Dakocytomation
S100	Rabbit	1:400	Dakocytomation
Synaptophysin	Mouse	1:20	Dakocytomation
Stro-1	Mouse	1:50	R&D Systems

3.3.3 Ultrastructural analysis

Following fixation with 2% glutaraldehyde in a 0.05M cacodylate buffer (pH 7.3), the fixative was gently aspirated with a glass pipette and the cells were post-fixed in 2% osmium tetroxide for 1 hour and stained with 2% uranyl acetate in 10% acetone for 20 min. Subsequently, the cell-seeded coverslips were put through dehydrating series of graded concentrations of acetone and embedded in araldite according to the pop-off method [133]. Ultra-thin sections (0.06µm) were mounted on 0.7% formvar-coated grids, contrasted with uranyl acetate and lead citrate, and examined in a Philips EM 208 transmission electron microscope operated at 80 kV.

3.3.4 Whole cell patch-clamp recordings

Changes in total membrane currents of hDPSC were studied at room temperature using the whole cell configuration of the patch clamp technique in voltage clamp conditions and continuous bath perfusion. The recording electrode was filled with intracellular solution containing (in mM): 135 KCl, 7.7 $MgCl_2$, 2 Mg^+ATP, 2 Na_2ATP, and 10 HEPES, pH 7.2. The extracellular solution used to record induced ionic currents contained (in mM): 145 NaCl, 1.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES and 10 glucose, pH 7.2 Glucose was added to all solutions in order to adjust the osmolarity to ~290 mOsm/kg. The experimental protocols and data acquisition were performed with a personal computer (Pentium III) controlled EPC-10 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Pipette electrodes were made from 1.5 mm (o.d.) borosilicate glass capillary tubes, and had a resistance between 3MΩ and 8 MΩ. Liquid junction (LJ) potentials were calibrated using the Junction Potential Calculator for Windows (JPCalcW, Peter H. Barry, Dept. of Physiology & Pharmacology, Australia & Axon Instruments, Inc., California, USA) and were taken into account at the start of each experiment. The LJ potential between the intracellular and extracellular solution was + 3.9 mV. Capacitive and leak currents were compensated for automatically by the Pulse program (HEKA Elektronik, Lambrecht, Germany) and residual capacitances and leak currents were eliminated by means of a P/6 protocol. The series resistance was compensated for 90%. The recording of whole-cell currents began 5 min after rupture of the

cell membrane, so that the cell interior was adequately equilibrated with the pipette solution. Data were filtered at 2.9 kHz, sampled at 20 kHz and stored on a computer hard disk for later analysis.

Chemicals

Monosodium glutamate (100μM) and γ-amino butyric acid (GABA) (100μM), Tetrodotoxin (TTX) (200nM) and tetraethyl ammonium (TEA) (35mM) were purchased from Sigma-Aldrich (GmbH, Seelze, Germany). The chemicals were applied topically throughout a fast application perfusion system (SF-77B, Warner Instruments, Holliston, MA, USA), by rapidly moving the solution interface across the cell surface.

Data analysis

Data obtained from whole-cell Patch Clamp recordings were analyzed with the PulseFit 8.77 software to assess current amplitudes (HEKA instruments). For the current-voltage relationship, data plots were fitted with the Goldman-Hodgkin-Katz (GHK) equation. Numerical data in the text are expressed as the mean ± SEM, n being the number of experiments.

3.4 Results

3.4.1 Morphology of hDPSC *in vitro* after neural differentiation

Neurosphere assay

hDPSC were grown in low cell binding surface plates in neural inductive media. Cells formed small spheres after 24h and these neurospheres continued to grow and fuse, creating large neurospheres after 1 week in culture (Figure 3.1 A-B). Neurospheres were plated onto coverslips pre-coated with poly-L-ornithine and fibronectin which allowed attachment of the neurosphere and the additional outgrowth of cells from the spheres (Figure 3.1 C).

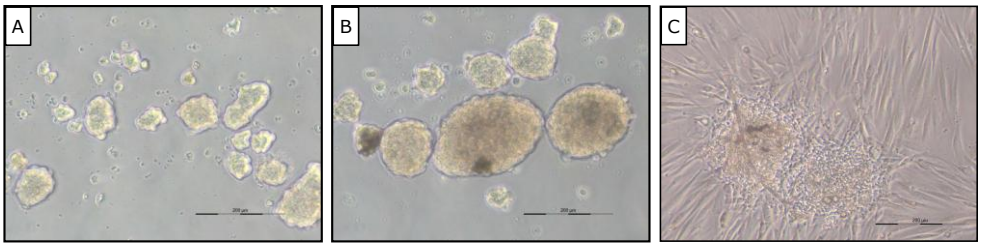


Figure 3.1: **Morphology of cells grown via neurosphere assay.** Neurospheres were grown in the presence of EGF and b-FGF in low cell binding plates. Small spheres were present after 24h and continued to grow, generating large colonies (B). Neurospheres were plated onto pre-coated coverslips with cells growing out of neurosphere (C). Scale bar = 200μm

Neural inductive media assay

Before differentiation, hDPSC cultures displayed a typical mesenchymal-like cell morphology, characterized by the presence of polygonal and spindle-shaped cells (Figure 3.2 A). After 10-14 days grown in neuronal medium, part of the population responded to the neurogenic induction. Cells grew processes and displayed stellate shaped forms (arrows), acquiering a neuron-like cell morphology. Cells that did not respond to the neurogenic induction retained their flat fibroblast-like morphology (Figure 3.2 B-C).

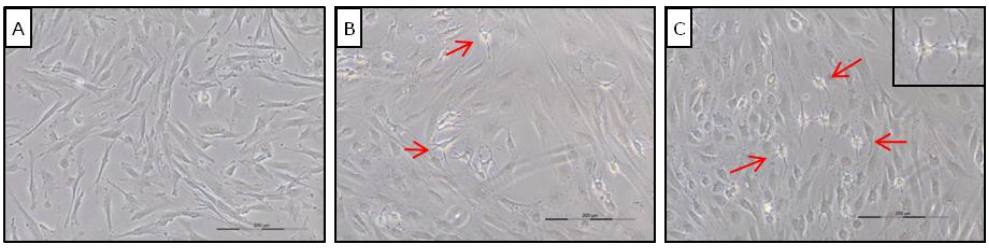


Figure 3.2: Morphology of cells grown via neural inductive media assay. Control hDPSC have a spindle shaped fibroblast-like morphology (A). hDPSC grown for 10-14 days in neuronal induction medium. Part of the population developed multiple neuronal processes (B-C, arrows + insert). Scale bar = 200µm

3.4.2 Immunocytochemistry

The protein expression of neuronal-associated markers expressed by hDPSC cultured in neuronal inductive conditions was assessed by means of immunocytochemical analysis.

Neurosphere assay

Human DPSC derived neurospheres were plated onto pre-coated coverslips and cultured for an additional 7 days. The expression of neuronal markers β-III-tubulin, neurofilament, synaptophysin, NeuN, GFAP and S100 was evaluated. No differences were seen in the expression pattern of β-III-tubulin, neurofilament and S100 after differentiation (data not shown). After staining of differentiated hDPSC with anti-synaptophysin, a subpopulation of the cells showed a polar distribution of vesicles between neighbouring cells (Figure 3.3 A-B). In addition, differentiated cells showed a positive immune-reactivity for NeuN and GFAP (Figure 3.3 C-D).

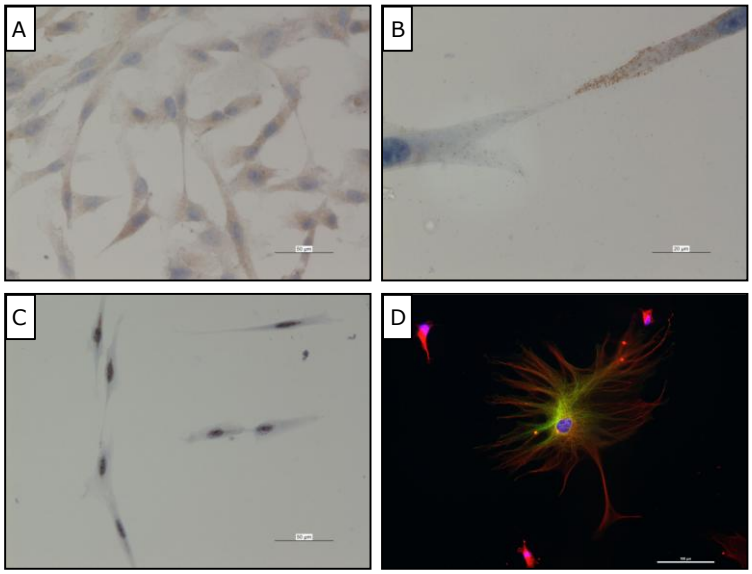


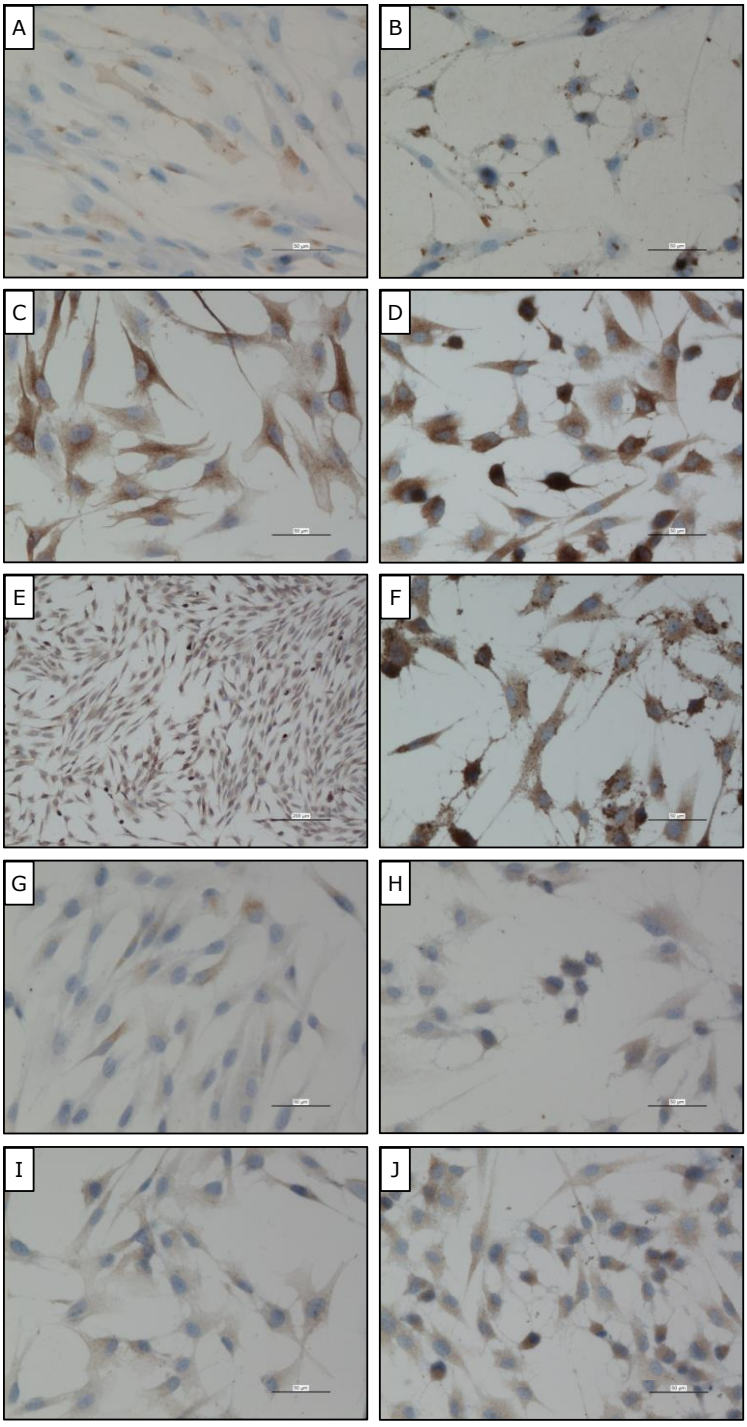
Figure 3.3: Immunocytochemical analysis of marker expression after neural differentiation (Neurosphere assay). A polar distribution of synaptophysin expression was observed after differentiation (A-B). Cells stained also positive for NeuN (C) and GFAP (D, red). Scale bar A,C = 50µm; Scale bar B = 20µm; Scale bar D = 100µm

Neural inductive media assay

After 10-14 days in culture with neuronal induction medium, the expression of Stro-1, β-III-tubulin, GalC, neurofilament and synaptophysin in both control and differentiated cells was evaluated. Control and differentiated hDPSC expressed the neuronal markers Stro-1, β-III-tubulin, GalC, neurofilament and synaptophysin. Compared with control hDPSC, cells were less positive for Stro-1 and more positive for neurofilament. However, hDPSC did not express NeuN and the glial markers MBP and GFAP after neurogenic differentiation (data not shown).

Figure 3.4: Immunocytochemical analysis of marker expression after neural differentiation (Neural inductive media assay). Control hDPSC: A,C,E,G,I; neural differentiated hDPSC: B,D,F,H,J.

A-B. Stro-1; C-D: β-III-tubulin; E-F: GalC; G-H: neurofilament; I-J: synaptophysin. Scale bar A-D, F-J = 50µm; Scale bar E = 200µm


A
B
C
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I
J

3.4.3 Ultrastructural analysis of hDPSC after neural differentiation

An ultrastructural analysis was performed to evaluate the morphological and structural changes upon neural differentiation.

Neurosphere assay

Neurospheres cultured for 7 days were plated onto pre-coated coverslips and kept in culture for an additional 7 days. Cells grown out of neurospheres, were fixed with 2% glutaraldehyde in 0.05M cacodylate buffer and further processed for electron microscopical analysis. Cells obtained an elongated bipolar cell morphology with an euchromatic nucleus and several cytoplasmatic organelles like mitochondria, rough endoplasmatic reticulum and Golgi apparatus (Figure 3.5 A-B). Compared to control hDPSC, numerous cell-cell contacts (Figure 3.5 C asterisk) and invaginations of the cell membrane, indicating the process of pinocytosis and/or exocytosis, were observed (Figure 3.5 D-E arrows). In addition, many vesicular bodies (Figure 3.5 F asterisk) were present within the cell cytoplasm and densification of the cell membrane, possible early synaptic membrane formation was observed (Figure 3.5 F arrow). Also dense core vesicles were present in the intercellular space (Figure 3.5 arrowhead).

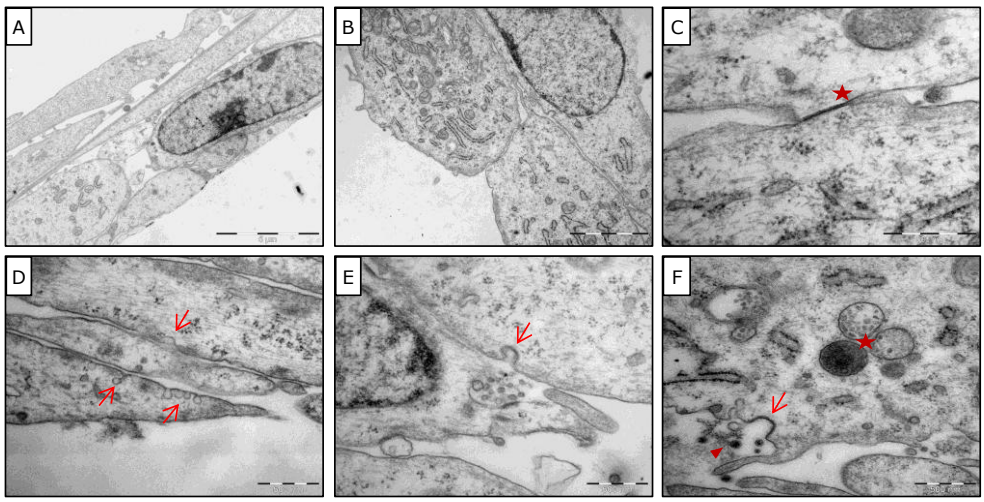


Figure 3.5: Transmission electron microscopical images of cells after neural differentiation via neurosphere assay. Cells grown out of plated neurospheres obtained a bipolar morphology with euchromatic nuclei (A-B). Numerous cell-cell contacts (asterisk C) and invaginations of the cell membrane (arrows D-E) were observed. Large granular vesicles (asterisk F) in the cell cytoplasm and small dense core vesicles (arrowhead F) within the intercellular space were present. Also, densification of the cell membrane was often visible. Scale bar A = 5µm; Scale bar B = 2µm; Scale bar C-F = 500nm

Neural inductive media assay

After 14 days in culture, cells grown on coverslips in neuronal induction medium, were processed for electron microscopical analysis. Differentiated hDPSC obtained a more bipolar cell morphology compared to control hDPSC. Cells had an oval euchromatic nucleus and numerous cell organelles were present within the cytoplasm, indicating a high metabolic cell activity (Figure 3.6 A-B). Furthermore, cell-cell contacts (Figure 3.6 C arrow) were frequently characterized by the alignment of small vesicles to the cell membrane (Figure 3.6 D arrows). At these sites, the vesicular content was exocytosed into the intercellular space. Sometimes these areas were flanked by synapse-like structures which were characterized by densification of the cellular membrane (Figure 3.6 E-F asterisk).

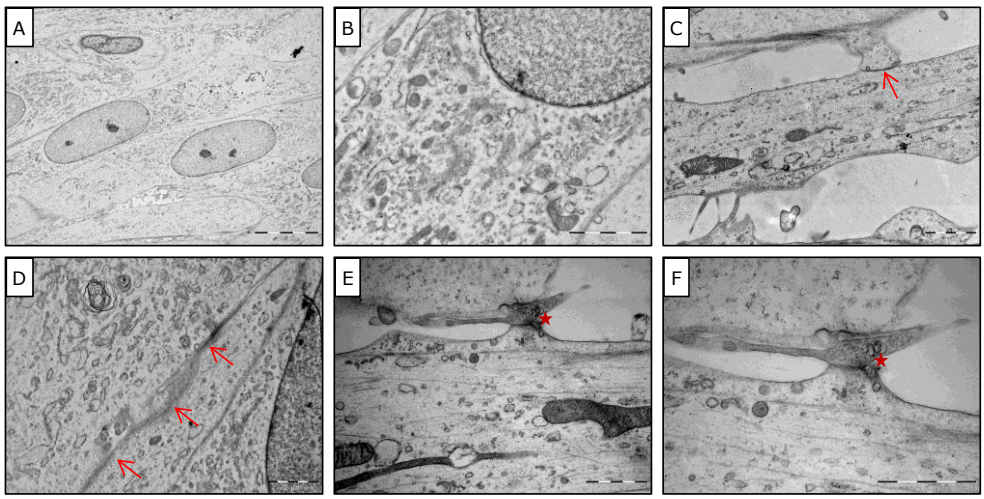


Figure 3.6: Transmission electron microscopical images of cells after neural differentiation via neural inductive media assay. Bipolar cells displayed an euchromatic nucleus containing numerous cell organelles (A-B). Also, cell-cell contacts aligned with small vesicles next to the cell membrane were often seen (C-D arrows). Occasionally, synapse-like structures were present (E-F). Scale bar A = 10µm; Scale bar B-D = 2µm; Scale bar E-F = 1µm

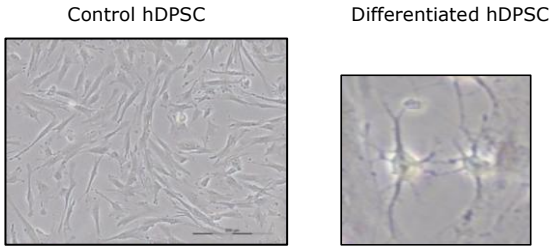
3.4.4 Electrophysiological measurements

To confirm that adult hDPSC were differentiating towards functionally active neurons, the presence of voltage-dependent sodium and potassium channels and their responsiveness to neurotransmitters glutamate and GABA, was evaluated by means of whole-cell patch-clamp recordings. Both control hDPSC and cells displaying a neuron-like morphology after 10-14 days of culture in neuronal inductive medium, were addressed for electrophysiological measurements (Figure 3.7 A). Only cells differentiated via the neural inductive media assay were analyzed.

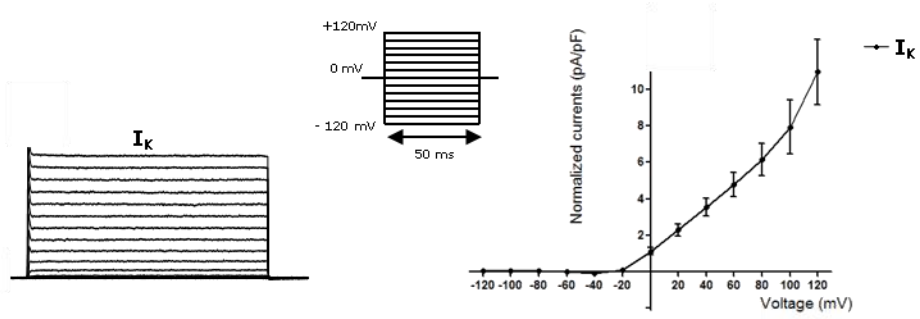
Whole-cell current traces of control hDPSC showed an outward voltage-gated potassium current (Figure 3.7 B) but no inward voltage-gated sodium current. On the other hand, whole-cell current traces of a differentiated cell showed an inward voltage-activated sodium current, which inactivated very rapidly,

followed by an outward voltage-activated potassium current. An I-V curve demonstrating Na^+ and K^+ currents, was generated by following protocol: Starting from a holding potential (VH) of -60 mV, voltage steps of 20 mV were applied and the currents were measured in intervals, ranging from -120 mV to +120 mV (Figure B-C). Voltage dependent K^+ currents of control hDPSC were shown to be sensitive for TEA (Figure 3.7 D). However, in differentiated cells, voltage-dependent Na^+ and K^+ currents were activated at -18.4 mV ±13.4 mV, and at +26.4 mV ±2.6 mV respectively (Figure 3.7 C). In addition, voltage-dependent Na^+ currents were shown to be sensitive for tetrodotoxin (TTX) and K^+ currents were demonstrated to be sensitive to tetraethyl ammonium (TEA) (Figure 3.7E). Application of 100 µM glutamate and 100 µM GABA onto the cell surface of differentiated hDPSC, voltage clamped at -60mV, a transient inward current that slowly desensitized was evoked (Figure 3.7 F).

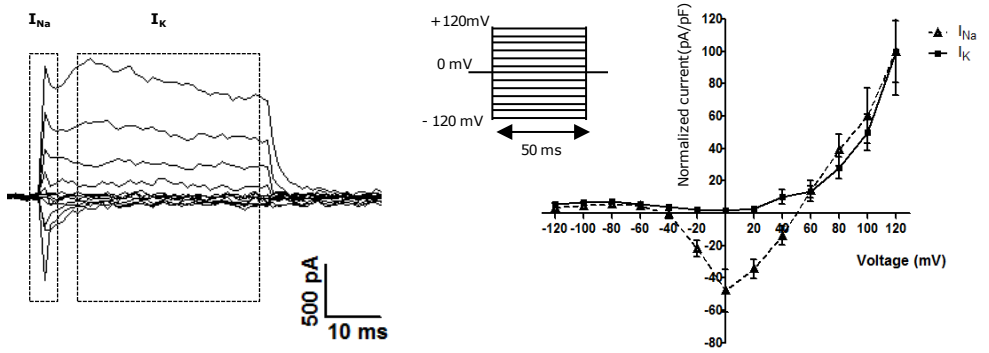
A.



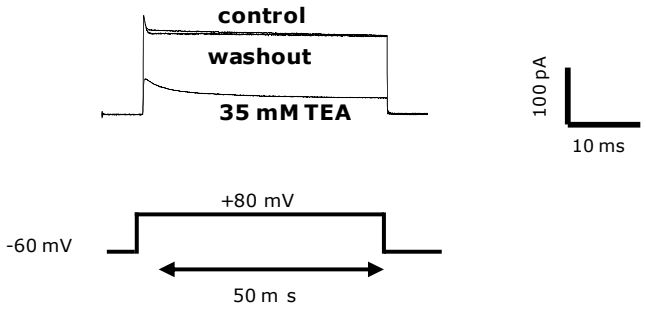
B.



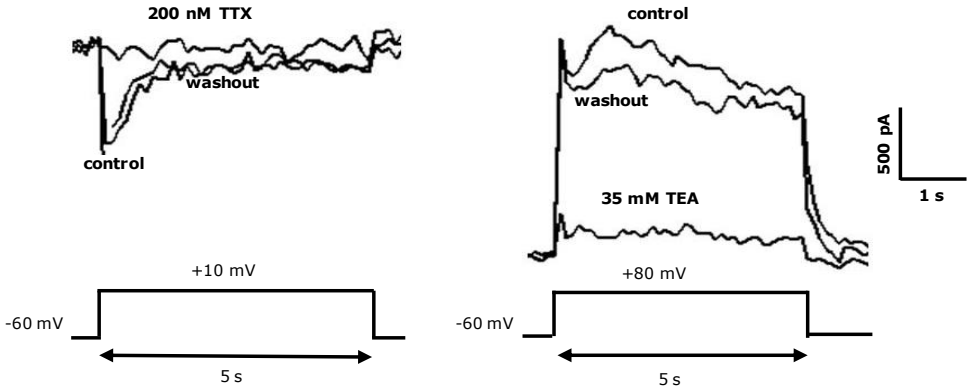
C.


I_{Na}
I_K
500 pA
10 ms
+120mV
0 mV
- 120 mV
50 ms
Normalized current (pA/pF)
Voltage (mV)
I_{Na}
I_K

D.


control
washout
35 mM TEA
100 pA
10 ms
+80 mV
-60 mV
50 m s

E.


200 nM TTX
washout
control
+10 mV
-60 mV
5 s
control
washout
35 mM TEA
500 pA
1 s
+80 mV
-60 mV
5 s

F.

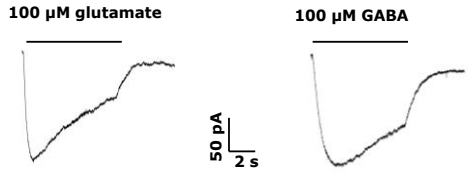


Figure 3.7: Whole cell patch clamp recordings on differentiated hDPSC via neural inductive media assay. Control and differentiated hDPSC with neuron-like cell morphology that underwent electrophysiological recordings (A). Voltage-activated potassium currents of control hDPSC (n = 6) (B). Voltage-activated currents of differentiated hDPSC (n = 10) (C). Starting from a holding potential (V_H) of -60 mV, voltage steps of 20 mV were applied and the currents were measured in intervals, ranging from -120 mV to +120 mV. The IV curve demonstrates voltage-dependent Na^+ and K^+ currents, activated at -18,4 mV for the Na^+ currents, and at +26,4 mV for the K^+ currents (C). Control hDPSC showed TEA sensitive K^+ currents (n=5) (D). Typical whole-cell patch-clamp recording of differentiated hDPSC. Traces represent the current elicited by a 5s voltage step to +10 mV (TTX) or +80 (TEA) from a holding potential of -60 mV and recorded in standard extracellular solution (control), in the presence of 200nM TTX or 35mM TEA, and in the standard extracellular solution after washout of the drug. The effects of TTX and TEA were measures in respectively 3 and 4 cells (E). Representative traces of responses to GABA (n=5) and glutamate (n=3) in differentiated hDPSC at (V_H) of -60 mV (F).

3.5 Discussion and Conclusion

Stem cell-based therapies are promising approaches for the treatment of several neurological disorders such as Alzheimer's and Parkinson's disease. In order to elucidate these therapies, the neural differentiation potential and the production and secretion of neurotrophic factors by stem cells must first be investigated *in vitro*. The neural differentiation potential of MSC has been widely investigated and yield great potentials. DPSC are much alike MSC and their multilineage differentiation potential and easy accessibility make them good candidates for cellular-based therapies.

In this study, human DPSC were evaluated regarding their neuronal differentiation potential. Neuronal differentiation was induced by means of two different protocols. The **neurosphere assay** resulted in the aggregation of hDPSC after 24h in culture forming sphere-like structures. Neurospheres continued to grow, forming larger spheres under influence from growth factors b-FGF and EGF. Both b-FGF and EGF have been known to be necessary in the formation of primary neurospheres derived from neural stem cells. Neural stem cells grown in media containing EGF alone did not efficiently generated neurospheres. When culturing secondary or tertiary neurospheres or inducing neuronal differentiation, the use of only b-FGF is reported to be sufficient [134-136]. On the other hand, the group of Sasaki *et al.* showed that neurosphere formation of DPSC was only dependent on b-FGF and not on EGF [83], suggesting that, dependent on the stem cell type, a combination of EGF and b-FGF or b-FGF alone is sufficient for neurosphere generation.

Several studies state that plating neurospheres on adhesive substrates, promotes a phenotypic neural differentiation of the cells [137-139]. Therefore, neurospheres were plated onto poly-L-ornithine and fibronectin pre-coated coverslips to investigate the neural marker expression upon neural differentiation after an additional culture period of 7 days in neural inductive medium. Although no differences were observed in the expression of β-III-tubulin, neurofilament and S100 after differentiation, a subpopulation of the differentiated cells showed a polar distribution of synaptophysin (Figure 3.3B).

The expression of synaptophysin, a glycoprotein characterizing neuronal synaptic vesicles in the cytoplasm or at synapses or cell junctions, can be found in neurons participating in synapse formation [140]. The NeuN positive immune-reactivity in part of the cell population is a further indication of cells differentiating into neuron-like cells. At the ultrastructural level, the presence of numerous cell-junctions, small and large granular vesicles containing neurotransmitters intra- and intercellular, and invaginations of the cell membrane indicating endo-/exocytosis of vesicles clearly confirms the differentiation towards neuron-like cells. However, the functionality of these cells has to be further investigated by means of whole cell patch-clamp recordings in order to confirm that cells are being fully differentiated into functionally active neurons. Also the content of the endo/exocytosis vesicles should be explored by means of immune-EM (e.g. with antibodies against dopamine).

The second approach to induce neuronal differentiation includes the change of standard culture medium into **neural inductive medium** containing b-FGF and EGF. Cells were cultured in this medium for an additional 10-14 days before evaluation. *In vitro*, part of the cell population acquired a neuronal-like cell morphology with multiple processes in response to growth factors EGF and b-FGF present in the neural inductive medium. Other cells preserved their flat morphology serving as an anchor for neuronal differentiated cells. Safford and co-workers demonstrated that pre-treatment of human adipose derived adult stem cells with EGF and b-FGF resulted in an improved neuronal phenotypic change with an increased expression of neuronal markers NeuN and intermediate filament M following 24h of neuronal induction [141]. Furthermore, it has been reported that mouse MSC differentiated toward neuron-like cells when treated with neuronal inductive media supplemented with b-FGF [142]. Media supplemented with EGF and b-FGF are used in several studies to commit stem cells to a neuronal fate [32, 55, 143]. However, this pre-treatment procedure of cell cultures needs to be followed by the addition of media supplemented with other growth factors (NGF, NT-3, cAMP, forskolin, isobutylmethylxanthine) in order to induce further neuronal differentiation and maturation [47, 48, 144].

Cells undergoing neuronal differentiation by means of the second differentiation method, did not express the neuronal marker NeuN. However, the increased expression of neurofilament and decreased expression of Stro-1, indicates that cells were differentiating towards neuronal-like cells or neural progenitor cells but further maturation steps are necessary to differentiate into fully matured neurons. Ultrastructural analysis revealed that differentiated hDPSC adopted a bipolar elongated cell morphology. Numerous cell junctions and small vesicles were frequently observed and in a few cells a densification of the cell membrane was seen, indicating a pre-synaptic membrane formation. However, no vesicles containing neurotransmitter or 'real' synapse formation were observed confirming the immature differentiation of hDPSC after the addition of neuronal inductive media for 10-14 days.

Besides morphological, immunocytochemical and ultrastructural evaluations of neuronal differentiated cells, electrophysiological characteristics of neurons such as depolarization in response to changes in membrane permeability to potassium and sodium and the induction of action potentials must be investigated. Therefore, electrophysiological measurements were performed on neuronal differentiated hDPSC to investigate the presence of voltage-gated Na^+ and K^+ channels and their responsiveness to neurotransmitters glutamate and GABA. Differentiated hDPSC were shown to demonstrate TTX sensitive inward voltage-gated sodium currents and TEA sensitive outward delayed rectifier potassium currents in accordance to the study of Király *et al.* in 2009 [48]. However, in control hDPSC, only voltage-gated potassium channels were present which were sensitive for TEA. In a previous study of Arthur *et al.* in 2008, no voltage-dependent potassium currents could be detected on neuronal differentiated hDPSC displaying voltage-dependent sodium currents [55]. Compared to our study and the study of Király *et al.,* a different neuronal induction protocol was performed, which might have resulted in a different stage of neuronal differentiation. When embryonic-derived neural stem cells were exposed to neuronal inductive media for only a few days, these cells already expressed voltage sensitive sodium currents. Moreover, voltage-gated delayed rectifier potassium currents and voltage-gated inward sodium currents have been shown to be present in neural progenitors [145-147]. Voltage dependent

sodium channels have been used as an electrophysiological marker to evaluate the stage of neuronal maturation in differentiating embryonic derived neural stem cells in generating fully functional neurons. Biella *et al.* were able to demonstrate a correlation between excitability and the sodium channel system during neuronal differentiation. Changes in the sodium channel system were associated with the ability of differentiating neural stem cells to generate action potentials [145]. It is known that voltage-dependent sodium and potassium channels are required for the generation and propagation of action potentials. Bone marrow derived MSC are also capable of undergoing neuronal differentiation. In the study of Wislet-Gendebien *et al.,* neuronal differentiated cells displayed sodium and potassium channels, were able to fire a single action potential and were responsive to neurotransmitters GABA and glutamate. Neuronal differentiated MSC in this study acquired several but not all electrophysiological characteristics of neuronal cells as trains of action potentials or synaptic activities after differentiation were never observed [84]. The group of Király *et al.*, did not evaluate the capacity of differentiated hDPSC to fire an action potential [48]. In the present study, differentiated hDPSC were responsive to neurotransmitters GABA and glutamate but were not able to fire a single action potential although voltage-dependent sodium and potassium channels were present. These results indicate that these differentiated hDPSC are undergoing neuronal differentiation but are still in an immature stadium of neural precursor cells.

We can conclude that the two different approaches to induce neuronal differentiation in hDPSC yield different results (see table 3.2). By using the neurosphere assay, differentiated cells expressing the mature neuronal marker NeuN were observed in the cell cultures. Furthermore, we were the first to prove at the ultrastructural level that these cells possessed neuronal characteristics such as numerous cell-cell contacts and the presence of neurotransmitter containing vesicles. To our knowledge, no ultrastructural analysis has been previously performed on neuronally differentiated DPSC. This technique can be an additional tool to determine the success of differentiation. However, the functionality of these differentiated hDPSC must be addressed by means of whole cell patch clamp recording before they can be addressed as fully

differentiated into functionally active neurons. In the second approach, differentiated cells were still in a neural progenitor phase indicating that longer culture periods and the addition of other growth factors such as cAMP and NT-3 is necessary for maturation of these cells. In conclusion, these results indicate that hDPSC can be differentiated into fully functional active neurons making them ideal candidates for studies in stem cell-based therapies for central or peripheral nervous diseases or injuries.

Table 3.2: Comparison of the results of different neuronal differentiation protocols

	Control	Neurosphere	NIM
Cell morphology	Fibroblast-like cells	Bipolar cells	Neuron-like cells: central cell body with multiple processes
Cell Markers			
β-III-tubulin	+++	+++	+++
GalC	+++	N.D.	+++
GFAP	-	+	-
MBP	-	-	-
NeuN	-	++	-
Neurofilament	+	+	++
S100	+++	+++	+++
Synaptophysin	+++	+++ (subpopulation gained polar distribution of vesicles)	+++
Stro-1	+/-	N.D.	+/--
Ultrastructural characteristics	Fibroblast-like cells, perinuclear organelle rich zone, no cell-cell contacts	Elongated bipolar cell morphology, organelles spread throughout cytoplasm, cell-cell contacts, invaginations of cell membrane, early synaptic membrane formation, dense core vesicles	Bipolar cell morphology, organelles spread throughout cytoplasm, cell-cell contacts, alignment of vesicles at cell membrane, densifications of cellular membrane
Electrophysiology	Outward voltage-gated K current	N.D.	Outward voltage-gated K current sensitive for TEA, inward voltage-gated Na current sensitive for TTX, sensitive for glutamate and GABA

NIM: neural inductive media; N.D.: not determined; +++: >80% ; ++: 50-80% ; + : <10-50%; +/-: <10%; +/--: <5%

4

Human dental pulp stem cells differentiate towards peripheral glial cells

4.1 Abstract

Surgical procedures, traumas or accidents can often result in peripheral nerve injury. Numerous techniques have been developed during the last several years, as treatment for peripheral nerve injury, but the results are far from the desired outcome. Recently, several groups have demonstrated the possible transdifferentiation of mesenchymal stem cells into Schwann-like cells having a positive effect on neurons and neurite outgrowth. In the present study, human DPSC are induced to undergo peripheral glial cell differentiation by means of growth media supplemented with different (growth) factors. Immunocytochemical analysis demonstrated the expression of glial cell marker GFAP and a decreased expression of nestin and Stro-1 after differentiation. Furthermore, hDPSC and differentiated hDPSC (SC-hDPSC) produced and secreted several (neuro)trophic factors which promoted the survival and neurite outgrowth in dorsal root ganglia cultures, with SC-hDPSC yielding a significantly better effect than naïve hDPSC. Although the capacity of hDPSC and SC-hDPSC to myelinate axons *in vitro* and *in vivo* has to be further elucidated, our results showed that hDPSC are able to undergo glial differentiation, proposing them to be good candidates for cell-based therapies as treatment for peripheral nerve injury.

4.2 Introduction

Numerous surgical procedures have been developed during the last several years as treatments for peripheral nerve injury. These techniques include anastomosis of the nerve ends with nerve or vein grafts but the overall clinical outcomes showed limited results. Therefore, other strategies have to be considered and further explored in order to develop new therapies as treatment for peripheral nerve injury.

Recent evidence propose that cell-based therapies might be a novel strategy to repair peripheral nerve injuries. The most obvious cells to use are Schwann cells. Schwann cells are the main glial cells in the peripheral nervous system as they are responsible for the formation and maintenance of the myelin sheath around axons. Furthermore, they are essential in peripheral nerve regeneration after nerve injuries by producing extracellular matrix molecules, integrins and (neuro)trophic factors providing guidance and trophic support for regenerating axons [148]. Recent *in vivo* studies already report the injection of Schwann cells as treatment for peripheral nerve injuries. However, the major drawback with the use of Schwann cells, is the loss and sacrifice of another peripheral nerve for harvesting Schwann cells. In addition, Schwann cells have a slow and time consuming growing capacity *in vitro*, leading to the need of alternative cell sources [96, 97].

A new and very promising strategy to improve peripheral nerve regeneration is the use of adult stem cells like bone marrow mesenchymal stem cells, adipose derived stem cells and skin neural crest-like precursor cells [97]. MSC have been shown to improve peripheral nerve regeneration by either secreting neurotrophic factors inducing axonal outgrowth *in vitro* and *in vivo*, or by differentiation into neurons and/or Schwann cells. Several *in vitro* studies report the transdifferentiation of mesenchymal stem cells into Schwann-like cells expression glial markers GFAP, S100 and NGFRp75 after differentiation. Transdifferentiation of the cells was induced by means of media containing several growth factors or by performing direct co-culture experiments with dorsal root ganglia [98, 99, 102, 149].

Another promising type of adult stem cells that recently has been discovered are the DPSC residing in dental pulp tissue [31]. DPSC can be easily isolated from extracted human third molars without ethical concerns. Furthermore, no tumorigenic effects have been reported by using DPSC in *in vivo* studies until now. As described in chapter 2, DPSC are multipotent stem cells capable of differentiating into cells of mesodermal lineages and expressing already neural markers like S100, β-III-tubulin and NGFRp75 in an undifferentiated state. Several *in vitro* studies also report the production and secretion of neurotrophic factors (BDNF, GDNF, NGF) by DPSC enhancing and guiding axonal outgrowth [56, 58].

In this chapter, the focus was on the transdifferentiation of human DPSC (hDPSC) towards a peripheral glial cell type by using culture media containing different stimulating (growth) factors. Not only the marker expression was evaluated via immunocytochemistry, but also an ultrastructural analysis was conducted. In addition, the secretion of neurotrophic factors in the media by naïve and differentiated hDPSC was investigated together with their influence on the survival and neurite outgrowth of dorsal root ganglia in *in vitro* cultures.

4.3 Materials & methods

4.3.1 Isolation and differentiation of human dental pulp stem cells into Schwann-like cells

Human dental pulp stem cells (hDPSC) were isolated and cultured as previously described in chapter 2 (2.3.1). At passage 2, Schwann cell differentiation [100, 101] was induced by replacing standard culture medium with aMEM containing 1mM beta-mercaptoethanol (BME) (Sigma-Aldrich, Bornem, Belgium) for 24h. Subsequently, cells were incubated with aMEM medium containing 10% FCS and 35ng/ml *all trans*-retinoic acid (RA) (Sigma-Aldrich, Bornem, Belgium). After 72h, medium was changed with growth medium supplemented with 5µM forskolin (Sigma-Aldrich, Bornem, Belgium), 10ng/ml basic fibroblast growth factor (b-FGF), 5ng/ml platelet derived growth factor AA (PDGFaa) and 200ng/ml Heregulin-β-1 (NRG) (Immunotools, Friesoythe, Germany). The cells were cultured in this supplemented medium for 2 weeks with medium changes every 2-3 days. hDPSC differentiated towards Schwann-like cells are further referred to as SC-hDPSC.

4.3.2 Human dental pulp stem cell conditioned medium

hDPSC were seeded at a density of 20,000 cells/cm² in standard hDPSC culture medium (see 2.3.1 of chapter 2). After 24h in culture, hDPSC were rinsed with PBS and incubated with standard culture medium supplemented with 0.1% FCS instead of 10% FCS. After 48h of incubation, the medium was collected and stored at -80°C. Furthermore, conditioned medium (CM) from Schwann-like differentiated cells was collected and stored at -80°C.

4.3.3 RNA extraction – cDNA synthesis - Reverse Transcriptase PCR

Total RNA was isolated from hDPSC, reverse transcribed into cDNA and RT-PCR reactions were performed as previously described in chapter 2 (2.3.4). Reverse Transcriptase PCR (RT-PCR) was performed by means of Taq DNA Polymerase (1U/µl) (Roche Diagnostics, Vilvoorde, Belgium). Primer sequences of pluripotency and neural crest genes can be found in table 4.2.
Ntera cells, which are pluripotent human testicular embryonal carcinoma cells, were used as a positive control for pluripotency markers.

4.3.4 Immunocytochemistry

To determine the expression of glial markers (table 4.1) of naïve and differentiated hDPSC, cells were seeded onto glass coverslips (5,000cells/cm²) and stained with the peroxidase-based EnVision System® (DakoCytomation, Glostrup, Denmark) as previously described in chapter 2 (2.3.2). The immune-reactivity of the cells was observed using a photomicroscope equipped with an automated camera (Nikon Eclipse 80i, Nikon Co., Japan).
A double staining for anti-GFAP and anti-S100 was performed in control and SC-hDPSC. Donkey-anti mouse Alexa555 (1:500) and donkey anti-rabbit Alexa488 (1:500) were used as secondary antibodies (Molecular probes). Slides were mounted using DAPI with Prolong Gold Antifade (Invitrogen/Molecular probes, Merelbeke, Belgium). Immune-reactivity was visualized using a fluorescence microscope (Nikon Eclipse 80i, Kingston, England).

Table 4.1: Primary antibodies for immunocytochemistry

	Species	**Dilution**	**Company**
Neural markers			
GFAP	Mouse	1:200	NovoCastra
NGFRp75	Mouse	1:50	DakoCytomation
S100	Rabbit	1:400	DakoCytomation
Other markers			
Nestin	Mouse	1:500	Chemicon
Stro-1	Mouse	1:50	R&D systems

Table 4.2: Primer sequences for Reverse Transcription PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Tm (°C)	Product length (bp)
Pluripotent markers				
Nanog	GAT-TTG-TGG-GCC-TGA-AGA-AA	AAG-TGG-GTT-GTT-TGC-CTT-TG	58	155
OCT4	AGC-CCT-CAT-TTC-ACC-AGG-CC	TGG-GAC-TCC-TCC-GGG-TTT-TG	56	456
Rex1	TGA-AAG-CCC-ACA-TCC-TAA-CG	CAA-GCT-ATC-CTC-CTG-CTT-TGG	56	554
SOX2	AGA-AGC-GGC-CGT-TCA-TCG-AC	TGC-TGA-TCA-TGT-CCC-GGA-GGT	56	570
Neural crest markers				
TF-AP2	TCC-CTG-TCC-AAG-TCC-AAC-AGC-AAT	AAA-TTC-GGT-TTC-GCA-CAC-GTA-CCC	50	396
ErbB3	GGT-GCT-GGG-CTT-GCT-TTT	CGT-GGC-TGG-AGT-TGG-TGT-TA	57	365
HNK-1	GTG-AGT-GCT-GGT-AAT-GAG-GAG-CCG	GGT-GTT-GTC-GTC-GTC-GGC-GA	60	642
NGFRp75	AGC-CAA-CCA-GAC-CGT-GTG-TG	TTG-CAG-CTG-TTC-CAC-CTC-TT	54,7	663
SOX 9	AGA-AGG-ACC-ACC-CGG-ATT-AC	CGG-CAG-GTA-CTG-GTC-AAA-CT	50	401
Housekeeping genes				
β actin	AAA-TCT-GGC-ACC-ACA-CCT-TC	AGA-GGC-GTA-CAG-GGA-TAG-CA	56	185
β2 microglobulin	CTC-ACG-TCA-TCC-AGC-AGA-GA	CGG-CAG-GCA-TAC-TCA-TCT-TT	56	213
Gus B	AGC-CAG-TTC-CTC-ATC-AAT-GG	GGT-AGT-GGC-TGG-TAC-GGA-AA	56	160

4.3.5 Ultrastructural analysis

Following fixation with 2% glutaraldehyde in a 0.05M cacodylate buffer (pH 7.3) at 4°C, the fixative was gently aspirated with a glass pipette and the cells were post-fixed in 2% osmium tetroxide for 1 hour and stained with 2% uranyl acetate in 10% acetone for 20 min. Subsequently, the cell-seeded coverslips were put trough dehydrating series of graded concentrations of acetone and embedded in araldite according to the pop-off method [133]. Ultra-thin sections (0.06µm) were mounted on 0.7% formvar-coated grids, contrasted with uranyl acetate and lead citrate, and examined in a Philips EM 208 transmission electron microscope operated at 80 kV.

4.3.6 Human Cytokine Antibody Array

A human cytokine antibody array was used to determine the secretion of cytokines and growth factors by hDPSC and SC-hDPSC (see table 4.3) according to manufacturer's protocol. Briefly, membranes were blocked using 1X blocking buffer for 1h at room temperature followed by overnight incubation at 4°C with media supplemented with 0.1% FCS or conditioned medium from hDPSC or SC-hDPSC. The following day, biotin-conjugated anti-cytokines solution was used as primary antibody and membranes were incubated for 2 hours at room temperature. Incubation with HRP-conjugated streptavidin for 2 hours was followed by the detection of signals by incubation with detection reagents and exposure to x-ray film.

Table 4.3: Cytokines and growth factors on human cytokine antibody array

Pos	Pos	Neg	Neg	BDNF	b-NGF	CNTF	EGF-R
Pos	Pos	Neg	Neg	BDNF	b-NGF	CNTF	EGF-R
ErbB2	ErbB3	FGF-9	GDNF	NCAM-1	NGF R	NT-3	NT-4
ErbB2	ErbB3	FGF-9	GDNF	NCAM-1	NGF R	NT-3	NT-4
PDGF R α	PDGF R β	SCF	SCF R	TGF α	TGF β1	TGF β2	TGF β3
PDGF R α	PDGF R β	SCF	SCF R	TGF α	TGF β1	TGF β2	TGF β3
Blank	Blank	Blank	Blank	Blank	Blank	Neg	Pos
Blank	Blank	Blank	Blank	Blank	Blank	Neg	Pos

Pos: positive control; Neg: negative control

4.3.7 Enzyme-linked Immunosorbent Assay

Enzyme-linked Immunosorbent Assays (ELISA's) were performed on hDPSC and SC-hDPSC conditioned medium (48h) in order to determine the concentration of BDNF and FGF-9 (RayBiotech, Inc., Boechout, Belgium). Experiments were performed in triplicate and absorbance was measured at 450nm by means of the FLUOstar Optima multifunctional microplate reader (BMG Labtech, Germany). Conditioned medium of 9 different patients was used and ELISA's were performed according to manufacturer's protocol.

4.3.8 Cell Proliferation assay

hDPSC and SC-hDPSC were seeded in a 96-well plate at a density of 5,000 cells/well for the detection of cells proliferation. After 24h, 48h and 72h a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. The cells were rinsed with PBS and incubated for 4 hours with culture media supplemented with MTT. After incubation, the MTT solution was removed and a DMSO-glycine solution was added in order to allow the reduction to formazan. The absorbance was measured at a wavelength of 540-550nm with a Benckmark microplate reader (Bio-rad Laboratories, Nazareth Eke, Belgium).

4.3.9 Dorsal root ganglia cell cultures

Dorsal root ganglia (DRG) were harvested from spinal cords of 5 days old Sprague–Dawley rat pups. Briefly, isolated DRG were dissociated with 0.025% collagenase at 37°C for 1h. Dissociated cells were plated at coverslips at a density of 25,000 cells/cm^2. Coverslips were pre-coated with poly-L-lysine (10µg/ml Sigma-Aldrich, Bornem, Belgium) for 1h. 1-2 hours after cell plating, medium was changed to remove non-adhering cells. Neurons were cultured for 24h in DMEM/F12 medium supplemented with Glutamax, 10% FCS and 100 U/ml Penicillin and 100 µg/ml Streptomycin.

4.3.10 Survival assay

Isolated DRG's were seeded in a 96-well plate at a density of 10,000 cells/well. After 24h in culture, conditioned medium from hDPSC and SC-hDPSC was added to the cells. After an incubation period of 48h, a MTT assay was performed as described in (4.3.8). Conditioned medium from 7 different patients was used.

4.3.11 Functional assay

DRG's, cultured for 24h, were incubated with conditioned medium of hDPSC and SC-hDPSC to analyze the neurite outgrowth of DRG's. After 48h, cells were fixed in 4% PFA and immunostained for anti-β-III-tubulin. Four independent experiments were carried out and neurite outgrowth was assessed by measuring the length of the longest neurite.

4.3.12 Statistical analysis

Statistical analysis was performed using Graphpad Prism 5 software (Graphpad, California, USA). Data from the survival assay and neurite outgrowth were first controlled for normality by means of a D'Agostino & Pearson omnibus normality test followed by making comparisons between control and experimental groups by means of a Kruskal-Wallis test, while applying a Dunn's multiple comparison post-hoc test. Data from ELISA were submitted to a D'Agostino & Pearson omnibus normality test, followed by an unpaired t-test. Data from the proliferation assays were compared by means of a two-way ANOVA followed by Bonferroni's multiple comparison test. P-values ≤ 0.05 were considered statistically significant (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). All data were expressed as mean ± Standard Error of the Mean (SEM).

4.4 Results

4.4.1 Morphology of differentiated hDPSC *in vitro*

Schwann cell differentiation was induced in hDPSC at passage 2. *In vitro*, undifferentiated hDPSC displayed a flattened fibroblast-like morphology (Figure 4.1 A). After 24h on differentiation medium containing 1mM BME, changes in cell morphology were observed in which cytoskeletal changes induced an elongated cell shape. Proceeding the induction protocol, cells acquired a bipolar morphology after 7-14 days due to the addition of different growth factors (PDGFaa, b-FGF, NRG) to the differentiation medium (Figure 4.1 B-C).

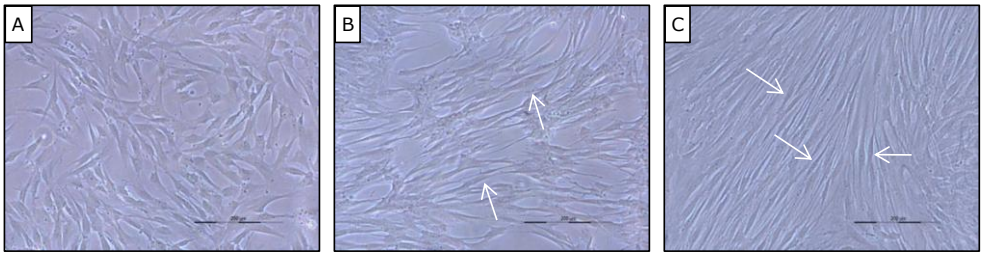
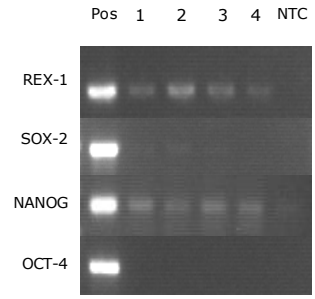


Figure 4.1: Phase contrast images of the morphological changes during differentiation. hDPSC displayed the fibroblast-like morphology (A). After starting the induction protocol, cell morphology changed to spindle-shaped bipolar cells (B-C). Scale bar = 200µm

4.4.2 Expression of pluripotency and neural crest markers in undifferentiated hDPSC

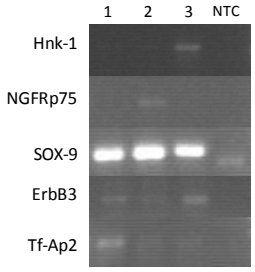
By means of RT-PCR, we investigated the expression of markers characteristic for pluripotency or for neural crest derived cells on the mRNA level in naïve hDPSC at passage 1. Ntera cells were used as a positive control to evaluate the expression of pluripotency markers. RT-PCR detected mRNA levels of REX-1, Sox-2, Nanog and OCT-4 in respectively 73%, 27%, 91% and 9% of the patients tested. Regarding to the transcript levels of neural crest markers, Hnk-1, NGFRp75, SOX-9, ErbB3 and Tf-AP2 expression was found in respectively 17%, 33%, 100%, 100% and 67% of the patients tested (Figure 4.2).

A.



	% of patients
Rex -1	73%
Sox-2	27%
Nanog	91%
Oct-4	9%

B.



	% of patients
Hnk-1	17%
NGFRp75	33%
SOX-9	100%
ErbB3	100%
Tf-Ap2	67%

Figure 4.2: Expression of pluripotency (A) and neural crest markers (B) in undifferentiated hDPSC on mRNA level. RT-PCR analysis of total RNA extracted from hDPSC. Ntera cells were used as a positive control for evaluating pluripotency markers. (NTC = no template control)

4.4.3 Immunophenotype of differentiated hDPSC

To evaluate the glial marker expression of SC-hDPSC, an immunocytochemical analysis was performed for GFAP, S100, nestin, NGFRp75 and Stro-1 in both hDPSC and SC-hDPSC. Differentiated cells showed a positive immune-reaction for GFAP and S100 (Figure 4.3 A-C). The level of co-expression of both markers differed between patients. In some cultures, cells co-expressed uniformly both GFAP and S100, in other cultures only a subpopulation of the cells expressed both makers (Figure 4.3 D-F). Furthermore, both nestin and Stro-1 expression was decreased in differentiated cell cultures meaning, less positively stained cells could be detected after differentiation (Figure 4.4). Concerning the expression of NGFRp75, no differences were observed before and after inducing differentiation into peripheral glial cells (data not shown).

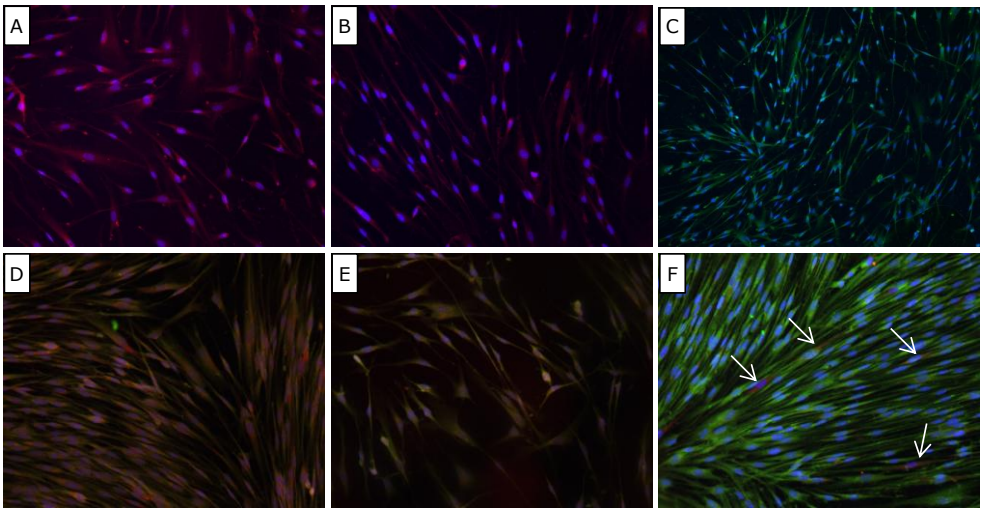


Figure 4.3: Immunofluorescence double staining of SC-hDPSC for glial markers GFAP and S100. SC-hDPSC expressed GFAP (A-B) and S100 (C). A uniformly co-expression of both markers was not found in all cell cultures. In some cultures, only a fraction of the cells expressed both GFAP and S100 (arrows) (F). C = 10x magnification; A,B,D-F = 20x magnification

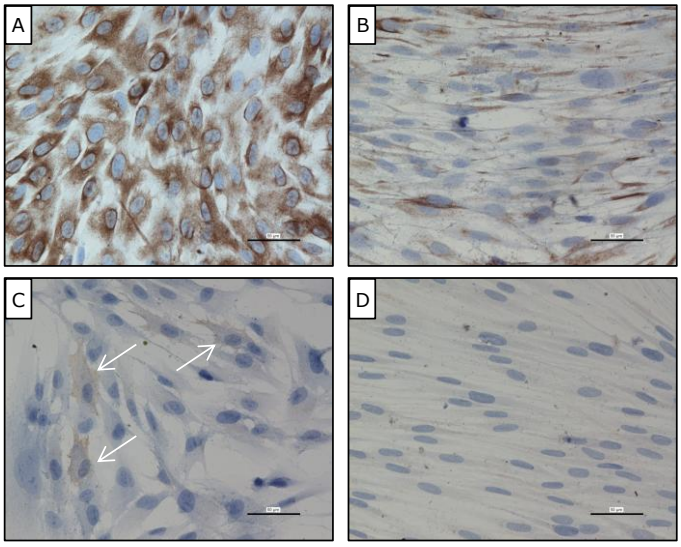


Figure 4.4: Immunocytochemical analysis of Nestin and Stro-1 expression in hDPSC (A,C) and SC-hDPSC (B,D). A decreased expression of both nestin (A-B) and Stro-1 (arrows C-D) was observed after differentiation towards peripheral glial cells. Scale bar = 50µm

4.4.4 Ultrastructural analysis of differentiated hDPSC

At the ultrastructural level, differentiated cells displayed a elongated spindle shaped morphology. Within the cell cytoplasm, numerous elongated mitochondria, granule particles (arrows Figure 4.5 A,B), Golgi apparatus and prominent rough endoplasmatic reticulum (RER) were observed. Unlike in cultures of naïve hDPSC, cell-cell contacts were often visible between neighbouring cells (Figure 4.5 C circle). In some cultures, the extracellular matrix contained striated collagen fibres (Figure 4.5 D asterix).

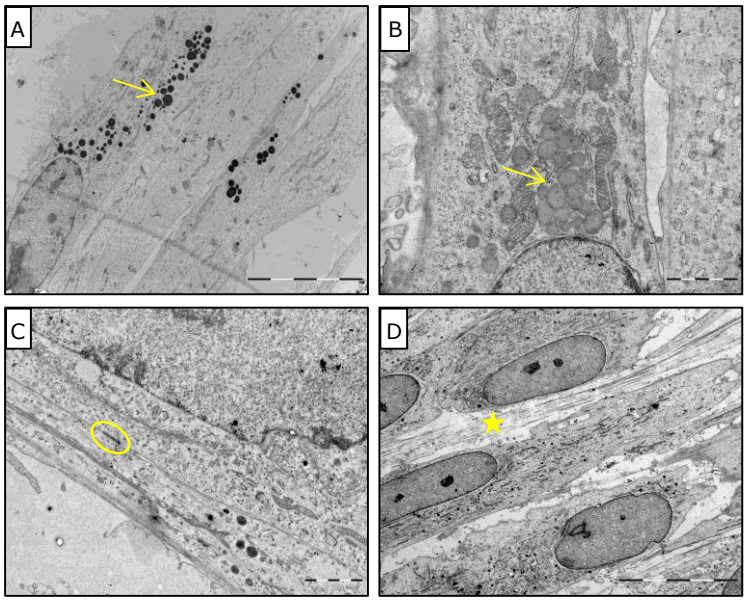


Figure 4.5: Transmission electron microscopic images of SC-hDPSC. Differentiated cells obtained a bipolar morphology with numerous mitochondria, prominent RER, Golgi apparatus and granule particles (arrows) spread throughout the cell cytoplasm. Cell-cell contacts (circle) and striated collagen fibers (asterix) were observed. Scale bar A = 10µm; Scale bar B = 5µm; Scale bar C-D = 2µm

4.4.5 Proliferation analysis

The proliferation rate of hDPSC before and after differentiation was determined in different patients over a period of 72h. The proliferation rate was assessed by means of a MTT assay. For each patient, SC-hDPSC showed a slightly higher proliferation rate compared to undifferentiated hDPSC. After 72h, the difference in proliferation rate between the two experimental groups was statistically significant in some patients (Figure 4.6).

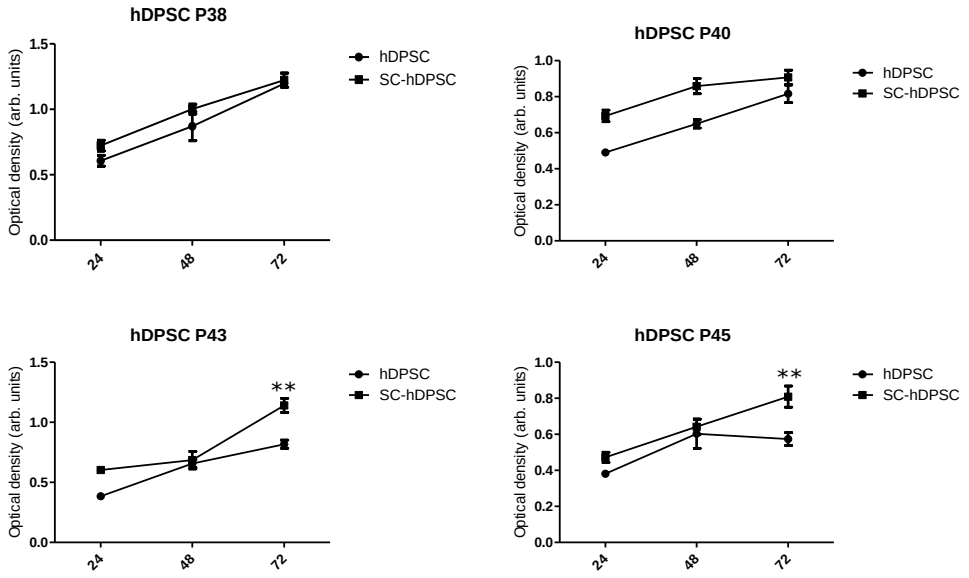


Figure 4.6: A comparison of the proliferation rates of hDPSC and SC-hDPSC in 4 different patients. For each graph, the lines show the proliferation of hDPSC and SC-hDPSC for a given patient over 72h. In all four patients SC-hDPSC have a better rate of proliferation compared with hDPSC in which 2 patients (SC-hDPSC P43 and SC-hDPSC P45) showed a significant increase at 72h (** $p \leq 0.01$)

4.4.6 Neurotrophic factors

In order to investigate the secretion of neural factors by hDPSC before and after differentiation towards a peripheral glial cell lineage, a cytokine array was conducted. The human cytokine antibody array revealed the detection of several cytokines and growth factors such as BDNF, CNTF, FGF-9, GDNF, TGF-beta 2 and TGF-beta 3 (Figure 4.7 A). The concentration of the secreted factors BDNF and FGF-9 in the conditioned medium form hDPSC and SC-hDPSC was determined by means of ELISA. The levels of BDNF increased significantly after differentiation from 6.751 ± 1.531ng/ml to 39.265 ± 8.792ng/ml. Regarding to the levels of FGF-9, no significant difference was observed before and after differentiation (Figure 4.7 B).

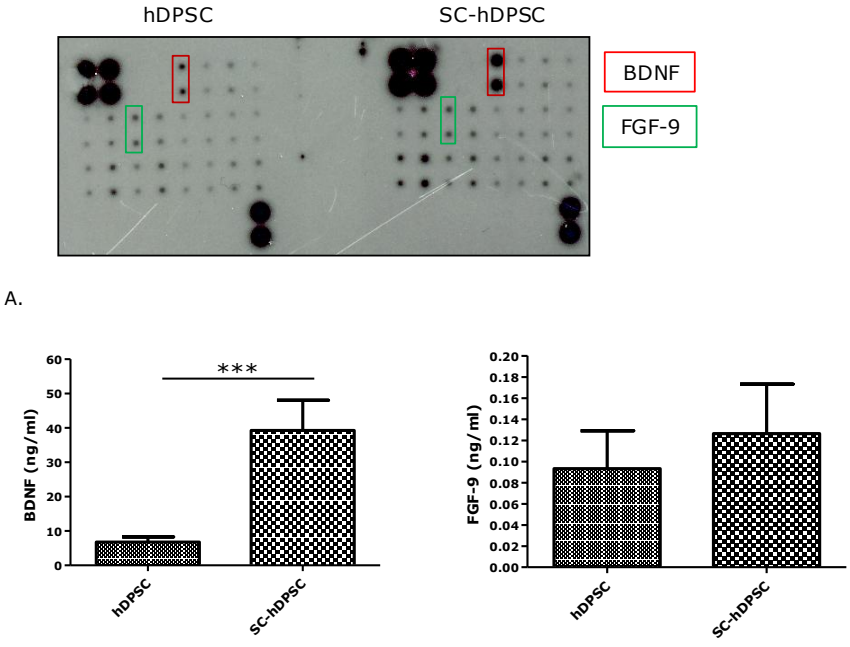


Figure 4.7: Human cytokine antibody array for neural factors (A). Increase in secreted factors after differentiation was detected for BDNF and FGF-9. **Concentrations of BDNF and FGF-9 secreted by hDPSC and SC-hDPSC (B).** A significant increase in BDNF levels was observed after differentiation. FGF-9 levels did not significantly increase. (*** $p \leq 0.001$)

4.4.7 Survival assay

DRG cultures were incubated with conditioned medium from hDPSC or SC-hDPSC for 48h. MTT assay resulted in a significant increase in the survival of DRG's after the addition of CM from hDPSC and SC-hDPSC. This indicates that secreted factors in the CM supported the survival of DRG's. Furthermore, compared with hDPSC, a higher percentage of survival was observed in DRG cultures after the addition of CM from SC-hDPSC (Figure 4.8).

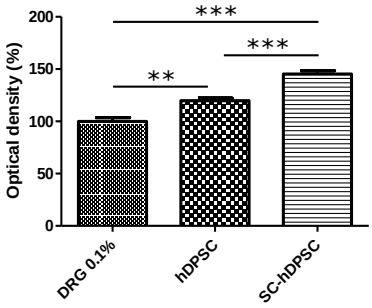
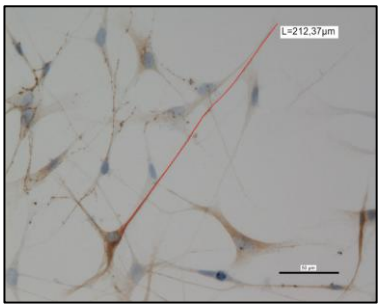


Figure 4.8: MTT analysis of the survival of DRG after addition of conditioned medium. A significant increase was observed in the survival of DRG's after adding CM from hDPSC and SC-hDPSC. (** $p \leq 0.01$; *** $p \leq 0.001$)

4.4.8 Neurite outgrowth analysis

Immunocytochemical staining for β-III-tubulin was performed to mark the neurite outgrowth of neurons in DRG cultures (Figure 4.9 A). The length of the longest neurite was measured following 48h of incubation with conditioned medium from hDPSC and SC-hDPSC. A significant increase in neurite length was observed after adding CM from hDPSC and SC-hDPSC. In addition, CM of SC-hDPSC had a greater positive influence on axonal outgrowth than CM of naïve hDPSC (Figure 4.9 B).

A. 

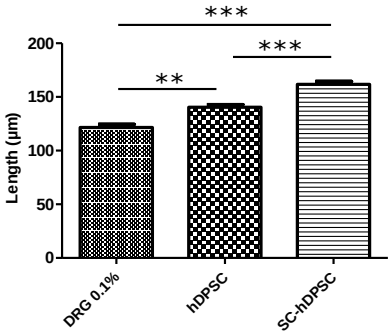
B. 

Figure 4.9: anti-β-III-tubulin immunostaining of DRG cultures (A). Scale bar = 50µm **Quantification of neurite outgrowth in DRG cultures after the addition of CM from hDPSC and SC-hDPSC (B).** CM from hDPSC and SC-hDPSC increased significantly the length of the longest neurite. A significant difference was observed between CM from hDPSC and SC-hDPSC. (** $p \leq 0.01$; *** $p \leq 0.001$)

4.5 Discussion and Conclusion

Several reports state that human third molars are an ideal source for the isolation of dental stem cells. Because third molars are the last teeth to develop, DPSC can be isolated from an early developmental tissue still possessing pluripotent and neural crest characteristics [150, 151].

In the first part of this study, the presence of pluripotency and neural crest markers at the mRNA level was evaluated in naïve hDPSC. The transcription factors Sox-2, Nanog and Oct-4 are crucial for maintaining the self-renewal capacity of stem cells [150]. According to previous studies, hDPSC contained transcript levels of Rex-1, Sox-2, Nanog and Oct-4 [79, 80]. However, due to patients variability, transcripts of these pluripotency genes were not found in all patients. It has previously been suggested that multiple stem cell niches are present in dental pulp tissue [152, 153]. Furthermore, different subpopulations of pluripotent stem cells have been isolated and characterized from dental pulp tissue, hence a different expression pattern in the different cell cultures [150]. hDPSC were found to express several neural crest markers, Hnk-1, NGFRp75, Sox-9, ErbB3 and transcription factor AP-2, indicating that hDPSC *in vitro* maintained some of the neural crest characteristics. Also in this case, patients variability may have led to the different results in transcript levels of these genes among the different patients/cell cultures. Furthermore, gene expression could have been lost during *in vitro* culture, in which cells could undergo asymmetric cell division, giving rise to a more differentiated daughter stem cell.

Dental pulp tissue originates from neural crest cells that give rise to odontoblasts, cementoblasts, periodontal cells and precursor cells of the tooth and to multiple other tissues including facial bone and cartilage, vascular smooth muscle and peripheral nervous system [132, 154-156]. Based on their neural crest origin, DPSC may possess a predisposition for differentiating in the peripheral glial cells under the correct environmental conditions. Some authors suggest that neural crest-derived dormant stem cells are residing within the dental pulp which can be isolated and *ex vivo* expanded while maintaining their neural crest multipotential properties [80]. Therefore, we investigated in the

second part of this study, the differentiation potential of hDPSC towards peripheral glial cells.

Differentiation of hDPSC towards peripheral glial cells was induced by the addition of various factors: BME, *all trans*-retinoic acid and growth media supplemented with a cocktail of growth factors PDGFaa, b-FGF, forskolin and NRG. BME is known to promote the formation of neurite-like outgrowth [123, 157] as seen in the cell cultures after 24h administration of BME. RA was used to further induce morphological cell changes as several reports state that RA together with BME can work as triggering factor that alters cell morphology. Furthermore, RA induces differentiation of embryonic stem cells into neural cells and regulates the expression of transcription factors which play a role in neural cell determination [100, 158]. An increase in cAMP, and thus, an elevated expression of mitogenic genes can be achieved when cells are treated with forskolin [159]. Taken together, BME and RA have altered cell morphology and further use of forskolin, b-FGF, PDGFaa and NRG did synergistically promoted differentiation of hDPSC into cells with Schwann cell characteristics [160]. The ultrastructural characteristics of undifferentiated hDPSC is previously described in Struys *et al* in 2010. Naïve hDPSC are fibroblast-like cells containing a perinuclear organelle-rich zone and a peripheral zone lacking any cell organelles. Furthermore no cell-cell contacts and extracellular matrix components were observed [52]. SC-hDPSC displayed a spindle shaped bipolar morphology with numerous organelles spread throughout the cell cytoplasm. In addition, the production and secretion of striated collagen fibers into the extracellular matrix was observed suggesting the formation of an endoneurium around the cell. No adequate ultrastructural data of Schwann cells was found in the literature to compare our data.

After differentiation, hDPSC expressed the glial marker GFAP and a decline in nestin and Stro-1 expression was observed via immunocytochemical analysis. The level of NGFRp75 expression was not changed after differentiation. Nestin expression is necessary for mesenchymal stem cells to develop towards a neural cell lineage [161]. When differentiation takes place, nestin expression is known to decrease, in accordance with the lower levels of nestin expression seen in SC-hDPSC in our cultures. However, based on the expression of the markers GFAP,

nestin and Stro-1 seen in SC-hDPSC, no conclusions can be made regarding a full differentiation into peripheral glial cells. The expression of early, immature SC markers such as cell-surface glycoprotein AN2, cell adhesion molecules L1 and N-CAM, and NGFRp75, have to be further explored.

To exclude that differentiation of hDPSC had an influence on cell viability, a proliferation assay on both naïve hDPSC and SC-hDPSC was conducted. According to the study of Brohlin *et al.* in 2009, the proliferation rate of SC-hDPSC was found to be higher compared to undifferentiated hDPSC. As they suggested, the presence of b-FGF in the differentiation media could be causing the higher proliferation rate observed in SC-hDPSC [98] as also seen in our study.

Several authors questioned the transdifferentiation capacity of MSC after chemical induction of differentiation because the expression of Schwann cell markers was not sufficient to indicate that these cells possessed the function of Schwann cells [149, 162]. In this study, it has been demonstrated that both undifferentiated hDPSC and SC-hDPSC were able to produce and secrete neurotrophic factors, promoting the survival and neurite outgrowth in DRG cultures. These results suggest that stem cells as such can give some benefits even before differentiation. It is known that Schwann cells produce growth factors as BDNF, GDNF, NGF and leukemia inhibitory factor which promote and guide axonal outgrowth or regeneration of nerve injury [163, 164]. We further investigated whether BDNF production of SC-hDPSC was similar to that of Schwann cells. In SC-hDPSC cultures, significantly greater amount of BDNF was detected compared to undifferentiated hDPSC cultures. Similar results were found in studies in which human umbilical cord Wharton's jelly-derived MSC or BM-MSC were differentiated into Schwann-like cells [160, 163]. SC-hDPSC promoted significantly more neurite outgrowth compared to undifferentiated hDPSC resulting from the significantly higher production and secretion of the growth factor BDNF by SC-hDPSC. BDNF, is known, together with other factors (GDNF, CNTF, FGF, NGF and VEGF) to promote neuronal rescue and survival, neurite outgrowth and guidance *in vitro* and *in vivo* [56-58, 160, 163]. Furthermore, a significantly higher survival percentage of DRG's was found with

the use of CM derived from SC-hDPSC cultures compared to CM of naïve hDPSC. Whether the higher survival rate of the DRG incubated with CM of SC-hDPSC is caused by the higher amount of BDNF present in this CM, has to be elucidated by addition of neutralizing antibodies against BDNF to the SC-hDPSC CM.
Together with the ability to produce and secrete neurotrophic factors, stem cells differentiated along a glial lineage must also be capable of (re)myelinating damaged axons and perform all Schwann cell characteristics. Therefore, direct co-cultures with neurons must be performed to elucidate the myelinating capacities of SC-hDPSC. Studies in which Schwann cell-like MSC were co-cultured with DRG's, reported that transdifferentiated MSC had similar morphological and phenotypic characteristics as Schwann cells and were able to invest axons and initiate myelination. However, no compact myelin was present in the *in vitro* co-cultures [101, 149]. Therefore, further research is necessary to elucidate the capability of SC-hDPSC to myelinate axons *in vitro* and *in vivo.* Our data indicate that SC-hDPSC have a better neuroprotective effect and promote neuronal outgrowth more than undifferentiated hDPSC *in vitro.* Further animal studies are required to investigate whether SC-hDPSC are also *in vivo* more neuroprotective and neurotrophic than naïve hDPSC.

In conclusion, naïve hDPSC still possessed pluripotency and neural crest related markers giving them a predisposition for peripheral glial cell differentiation. hDPSC were able to differentiate into a Schwann cell-like cell type obtaining typical Schwann cell characteristics on the morphological, immunocytochemical and ultrastructural level. Furthermore, SC-hDPSC produced significantly more BDNF than undifferentiated stem cells and CM of SC-DPSC significantly increased the survival and neuronal outgrowth of DRG *in vitro*. These data indicate that hDPSC are good candidates for cell-based therapies in the regeneration of peripheral nerve injuries.

5
Stem cells from apical papilla

5.1 Abstract

During the last 10 years, several stem cell populations, resembling mesenchymal stem cells, have been characterized in dental tissues including stem cells from apical papilla (SCAPs). The apical papilla is considered to be an immature tissue containing stem cells with a high proliferative capacity and multi-lineage differentiation potential.

In this study, SCAPs were characterized by means of immunocytochemical stainings and ultrastructural analysis. The basal levels of mesenchymal and neural marker expression in undifferentiated SCAPs was determined at the mRNA and protein level. Undifferentiated SCAPs expressed the mesenchymal marker CD29, CD44, CD105, CD117, CD146 and Stro-1. In addition, a positive immune-reactivity for β-III-tubulin, GalC, neurofilament, S100, synaptophysin, nestin and vimentin was found in the cultures.

Under the appropriate culture conditions, SCAPs differentiated towards adipogenic, chondrogenic or osteogenic lineages. After adipogenic induction, the expression of fatty acid binding protein-4 was observed in differentiated cells. Furthermore, the cell cytoplasm contained numerous lipid droplets which could be stained with Oil Red O staining and their presence was confirmed at the ultrastructural level. After chondrogenic induction, cells were immune-reactive for aggrecan and at the ultrastructural level, vesicles containing extracellular matrix components and numerous striated collagen fibers were present. SCAPs cultured in osteogenic medium showed expression of ALP. In addition, Alizarin Red S positive mineralized deposits were detected in the cultures. Transmission electron microscopy analysis showed the presence of collagen fibers, extracellular vesicles and mineralization nuclei. These results demonstrate that SCAPs are a mesenchymal-like stem cell population with a multi-lineage differentiation potential which opens perspectives for the use of these stem cells for tissue regeneration. This is the first study that provides ultrastructural evidence that SCAPs are able to differentiate into adipogenic, osteogenic and chondrogenic lineage cells.

5.2 Introduction

Over the last years, mesenchymal stem cells (MSC) have gained increasing interest due to their self-renewal capacities and their multi-lineage differentiation potentials. MSC yield a high proliferation rate and are able to undergo adipogenic, osteogenic, chondrogenic, myogenic and neurogenic differentiation. These abilities suggest that MSC are suitable candidates for tissue engineering and regeneration especially in endodontics. MSC are not only present in bone marrow, but can also be found in a variety of tissues and organs such as liver, nerve, muscle, skin, synovium, and cartilage [5, 7, 165-168]. Due to the fact that harvesting bone marrow is a rather painful and invasive procedure, a limited amount of cells can be isolated and the number and differentiation potential of bone marrow MSC significantly decreases with age, alternative sources of stem cells are needed [169].

Recently, adult human stem cells have been identified in dental tissues. In 2000, Gronthos *et al*. were the first to describe the presence of DPSC in human dental pulp tissue. During the following years, stem cells from apical papilla (SCAPs), periodontal ligament stem cells, dental follicle precursor cells and stem cells from human exfoliated deciduous teeth have been isolated and characterized [31-36, 42, 78]. SCAPs are a multipotent stem cell population and are present in the apical region of the dental papilla, the apical papilla, which is loosely attached to the apex of the developing tooth. The apical papilla is considered to be an immature tissue containing a cell population playing a role in root development, apexogenesis, pulp-dentin regeneration and bioroot engineering [34, 35, 78, 170].

When SCAPs are compared with DPSC, SCAPs possess a higher proliferation potential and a higher population doubling capacity because they are derived from a developing tissue while the dental pulp is considered to be a more mature tissue. Furthermore, SCAPs have a higher mineralization rate after induction of differentiation [75]. Under the correct conditions, this cell population is also capable of differentiation into osteogenic, odontogenic, adipogenic and neurogenic lineages *in vitro*. *In vivo* studies show that, when transplanted subcutaneously into immunocompromised mice, SCAPs are able to differentiate into odontoblasts and osteoblasts and can form ectopic pulp-

dentine like tissue complexes [54]. Furthermore, in a pilot study, using minipigs, it is suggested that SCAPs are the ideal cell source for primary odontoblasts to complete root formation in contrast to DPSC which are involved in reparative dentin formation [34]. In addition, SCAPs display immunomodulatory capacities by suppressing T-cell proliferation *in vitro* through an apoptosis-independent mechanism [171]. This stem cell population can also be cryopreserved without affecting their biological and immune properties [172]. The above mentioned capacities of the SCAPs makes them suitable candidates for cell based regeneration like dentin root formation studies.

Although the marker expression and differentiation potential of SCAPs are well described in the literature, an ultrastructural analysis of the differentiation potential has not been performed. Furthermore, the chondrogenic differentiation has not been determined [173]. This chapter will investigate the characterization and differentiation potential (adipogenic, chondrogenic and osteogenic) of SCAPs *in vitro*. The main focus will be on the morphological changes during differentiation at the ultrastructural level. It must be taken into account that an ultrastructural analysis is necessary to evaluate the morphological changes upon differentiation and therefore determine the success of a 'real' differentiation.

5.3 Materials & methods

5.3.1 Isolation and cell culture of stem cells from apical papilla

Normal human third molars were collected from patients (18-24 years old) at the Ziekenhuis Oost Limburg, Genk with the patient's informed consent. The apical papillae were separated from the teeth and collected in culture medium consisting of alpha MEM (GIBCO Invitrogen Corp, Paisly, Scotland, UK) supplemented with 10% heat inactivated fetal calf serum (FCS) (Biochrom AG (Berlin, Germany)), 2mM L-Glutamine, 100 U/ml Penicillin and 100µg/ml Streptomycin (GIBCO Invitrogen Corp). Apical papillae were cut into pieces of 1mm² and placed in a 6-well plate containing culture medium. The explants were kept in culture for 14 days to allow cells to grow out of the explants. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 with medium change twice a week. After 10-14 days, confluency was reached and cells were subcultured. For all experiments, cells between passage 1 and 5 were used.

5.3.2 Immunocytochemistry

To determine the expression of mesenchymal and neural markers of the SCAPs, cells were seeded onto glass coverslips (5,000cells/cm²) and stained with the peroxidase-based EnVision System® (DakoCytomation, Glostrup, Denmark). When confluent, cells were fixed with paraformaldehyde 4% for 20 minutes and washed with Phosphate Buffered Saline (PBS). When necessary, cells were permeabilized with Triton-X 0.05% for 30 minutes at 4°C and washed with PBS. To block non-specified binding sites, cells were incubated with 10% normal goat serum or normal donkey serum at room temperature for 20 minutes. After washing with PBS, cells were incubated with primary antibody (table 5.1) for 1h, followed by incubation with goat anti-mouse, goat anti-rabbit, or donkey anti-goat horseradish peroxidase-conjugated secondary antibodies for 30 minutes.
To visualize the peroxidase, diaminobenzidine chromogenic substrate was used. Cells were counterstained with Mayer's hematoxylin and mounted using an

aqueous mounting medium (Aquatex, Merck, Darmstadt, Germany). The immune-reactivity of the cells was observed using a photomicroscope equipped with an automated camera (Nikon Eclipse 80i, Nikon Co., Japan).

Table 5.1: Primary antibodies for immunocyto- and immunohistochemistry

	Species	**Dilution**	**Company**
Mesenchymal stem cell markers			
CD29	Mouse	1:35	Abcam
CD34	Mouse	1:50	Abcam
CD44	Mouse	1:200	Abcam
CD105	Mouse	1:100	Abcam
CD117 (c-Kit)	Mouse	1:100	Santa Cruz
CD146	Rabbit	RTU*	Abcam
Cytokeratin	Mouse	RTU*	Dakocytomation
Stro-1	Mouse	1:50	R&D systems
Neural markers			
β-III tubulin	Mouse	1:2000	Sigma Aldrich
GalC	Mouse	1:20	Dr. V.W.Yong Calgary
GFAP	Mouse	1:200	NovoCastra
MBP	Mouse	1:200	Serotec
Neurofilament	Mouse	1:100	Dakocytomation
NGFRp75	Mouse	1:50	Dakocytomation
S100	Rabbit	1:400	Dakocytomation
Synaptophysin	Mouse	1:20	Dakocytomation
Other markers			
Nestin	Mouse	1:500	Chemicon
Vimentin	Mouse	1:500	Dakocytomation
FABP-4	Goat	1:100	R&D systems
Aggrecan	Goat	1:100	R&D systems
Osteocalcein	Mouse	1:100	R&D systems

*RTU : Ready to use

5.3.3 Immunohistochemical analysis of markers in apical papilla

Apical papilla were fixed with unifix for 2 hours, dehydrated in graded ethanol, embedded in paraffin and serially sectioned at 5µm. After rehydratation of the tissue sections, antigen retrieval was performed by microwaving the slides in 10mM citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched by incubating the slides in 0.5% H_2O_2 for 30 minutes. Next, nonspecific binding sites were blocked with 3% normal goat serum for 20 minutes. Subsequently, tissues slides were washed 3 times in PBS and incubated with the primary antibody (table 5.1) for 1 hour. Next, slides were rinced in PBS and incubated with peroxidase-labelled secondairy antibody for 30 minutes and subsequently visualized using the Dako Envision® System. Sections were counterstained with hematoxylin, coverslipped with an aqueous mounting medium and examined using a photomicroscope equipped with an automated camera (Nikon Eclipse 80i, Nikon Co., Japan).

5.3.4 RNA extraction – cDNA synthesis - Reverse Transcriptase PCR

Total RNA was isolated using the RNeasy Mini plus kit (Qiagen, Venlo, The Netherlands) following manufacturer's protocol. This kit ensures additional elimination of genomic DNA. The concentration and purity of total RNA was determined by measuring the optical density at 260nm and the 260/280nm ratio using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). 700ng of total RNA was reverse transcribed into cDNA according to the manufacturer's instructions of the Reverse Transcription System (Promega, Leiden, the Netherlands). Samples were stored at -20°C until further analysis. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed by means of Taq DNA Polymerase (1U/µl) (Roche Diagnostics, Vilvoorde, Belgium) (table 5.2).
PCR reactions were performed by a BioRad Thermal cycler (Biorad, Hercules, CA, USA) under the following conditions: a denaturation step at 94°C for 5 minutes was followed by amplification over 35 cycles of denaturation (94°C for 1

minute), annealing (temperature dependent on the primers for 60 seconds) and elongation (72°C for 2 minutes). Sequences of the primers (Eurogentec S.A. Seraing, Belgium) can be found in table 5.3. The housekeeping genes β-actin, GusB and β2-microglobulin were used as a control for the PCR reaction. PCR products were separated on a 1.5% agarose gel (Invitrogen, Merelbeke, Belgium) and visualized with gel red.

Table 5.2: Composition of PCR reaction mix and applied PCR program

PCR mix per sample (µl)	
10X PCR buffer	2.5
Forward primer (25µM)	1
Reverse primer (25µM)	1
dNTPs (2mM)	0.25
Taq Polymerase (1U/µl)	0.75
MilliQ	19.1
cDNA	1

Table 5.3: Primer sequences for Reverse Transcription Polymerase Chain Reaction

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Tm (°C)	Product length (bp)
Pluripotent markers				
Nanog	GAT-TTG-TGG-GCC-TGA-AGA-AA	AAG-TGG-GTT-GTT-TGC-CTT-TG	58	155
OCT4	AGC-CCT-CAT-TTC-ACC-AGG-CC	TGG-GAC-TCC-TCC-GGG-TTT-TG	56	456
Rex1	TGA-AAG-CCC-ACA-TCC-TAA-CG	CAA-GCT-ATC-CTC-CTG-CTT-TGG	56	554
SOX2	AGA-AGC-GGC-CGT-TCA-TCG-AC	TGC-TGA-TCA-TGT-CCC-GGA-GGT	56	570
Neural crest markers				
TF-AP2	TCC-CTG-TCC-AAG-TCC-AAC-AGC-AAT	AAA-TTC-GGT-TTC-GCA-CAC-GTA-CCC	50	396
ErbB3	GGT-GCT-GGG-CTT-GCT-TTT	CGT-GGC-TGG-AGT-TGG-TGT-TA	57	365
HNK-1	GTG-AGT-GCT-GGT-AAT-GAG-GAG-CCG	GGT-GTT-GTC-GTC-GTC-GGC-GA	60	642
P75 (NGFR)	AGC-CAA-CCA-GAC-CGT-GTG-TG	TTG-CAG-CTG-TTC-CAC-CTC-TT	54,7	663
SOX 9	AGA-AGG-ACC-ACC-CGG-ATT-AC	CGG-CAG-GTA-CTG-GTC-AAA-CT	50	401
Neural markers				
β-III-tubulin	CAT-CCA-GAG-CAA-GAA-CAG-CA	GCC-TGG-AGC-TGC-AAT-AAG-AC	56	566
GalC	GCC-AAG-CGT-TAC-CAT-GAT-TT	GCA-GAG-ATG-GAC-TCC-CAG-AG	58	161
GFAP	GCC-AAG-CCA-GAC-CTC-ACC-GC	GTG-TCC-AGG-CTG-GTT-TCT-CGA-ATC	60	509
MBP	CCC-CGT-AGT-CCA-CTT-CTT-CA	TCC-CTT-GAA-TCC-CTT-GTG-AG	56	178
Nestin	AAC-AGC-GAC-GGA-GGT-CTC-TA	TTC-TCT-TGT-CCC-GCA-GAC-TT	56	220
Neurofilament H	AGG-AAC-CAG-ATG-ATG-CCA-AG	TGG-AGG-CTT-GCT-GTC-TTT-TT	58	233

S100 A6	TGA-TCC-AGA-AGG-AGC-TCA-CC	CCC-TTG-AGG-GCT-TCA-TTG-TA	56	160
Vimentin	GAG-AAC-TTT-GCC-GTT-GAA-GC	TCC-AGC-AGC-TTC-CTG-TAG-GT	56	170
Housekeeping genes				
β actin	AAA-TCT-GGC-ACC-ACA-CCT-TC	AGA-GGC-GTA-CAG-GGA-TAG-CA	56	185
β2 microglobulin	CTC-ACG-TCA-TCC-AGC-AGA-GA	CGG-CAG-GCA-TAC-TCA-TCT-TT	56	213
Gus B	AGC-CAG-TTC-CTC-ATC-AAT-GG	GGT-AGT-GGC-TGG-TAC-GGA-AA	56	160

5.3.5 Fluorescent activated cell sorting analysis

Cells at passage 2-3 were seeded in 25cm² culture flask and were harvested by trypsinization after 7 days. Cells were incubated for at least 1h at room temperature in PBS with 2% FCS to allow re-expression of receptor proteins at the cell surface. For intracellular stainings, cells were first fixed and than permeabilized with the cytofix/cytoperm kit (Becton–Dickinson, San Jose, CA) according to the protocol provided by the manufacturer. Then, 0.5×10^5 cells were washed once with PBS containing 2% FCS and were incubated with primary antibody (see table 5.1) for 30 min at room temperature. As a negative control for non-specific background staining, also appropriate isotype controls were included in the study. Thereafter, the cells were washed three times with PBS and incubated with secondary antibody (FITC-labeled goat anti-rabbit (eBioscience, San Diego, CA) or PE-labeled anti-mouse IgG (Invitrogen)) for 45 min at room temperature. Samples were analyzed on a FACScalibur™ flow cytometer equipped with CellQuest software (BD Biosciences, Erembodegem, Belgium).

5.3.6 Differentiation procedures

Adipogenic differentiation

To induce adipogenic differentiation, cells were seeded onto 12-mmø glass coverslips or onto plastic coverslips (Thermanox®, Electron Microscopy Sciences) at a density of 17,500 cells/cm² for light and transmission electron microscopical analysis. After 24h, culture medium was replaced with adipogenic induction medium (R&D systems, UK). Medium was changed every 72h. After 3 weeks, cells seeded on glass coverslips were washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were stained with 0.3% Oil red O to visualize intracellular lipid droplets or stained with an antibody against fatty acid binding protein-4 (FABP-4). Cells seeded on plastic coverslips were fixed with 2% glutaraldehyde in 0.05M cacodylate buffer (pH 7.3) at 4°C and further processed for transmission electron microscopical analysis.

Chondrogenic differentiation

Chondrogenic differentiation was performed with the Stempro Chondrogenesis differentiation kit (Invitrogen) according to the manufacturer's instructions. After three weeks of incubation, cells were stained with an antibody against aggrecan or were processed for transmission electron microscopical analysis.

Osteogenic differentiation

SCAPs were seeded onto 12-mmø glass coverslips or onto Thermanox coverslips at a density of 5,000 cells/cm². When 50-70% confluence was reached, medium was replaced with osteogenic differentiation medium (R&D Systems, UK). Medium was changed every 3 days. After 3 weeks, cells seeded on glass coverslips were washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were stained with Alizarin red S to visualize calcified deposits in the cell cultures. Furthermore, immunocytochemistry with an antibody against alkaline phosphatase (ALP) was performed. Cells seeded on plastic coverslips were fixed with 2% glutaraldehyde in 0.05M cacodylate buffer (pH 7.3) overnight at 4°C.

5.3.7 Ultrastructural analysis

Following fixation, the fixative was gently aspirated with a glass pipette and the cells were post-fixed in 2% osmium tetroxide for 1 hour and stained with 2% uranyl acetate in 10% acetone for 20 min. Subsequently, the cell-seeded coverslips were put through dehydrating series of graded concentrations of acetone and embedded in araldite according to the pop-off method [133]. Ultra-thin sections (0.06µm) were mounted on 0.7% formvar-coated grids, contrasted with uranyl acetate and lead citrate, and examined in a Philips EM 208 transmission electron microscope operated at 80 kV.

5.4 Results

5.4.1 Morphology of SCAPs *in vitro*

SCAPs were cultured via the explant method and after a few days, cells started to migrate out of the tissue explant. After 10-14 days, confluency was reached and cells were subcultured (Figure 5.1 A-B). *In vitro*, SCAPs are a heterogeneous cell population containing a polygonal or spindle-shaped fibroblast-like morphology. SCAPs display mesenchymal stem cell characteristics such as adherence to plastic surfaces and the capability to form colonies *in vitro* (Figure 5.1 C-D).

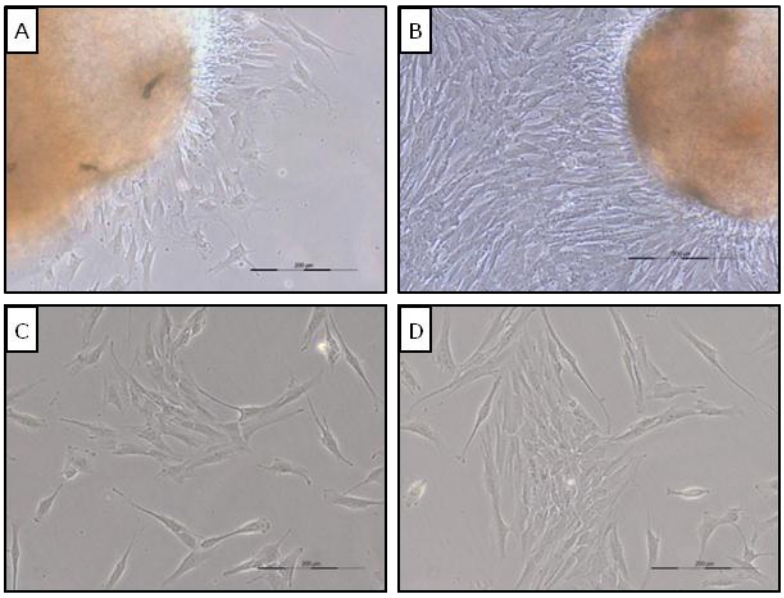


Figure 5.1: Phase contrast images of SCAPs in culture. SCAPs are isolated via the explant method. Cells migrate out of tissue explants and after 10-14 days, confluency is reached (A-B). SCAPs display a fibroblast-like morphology, are an adherent cell type and are able to form colonies *in vitro* (C-D). Scale bar = 200µm

5.4.2 Ultrastructural analysis of undifferentiated SCAPs

At the ultrastructural level, cells had a bipolar morphology and displayed an oval euchromatic nuclei with multiple prominent nucleoli. Cell-cell contacts were often visible. The cell cytoplasm was characterized with organelles widely spread throughout the cytoplasm. The content of cell organelles consisted of rough endoplasmatic reticulum (RER) cisternae, Golgi apparatus and numerous elongated mitochondria. Furthermore, the cell cytoplasm contained intermediate filaments and some vacuoles and vesicles. No production of extracellular matrix components was detected. Often, the cell surface showed filopodia (Figure 5.2 A-D).

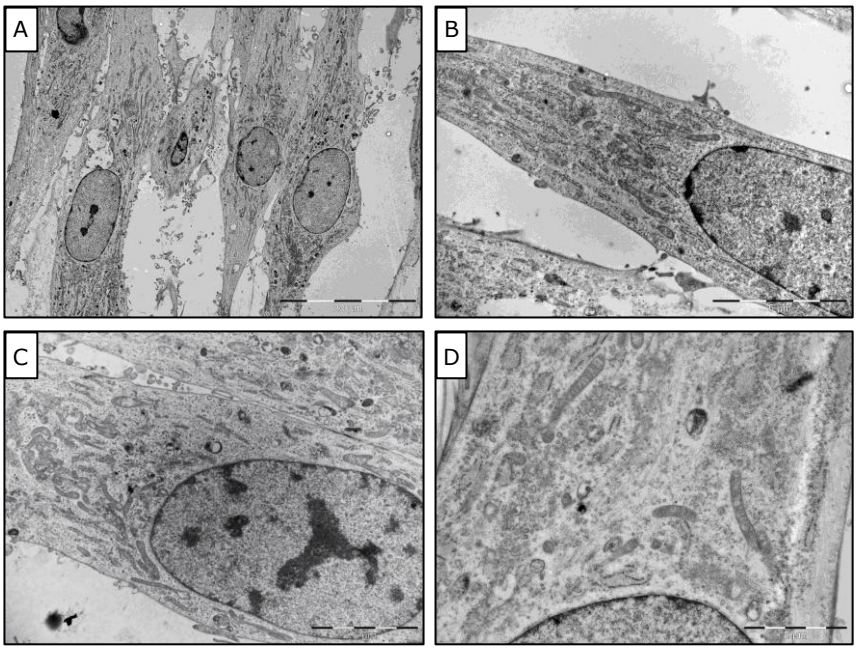


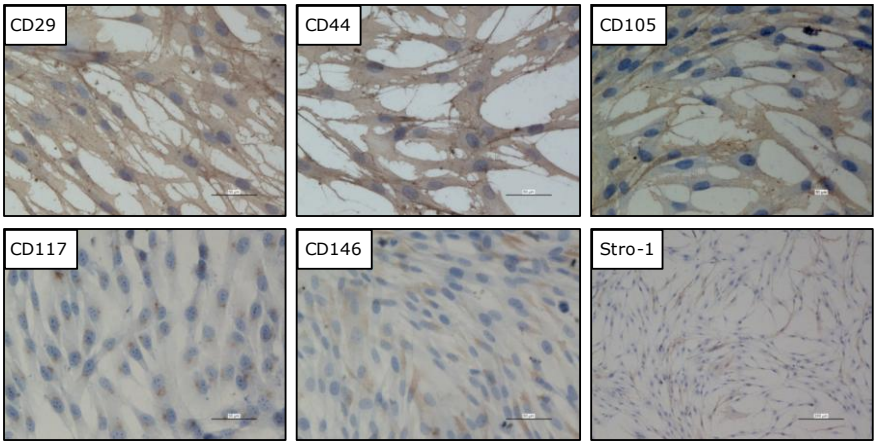
Figure 5.2: Transmission electron microscopic images of undifferentiated SCAPs. SCAPs showed a bipolar morphology. Cells contained a euchromatic nuclei with prominent nucleoli (A). Cell cytoplasm contained mitochondria, RER, some Golgi apparatus and vesicles. No production of extracellular matrix components were visible (B-C). Some cell-cell contacts were detected (C). Intermediate filaments could be found in the cell cytoplasm (D). Scale bar A = 20µm; Scale bar B-C = 5µm; Scale bar D = 2µm

5.4.3 Immunophenotype of undifferentiated SCAPs.

Basal mesenchymal marker expression

To evaluate the mesenchymal marker expression of undifferentiated SCAPs, immunocytochemistry was performed. SCAPs stained uniformly for the surface markers CD29, CD44 and CD105. A perinuclear expression pattern of CD117 (c-kit) was observed in all SCAPs. Furthermore, only a subpopulation of the cells showed a positive immune-reactivity for CD146, Stro-1 and cytokeratin.

Flow cytometric analysis of cultured SCAPs of passage 4 confirmed the expression of Stro-1, CD34, CD44 and cytokeratin. In addition, no expression of CD45 could be detected. Values are presented as means of at least 3 different patients ± SEM (Figure 5.3).

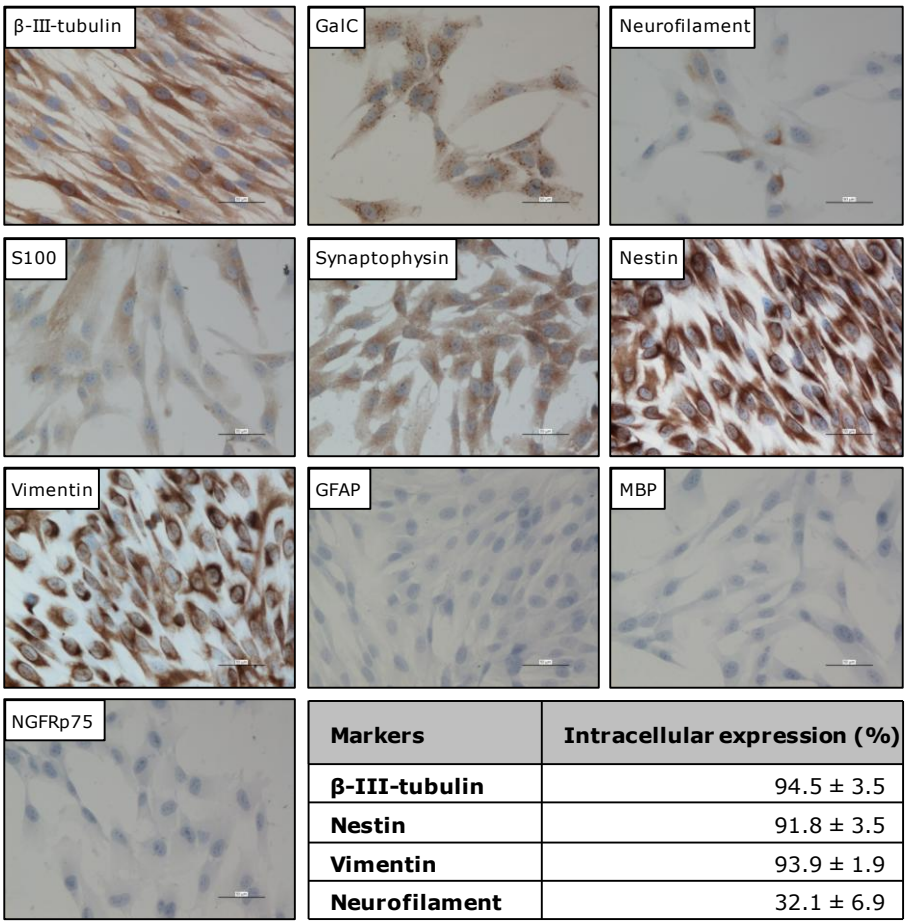


Markers	Surface expression (%)
Stro-1	4.4 ± 2.2
CD34	5.2 ± 3.0
CD45	0.1 ± 0.05
CD44	96.1 ± 2.0
Cytokeratin	2.3 ± 1.0

Figure 5.3: Mesenchymal marker expression of undifferentiated SCAPs. Immunophenotypic profile of cultured SCAPs. SCAPs stained positive for CD29, CD44, CD105, CD146 and CD117. Only a subset of the population are positive for Stro-1 and cytokeratin. Flow cytometric analysis of cultured SCAPs revealed the expression of Stro-1, cytokeratin, CD34 and CD44 but no expression of CD45. Values are presented as means of at least 3 different patients ± S.E Scale bar = 50µm; Scale bar (Stro-1) = 200µm

Basal neural marker expression

The basal expression of neural markers was determined in cultured SCAPs via an immunocytochemical analysis. SCAPs stained almost uniformly for β-III-tubulin, GalC, S100 and synaptophysin. Only a fraction of the cells were immune-reactive for neurofilament, respectively 32.1%. Furthermore, all SCAPs were expressing the cytoskeleton markers vimentin and nestin. No expression of GFAP, MBP and NGFRp75 was observed. The high expression of β-III-tubulin, neurofilament, nestin and vimentin was confirmed via flow cytometric analysis. Values are presented as means of at least 3 different patients ± SEM (Figure 5.4).



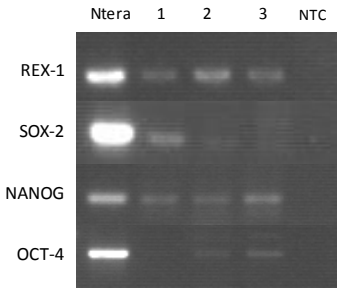
Markers	Intracellular expression (%)
β-III-tubulin	94.5 ± 3.5
Nestin	91.8 ± 3.5
Vimentin	93.9 ± 1.9
Neurofilament	32.1 ± 6.9

Figure 5.4: Neural marker expression of undifferentiated SCAPs. SCAPs stained positive for β-III-tubulin, GalC, S100, synaptophysin, nestin and vimentin, while they are negative for GFAP, MBP and NGFRp75. Only a subset of the population is positive for neurofilament. Flow cytometric analysis of cultured SCAPs revealed the expression of the neural markers β-III-tubulin, nestin and vimentin. Only respectively 32.1 % of the cells expressed neurofilament. Values are presented as means of at least 3 different patients ± S.E. Scale bar = 50μm

5.4.4 Expression of pluripotency and neural crest markers in undifferentiated SCAPs

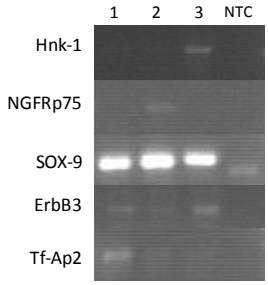
By means of RT-PCR, we investigated the expression of markers characteristic for pluripotency or for neural crest derived cells on the mRNA level in SCAPs at passage 1. Ntera cells (pluripotent human testicular embryonic carcinoma cell line) were used as a positive control to evaluate the expression of pluripotency markers. RT-PCR detected mRNA levels of REX-1, Sox-2, Nanog and OCT-4 in respectively 80%, 45%, 80% and 30% of the patients tested. Regarding to the transcript levels of neural crest markers, Hnk-1, NGFRp75, SOX-9, ErbB3 and Tf-AP2 expression was found in respectively 17%, 33%, 100%, 100% and 67% of the patients tested (Figure 5.5).

A.



	% of patients
Rex-1	80%
Sox-2	45%
Nanog	80%
Oct-4	30%

B.



	% of patients
Hnk-1	17%
NGFRp75	33%
SOX-9	100%
ErbB3	100%
Tf-Ap2	67%

Figure 5.5: Expression of pluripotency (A) or neural crest markers (B) in SCAPs on mRNA level. RT-PCR analysis of total RNA extracted from SCAPs. Ntera cells (pluripotent human testicular embryonic carcinoma cell line) were used as a positive control for evaluating pluripotency markers. (NTC = no template control)

5.4.5 Immunohistochemical analysis of marker expression in apical papilla

An immunohistochemical staining on human apical papilla was performed to evaluate the expression pattern of mesenchymal and neural markers in apical tissue (Figure 5.6).

Expression of the mesenchymal markers CD29, CD44, and CD146 was spread throughout the whole tissue. In contrast to the results of the immunocytochemical staining *in vitro,* no expression of CD105 and Stro-1 could be observed. Upon staining with anti-CD117, blood vessels showed a weak immune-reactivity. Furthermore, apical papilla tissue was homogenously stained with anti-nestin and anti-vimentin. A weak expression of S100 by the cells was observed throughout the tissue in contrast to the strong S100 positively stained neural structures. β-III-tubulin and GalC positive cells were also related to neural structures. In addition, nerve fibers were positively stained with anti-neurofilament, which was comparable with β-III-tubulin staining.

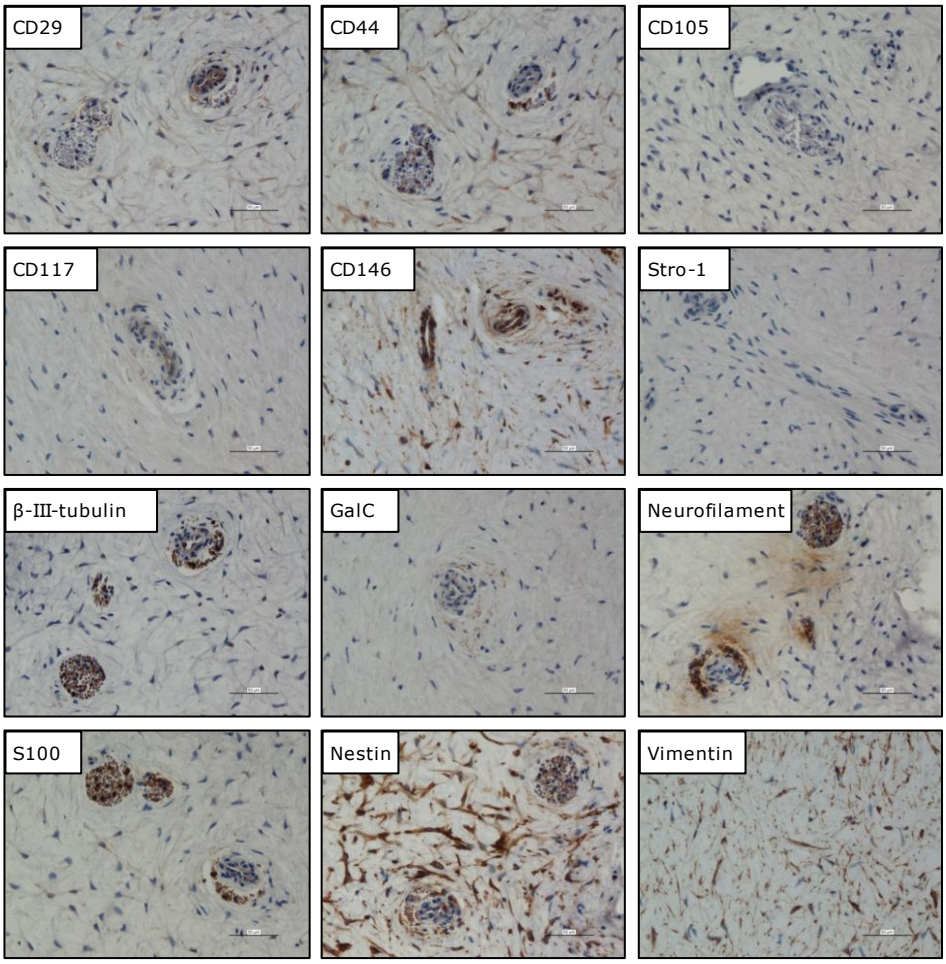


Figure 5.6: Mesenchymal and neural marker expression in apical papilla tissue. Scale bar = 50μm

5.4.6 Differentiation potential

The mesodermal differentiation potential of SCAPs was investigate by exposing the cells to different differentiation media to undergo adipogenic, chondrogenic and osteogenic differentiation.

Adipogenic differentiation

To induce adipogenic differentiation, SCAPs were incubated for 3 weeks with culture media containing adipogenic supplement. The results demonstrated the presence of multiple lipid droplets stained with Oil-red O (ORO) staining (Figure 5.7 A-C). Lipid droplets were mainly located at the periphery of the cell. In some cells, lipid droplets fused to form larger ones. However, not all cells underwent adipogenic differentiation (Figure 5.7A). In undifferentiated cells, no lipid droplet accumulation could be observed. (Figure 5.7D).

To confirm adipogenic differentiation, immunostaining with anti-Fatty Acid Binding Protein-4 (FABP-4) was performed. In cultures of differentiated SCAPs, FABP-4 positive cells were present (Figure 5.8 A-B). Undifferentiated SCAPs stained negative for FABP-4 (Figure 5.8C).

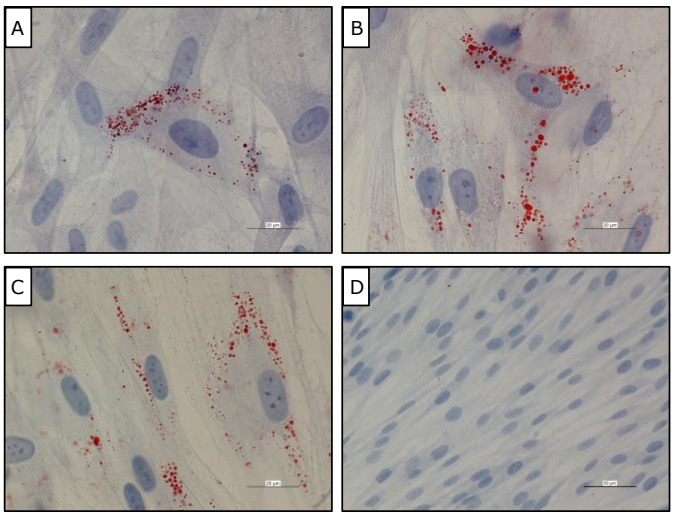


Figure 5.7: Adipogenic differentiation of SCAPs after 3 weeks. Oil-Red-O staining. Lipid vacuoles are stained with ORO staining (A-C). Undifferentiated SCAPs did not show presence of lipid droplets (D). Scale bar A-C = 20µm; Scale bar D = 50µm

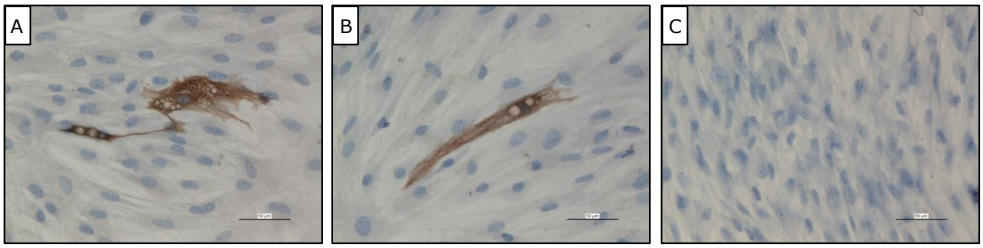


Figure 5.8: Adipogenic differentiation of SCAPs after 3 weeks. Immunocytochemical staining against FABP-4. Differentiated SCAPs expressed FABP-4 (A-B). No FABP-4 expression was observed in undifferentiated SCAPs (C). Scale bar = 50µm

To further elucidate the adipogenic differentiation potential of the SCAPs, a transmission electron microscopic analysis was performed. At the ultrastructural level, some morphological changes of SCAPs into adipocytes-like cells could be observed, confirming the adipogenic differentiation potential. The cell cytoplasm contained numerous lipid droplets located at the periphery of the cell (Figure 5.9 C,D). Furthermore, dilated rough endoplasmatic reticulum was present, mostly located at the cell border (Figure 5.9 A-C). Not all cells responded to adipogenic differentiation signals, meaning that in some cells no signs of dilated RER or lipid droplets could be detected.

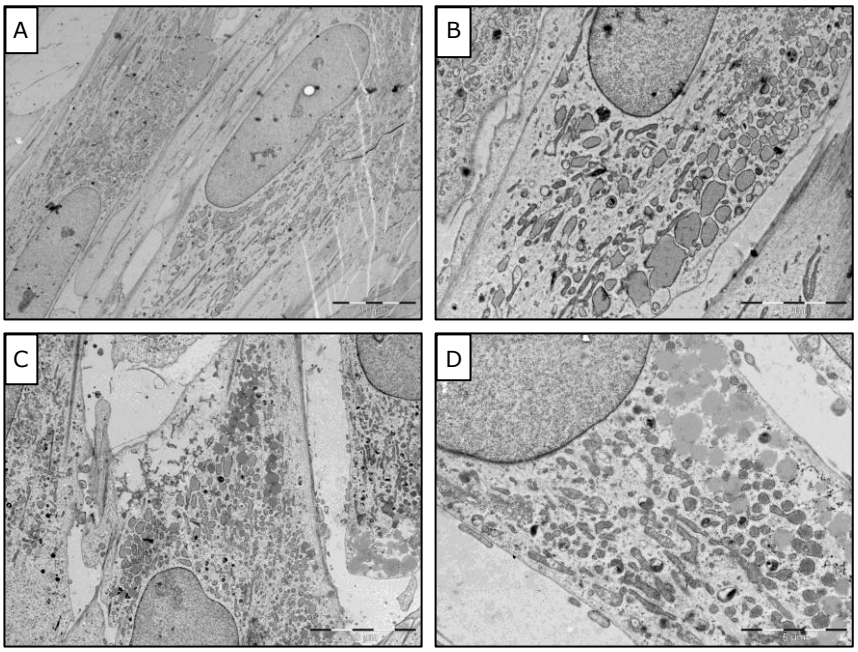


Figure 5.9: Adipogenic differentiation of SCAPs after 3 weeks. Transmission electron microscopic images. The presence of dilated RER and some lipid droplets confirmed the differentiation into adipocyte-like cells (A-D). Scale bar A-C = 10µm; Scale bar B-D = 5µm

Chondrogenic differentiation

Chondrogenic differentiation of the SCAPs was induced by incubating the cells for 3 weeks with 'Stempro Chondrogenesis Osteogenic differentiation medium (Invitrogen). Chondrogenic differentiated SCAPs highly expressed aggrecan, a structural glycoprotein of cartilage, while undifferentiated cells shown no immune-reactivity for the anti-aggrecan antibody (Figure 5.10). Ultrastructural examination of the chondrogenic SCAPs revealed a high accumulation of vesicles with extracellular matrix components in the cytoplasm of these cells. Another confirmation of the acquired chondrogenic phenotype was the massive production of collagen microfibers in the extracellular space (Figure 5.11). However, unlike for chondrogenic differentiated hDPSC, no cartilage-like fragments could be found in light or ultramicroscopical images, which may indicate that the chondrogenic differentiation of the SCAP was incomplete.

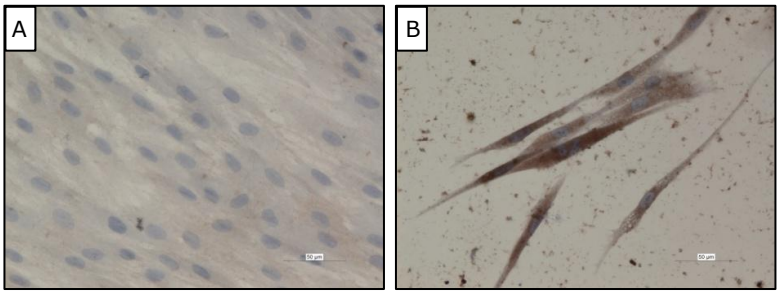


Figure 5.10: Chondrogenic differentiation of SCAPs after 3 weeks. Immunocytochemical staining against aggrecan. Differentiated cells showed a very high expression of aggrecan (B), while undifferentiated, control SCAPs are negative (A). Scale bar = 50 µm

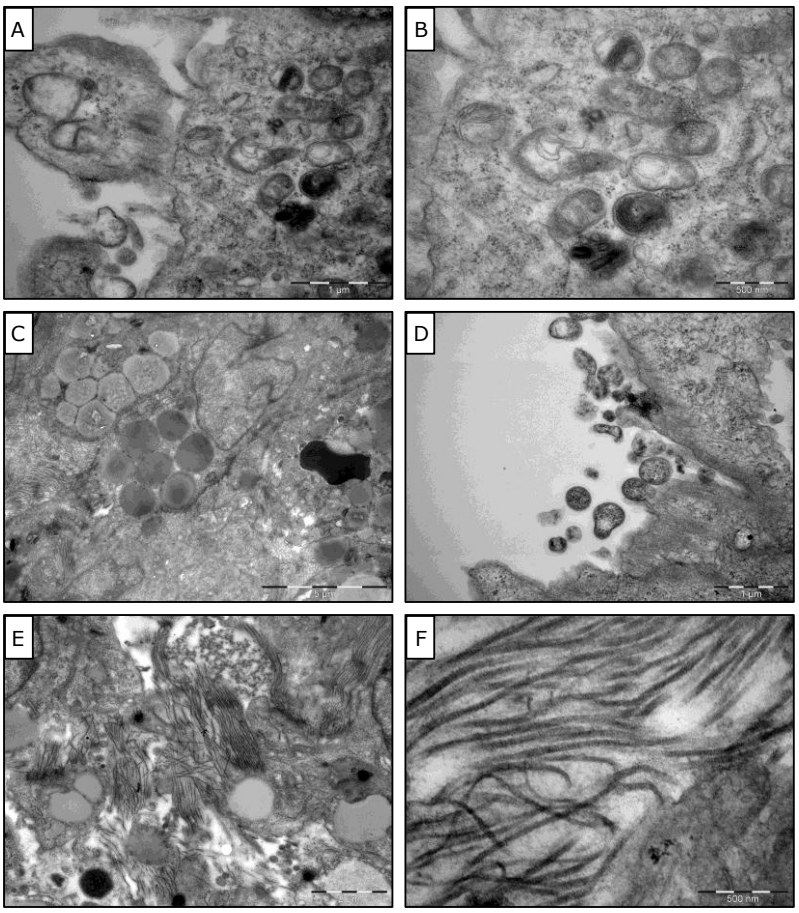


Figure 5.11: Chondrogenic differentiation of SCAPs after 3 weeks. Transmission electron microscopic images. Typically, the cytoplasm of chondrogenic differentiated SCAPs contained lamellar vesicles (A,B) and excessive amounts of vesicles with extracellular matrix components (C). These vesicles were secreted into the extracellular space by means of exocytosis (D). In the extracellular space the secreted vesicles interacted with surrounding collagen fibers (E). High magnification showed the typical banded pattern of collagen fibers in the extracellular space (F). Scale Bar A,D = 1µm; Scale bar B,F = 500nm; Scale bar C = 5µm; Scale bar E = 2µm

Osteogenic differentiation

Osteogenic induction of the SCAPs resulted in a high *de novo* expression of ALP, while control, undifferentiated SCAPs showed no sign of ALP expression (Figure 5.12 A-B). Moreover, numerous mineralized depositions could be detected by Alizarin red staining in osteogenic induced SCAPs monolayer's. This is an early step in matrix mineralization but a crucial step towards the formation of bone tissue (Figure 5.12 C-D).

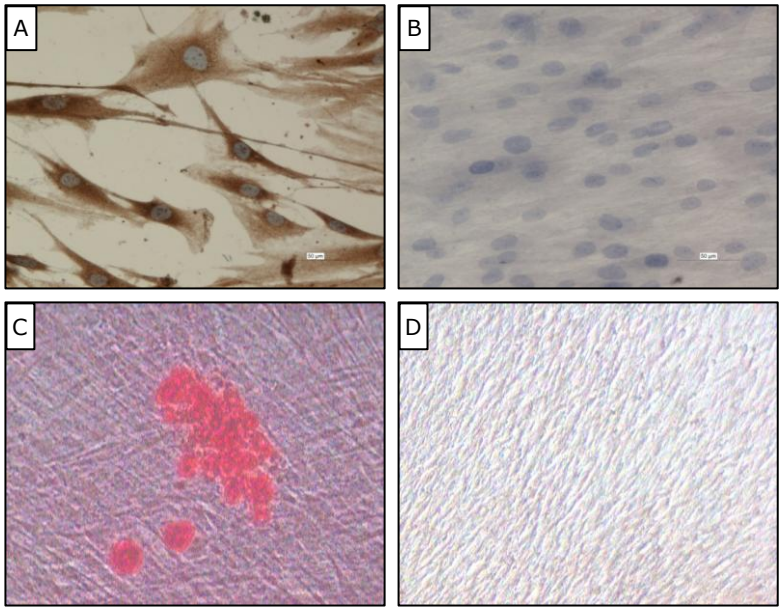


Figure 5.12: Osteogenic differentiation of SCAPs after 3 weeks. ALP staining / Alizarin Red S staining. Immunocytochemical staining against ALP (A-B). SCAPs incubated with osteogenic differentiation medium for 3 weeks have a high de novo expression of ALP, while no expression of ALP could be observed in undifferentiated SCAPs. Alizarin red staining shows the presence of multiple mineralized (calcified) nodules in the monolayer of osteogenic induced SCAPs (C) but none in the control, undifferentiated SCAPs (D). Scale bar A-B = 50µm; C-D = 40x magnification

Osteogenic differentiation was confirmed at the ultrastructural level where prominent morphological changes could be detected. Osteogenic induced SCAPs have acquired an osteoblast-like phenotype with an eccentric nucleus (Figure 5.13 A). The cytoplasm of these cells was characterized by high amount of mitochondria and dark-stained vesicles containing extracellular matrix components (Figure 5.13 B-C). The extracellular space surrounding the differentiated SCAPs contained mainly collagen fibres and extracellular vesicles which play a key role in the mineralization process. Also numerous mineralized nuclei containing hydroxyapatite needles could be found in the extracellular space. These mineralized nuclei are inevitable evidences for mineralization and bone formation (Figure 5.13 D-F).

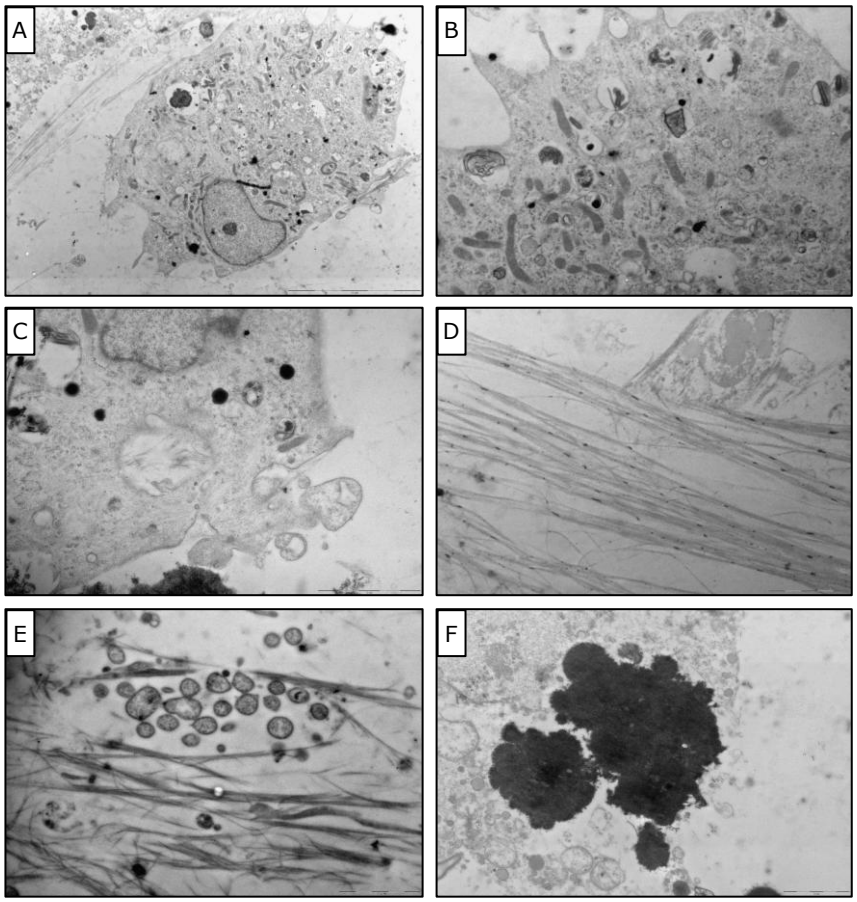


Figure 5.13: Osteogenic differentiation of SCAPs after 3 weeks. Transmission electron microscopic images. A-B) Osteogenic induced SCAPs showed an osteoblast-like phenotype with an eccentric nucleus and a cytoplasm containing some lamellar vesicles and a high amount of mitochondria. Furthermore, dark-stained vesicles containing extracellular matrix components could be observed. The content of these vesicles is released into the extracellular space via exocytosis (C). The extracellular space surrounding the differentiated SCAPs contained mainly collagen fibres (D) and extracellular vesicles (E) which play a role in the mineralization of the extracellular matrix. Also numerous mineralized nuclei consisting of fused hydroxyapatite needles could be found in the extracellular space (F). Scale bar A = 50 µm; B-C, F = 2µm; scale bar D-E = 500nm

5.5 Discussion and Conclusion

Many adult tissues contain a population of stem cells that have the capacity for self-renewal after trauma, disease or aging. Recently, adult stem cells have been isolated and characterized from dental tissues.
The human tooth with an immature apex is a developing organ, containing self-renewal and differentiating stem cells, which are activated to make a complete tooth. Root formation starts as the epithelial cells from the cervical loop proliferate apically, and influence the differentiation of odontoblasts from undifferentiated mesenchymal cells and cementoblasts from follicle mesenchyme. It is generally known that the dental papilla contributes to tooth formation and eventually converts to pulp tissue within in the pulp chamber. As the root continues to develop after the bell stage, the location of the dental papilla becomes apical to the pulp tissue [20, 74, 174-176]. Therefore, adult stem cells could be present in the apical papilla of teeth with an immature apex. Third molars with immature apex are frequently extracted for orthodontic reasons and are a valuable developing human tissue that is constantly available.
SCAPs can be isolated via enzymatic digestion or via the explant method [34, 35, 76, 78, 177]. In this study, the explant method is used to isolate cells from human apical papilla. Isolated cells displayed typical MSC characteristics: adherence to a plastic surface; a spindle shaped fibroblast-like morphology and the capacity to form colonies *in vitro*. This *in* vitro morphology is similar to previous studies in which enzymatic digestion or explant culture is used as isolation method [34, 35, 76, 177].

By using transmission electron microscopy, the ultrastructural characteristics of SCAPs can be analyzed. Undifferentiated cells showed a bipolar morphology with euchromatic nuclei, cell organelles spread throughout the cell cytoplasm and no extracellular matrix depositions. A previous ultrastructural study on DPSC showed a similar morphology of bipolar cells with euchromatic nuclei. However, in contrast to SCAPs, undifferentiated DPSC displayed an organelle-rich perinuclear zone and a peripheral zone with intermediate filaments but lacking any organelles [52]. To our knowledge, this study is the first one in which an ultrastructural characterization of undifferentiated SCAPs is described.

The analysis of mesenchymal surface markers in undifferentiated SCAPs revealed the expression of CD29, CD44, CD105, and CD117. A subpopulation of the cells expressed the markers CD146 and Stro-1 [34, 75, 178]. Furthermore, in some cell cultures, a few cytokeratin positive cells could be detected (2.3% of the cell population) probably originating from not properly removed epithelial cells surrounding the apical papilla during the isolation. Vimentin, an intermediate filament found in cells of mesodermal origin was also present in undifferentiated cells [179]. To investigate the neural differentiation potential of SCAPs, the basal neural marker expression of the cells has to be evaluated. Undifferentiated SCAPs expressed neural markers β-III-tubulin, GalC, S100, and synaptophysin. A subset of the cells are immune-reactive for neurofilament. The expression of neural markers in undifferentiated SCAPs can be explained by their neural crest origin, giving the SCAPs a predisposition of differentiation towards neural lineages. Due to their origin, SCAPs might still possess transcripts of neural markers. In addition, the expression of neural markers *in vitro* can be up-regulated by using culture media containing high levels of serum. Similar to SCAPs, DPSC also express several neural markers when grown in culture medium containing high levels of serum (10% FCS). However, when grown in low-serum conditions (2% FCS), DPSC did not express β-III-tubulin and S100 [80]. Therefore, when investigating neural differentiation, basal levels of neural marker expression in non-differentiated cells must be taken into account.

SCAPs are derived from a developing tissue and may represent a population of early stem/progenitor cells. Considering the neural crest derived origin of the apical papilla, the expression of neural crest markers in SCAPs was evaluated. Hnk-1, NGFRp75, Sox-9, ErbB3 and transcription factor AP-2 mRNA transcripts were found in undifferentiated SCAPs, confirming their neural crest derived origin. However, since NGFRp75 expression could not be detected after immunocytochemical staining, the presence of neural crest markers on the mRNA level does not imply the expression on the protein level. SCAPs isolated in this study could have lost only part of their neural crest derived characteristics, since the cells showed a positive immune-reactivity for nestin which is considered to be a neuroectodermal marker [180, 181]. Furthermore, the expression of Rex-1, Sox-2, Nanog and Oct-4 transcripts in undifferentiated

SCAPs suggests a pluripotent profile. However, no expression of the pluripotent markers Sox-2, Nanog and Oct-4 was detected after FACS analysis (data not shown), suggesting that SCAPs possess the transcripts of pluripotent markers at mRNA level but have lost the expression of markers at the protein level during development of the tooth or during the isolation and culture procedures. However, several studies already indicate the differentiation potential into adipogenic, osteogenic, myogenic and neurogenic lineages, indicating that the differentiation potential of SCAPs is not restricted to mesodermal lineages. Nevertheless, further investigation is needed to confirm the pluripotency of SCAPs regarding their marker expression and differentiation potential.

There is no exact location of SCAPs in apical papilla tissue described in the literature. By means of immunohistochemistry, the expression of several mesenchymal and neural markers *in situ* have been analyzed. CD29, CD44, CD146, nestin and vimentin expression was found throughout the whole tissue. To identify the niche of SCAPs, mesenchymal markers can be used in combination with for instance CD24, a marker specific for SCAPs. Neural marker expression was related to neural structures, suggesting that *in vitro* cell cultures gain their neural marker expression after isolation and via culture media containing high serum levels [80].

SCAPs exhibited a multilineage potential *in vitro,* differentiating into adipogenic, chondrogenic and osteogenic lineages when grown under the appropriate culture conditions. Adipogenic differentiation resulted in the accumulation of lipid droplets in the cytoplasm, stained with Oil-Red-O staining, and the expression of fatty acid binding protein-4. The ultrastructural analysis confirmed the presence of cytoplasmatic lipid droplets in differentiated cells. In the absence of lipid droplets, dilated rough endoplasmatic reticulum was observed. However, not all cells differentiated into a adipocyte-like phenotype.

In contrast to previous studies in which DPSC undergo chondrogenic differentiation, monolayer's of SCAPs were used instead of 3D-sphere cultures. Upon chondrogenic differentiation, cells expressed aggrecan, a structural glycoprotein of cartilage, but no sign of cartilage fragments could be observed at

the ultrastructural level. However, a high accumulation of vesicles containing extracellular matrix components and collagen fibers in the extracellular space suggests an incomplete chondrogenic differentiation of the cells.
When cells were submitted to osteogenic inducing media, calcium deposits were observed after 2-3 weeks in all cell cultures. Furthermore, *de novo* expression of ALP was seen in differentiated cells. At the ultrastructural level, cells obtained an osteoblast-like morphology with an eccentric nucleus. Several striated collagen fibres and mineralized nuclei were located in the extracellular space. The globular secretion of mineralized nuclei together with the presence of hydroxyapatite needles confirms a real mineralization in the cell culture.

The present study characterizes SCAPs both at the immunocytochemical and ultrastructural level. We found a mesenchymal stem cell population residing inside the apical papilla capable of undergoing multilineage differentiation into adipocytes, chondrocytes and osteoblasts. We emphasize the use of (transmission) electron microscopy to evaluate a real differentiation of the cells through morphological/structural changes. SCAPs are derived from an immature tissue, the apical papilla, and can be easily isolated from human third molars that are frequently removed for orthodontic reasons. SCAPs could be a excellent resource of stem cells with a high potential in future tissue regeneration studies.

6

Summary and General Discussion

In the last decade, cell-based therapies are emerging as a novel therapeutic option in a wide variety of neurological disorders such as Alzheimer's and Parkinson's disease [182]. These neurodegenerative disorders are characterized by the loss or degeneration of neurons leading to functional impairment. As treatment, therapies are based on delaying the deleterious effects from the loss of neurons or other neural cell types [183]. Neural stem cells are the most ideal cell source to be applied in stem cell-based therapies for neurological disorders, considering their self-renewal capacity and ability to differentiate into neural cell types such as neurons, astrocytes and oligodendrocytes. However, neural stem cells are difficult to harvest from the adult brain and therefore, other stem cell sources are needed. Several stem cell populations can be found within the adult human body. Among them are the MSC which have been proven to be a multipotent stem cell population being able to differentiate into cells of adipogenic, chondrogenic, osteogenic, myogenic, and neurogenic lineages [7]. In addition, MSC secrete a broad range of bioactive molecules, such as growth factors, cytokines and chemokines, which play an important biological role during injury [6]. MSC can be found in a wide variety of tissues and organs such as bone marrow, umbilical cord blood or tissue, adipose tissue and dental tissues. Several dental tissue-derived MSC-like populations have been isolated and characterized during the last several years such as DPSC. DPSC are considered to originate from the neuroectoderm, leading to the presence of neural characteristics in DPSC [21, 31]. Similar to DPSC, stem cells from apical papilla are found to be a multipotent stem cell population with a high proliferative capacity and being able to differentiate into multiple cell types [34, 35].

In this thesis, we aimed at elucidating the neuronal and glial characteristics of DPSC. Before inducing neuronal or glial cell differentiation, we determined the expression of pluripotent, neural crest, mesenchymal, and neural markers in undifferentiated hDPSC to unravel the marker expression before differentiation. Next, we focused on the neuronal and glial cell differentiation of DPSC and the production of neurotrophic factors before and after glial cell differentiation. In the last chapter of the thesis, we described the isolation and characterization of stem cells from apical papilla. We investigated the pluripotency and neural crest origin of this stem cell population in order to gain insight into their origin,

characteristics and differentiation potential. Therefore, a morphological, immunocytochemical and ultrastructural characterization was performed of *in vitro* cell cultures. In addition, their multilineage differentiation potential towards adipogenic, chondrogenic and osteogenic lineages was determined via immunocytochemical and ultrastructural analysis. The obtained results of this thesis are summarized and discussed in the following paragraphs.

The expression of mesenchymal and neural markers in undifferentiated hDPSC

In most studies, the success of neural differentiation by stem cells is determined by morphological changes and the expression of neural specific makers such as neurofilament and β-III-tubulin. However, the expression of neural markers in undifferentiated hDPSC has to be elucidated before inducing neural differentiation. In that way, the differences found in neural marker expression before and after differentiation will give an indication of the success of differentiation.

In order to gain insight into the neural marker expression of undifferentiated hDPSC, we performed reverse transcriptase PCR and immunocytochemistry on *in vitro* cell cultures. The results obtained in **chapter 2** demonstrated that naïve hDPSC do express neural markers like β-III-tubulin, synaptophysin, neurofilament, and S100 and a subpopulation of the cultures stained positive for GalC and NGFRp75. The expression of neural markers by hDPSC in an undifferentiated state might be explained by their neural crest origin. However, when using culture media with low serum levels (2% compared to 10% used in our experiments), the neural marker expression is lost [80]. Therefore, the use of culture media containing high serum levels, could explain the expression of neural markers in undifferentiated hDPSC cultures due to the presence of several growth factors within serum. Culture media with high concentrations of serum (≥10%) are widely used in DPSC cultures, to ensure cell adhesion during the initial isolation of the cells [31, 64, 184]. Nevertheless, it has recently been shown, that DPSC cultured under low serum conditions (2%), maintained their *in vitro* differentiation capacities toward osteogenic, chondrogenic, endothelial, smooth muscle and neural lineages. However, low serum conditions resulted in

an incomplete neural differentiation with the expression of both immature and mature neural markers. In addition, the efficient growth of DPSC on three-dimensional structures as neurospheres, was achieved only in the presence of serum [80]. Since certain cell culture conditions may interfere with the differentiation potential of hDPSC, the medium composition and/or cell culture conditions have to be carefully considered before investigating the neural differentiation potential *in vitro*. Further research is necessary to determine whether DPSC, grown in low serum conditions, will be able to differentiate into fully matured and functional neurons *in vitro*. In addition, the success of differentiation must not only be based on morphological changes and the expression of neural makers after differentiation, but also functional studies must be performed to determine a successful neural differentiation *in vitro*. Also, immunohistochemical stainings can be performed on dental pulp tissue in order to elucidate the expression of neural markers in a model closer to the *in vivo* situation.

In order to explore the location of stem cell niches in dental pulp tissue, immunohistochemical stainings were performed with antibodies directed against the same neural and mesenchymal markers used in *in vitro* cultures **(chapter 2)**. In our experiments, stainings were executed on young dental pulp tissues meaning that dental pulp tissue was isolated from teeth in a less than 50% root formation stage, and therefore still being an immature tissue. Immunohistochemical staining revealed the presence of two possible stem cells niches: at perivascular regions and at the cementum-enamel border. Stem cells present in the second niche, seemed to migrate apically, suggesting the migration of stem/progenitor cells from the dental papilla to the apex of the tissue. The migration of the dental papilla is known to take place during root formation. In addition, the obtained results indicated that the stem cell niche at the cementum-enamel border disappeared during maturation of the tooth, since only a perivascular niche could be observed in adult dental pulp tissue. This could explain the presence of a migration pattern, in immature dental pulp tissue, seen from the cementum-enamel border to the centre of the tissue. The presence of a perivascular niche in adult dental pulp tissue has been confirmed by a previous study of Shi *et al.* in which cells were labeled with a combination

of markers (stem cell marker Stro-1, the perivascular cell marker CD146, alpha smooth muscle actin and the pericyte associated antigen 3G5) [113]. A recent study also suggested the existence of multiple stem cell niches within the dental pulp containing distinct multipotent DPSC [80] instead of only one perivascular stem cell niche. Therefore, further investigations are needed to elucidate the DPSC niche in more detail and whether one or more stem cell niches are present within dental pulp tissue. In addition, the identification of the exact location of the niches could be helpful in understanding if the isolated multipotent stem cells are derived from one highly proliferative multipotent stem cell population or from committed progenitors belonging to distinct lineages.

Characterization of the neuronal differentiation potential of hDPSC

In **chapter 3**, we focused on the neuronal differentiation potential of hDPSC. Several protocols are available to induce neuronal differentiation of neural and mesenchymal stem cells and can be subdivided into two categories: (1) differentiation via the neurosphere assay meaning the generation of three-dimensional structures called spheres (mainly used for differentiation of neural stem cells), or (2) neuronal induction and maturation via the addition of a cocktail of growth factors on monolayer cell cultures. We used both approaches to induce neuronal differentiation in human DPSC and compared the obtained results based on morphology, expression of neuronal specific markers and ultrastructural characteristics.

The neurosphere and neuronal inductive media assay resulted in different outcomes. Cells grown out of plated neurospheres acquired a bipolar cell morphology similar to neural progenitor cells and even more mature bipolar neurons since a subpopulation of the cultures expressed the neuronal marker NeuN. This expression indicated that the differentiated cells were postmitotic and in a maturation phase [185]. In addition, a polar distribution of synaptophysin positive vesicles was seen between neighbouring cells suggesting a synapse formation between cells. The presence of vesicles at synapses was also observed at the ultrastructural level. In addition, further neuronal characteristics such as the presence of neurotransmitter containing vesicles and numerous cell-cell contacts confirmed the neuronal differentiation potential of

hDPSC. In the future, immune-electron microscopy should be performed to reveal the content of these vesicles. This technique can identify the specific type of neuron, generated after the neuronal differentiation of hDPSC. Also electrophysiological measurements are required to determine the functionality of hDPSC-derived neuron-like cells, obtained via the neurosphere assay. The presence of voltage-dependent sodium and potassium currents and the generation of action potentials are an indication of neuron functionality which can be determined via whole cell patch clamp recordings. By this method, the success rate of hDPSC differentiated into functionally active neurons can be determined into more detail.

The second differentiation approach, meaning differentiation induced via neuronal induction media, showed that part of the cells obtained a typical neuron-like morphology: the cells have a polar cell body with an axon and multiple dendrites. Other cells preserved their flat appearance, possibly serving as an anchor for neuronal differentiated cells. Although differentiated cells acquired a neuron-like morphology, no mature neuronal marker expression such as NeuN was observed in these cells. Nevertheless, the increased expression of neurofilament and decreased expression of Stro-1 indicated that cells were differentiating towards cells of the neural lineage, becoming neural progenitor cells. Ultrastructural observations confirmed the immature neuronal differentiation of hDPSC after the addition of neuronal inductive media. Furthermore, electrophysiological recordings demonstrated the presence of voltage-gated sodium and voltage-gated potassium channels on differentiated cells of which currents were sensitive for TTX and TEA respectively. However, no action potentials could be generated, meaning that no functional active neuron-like cells were generated after differentiation. It has been reported that voltage-gated delayed outward rectifier potassium currents and voltage-gated inward sodium currents are present in neural progenitors [145-147]. These results are another indication that neuronally differentiated cells were not differentiated into fully functional mature neurons, but developed into neural progenitor cells. Further experiments and optimization of the neuronal inductive media are necessary to differentiate hDPSC into more mature functional neurons.

Maturation of the differentiated cells can be achieved by the addition of other growth factors such as cAMP and NT-3. It is known that cAMP signaling pathways promote neuron and glial differentiation [186-189]. Human BM-MSC were also shown to differentiate into neural progenitors in response to PKC activation and increased intracellular cAMP level [157, 190]. In addition, NT-3, together with nerve growth factor, has been demonstrated to play a crucial role during neuronal maturation. Other factors like b-FGF, EGF and retinoic acid were reported to be regulators of cell proliferation and neural commitment [191, 192]. Taken together, several growth factors play a role in the maturation of neural progenitor cells and these factors can be used in our future experiments to develop mature neuron-like cells. The group of Varga *et al.* established a optimal protocol in which cAMP and NT-3 are used, for the differentiation of human DPSC into fully functional neurons. This protocol consist of first a epigenetic reprogramming step using 5-Azacytidine and b-FGF followed by the neuronal induction in which b-FGF, NGF, cAMP and NT-3 are used. The final step includes the maturation step with increased levels of cAMP, continuously addition of NT-3 and other neuroprotective growth factors. After neuronal differentiation of hDPSC, cells which acquired a specific neuron-like morphology after the first step of their protocol were observed. In the final step, the maturation phase, cells expressed increased levels of neuron specific enolase, N-tubulin and GFAP, and also NeuN immunoreactivity was detected. Furthermore, voltage dependent sodium and potassium channels were present, but the generation of action potentials was not investigated [47, 48]. However, no ultrastructural analysis on the cells was conducted, and thus there is no information of the neuronal morphology (like cell-cell contacts, synapse formation) of the obtained cells. Nevertheless, this method of neuronal differentiation established by Király *et al.,* might provide us an efficient protocol for the differentiation of hDPSC into functional active neurons for further *in vivo* studies. However, before applying these pre-differentiated cells in an animal model, the generation of action potentials should be investigated since this is a basic criteria for fully matured functional neurons. Also, in order to gain more insight into the morphological changes during differentiation and to confirm the formation of synapses, transmission and scanning electron microscopy will be performed. Compared to the neurosphere assay, the differentiation protocol of

Király *et al.* is described to be relative easy and rapid to perform, and highly reproducible.

Several *in vivo* studies showed that non-differentiated DPSC primarily promoted the recruitment, proliferation and neuronal differentiation of endogenous cells of the host organism by secreting neurotrophic factors. However, little or no evidence is available if these cells differentiated into neural cells *in vivo* [55-57, 81, 82]. Therefore, further research is needed to explore (1) if predifferentiated cells can be used as transplants into brain tissue after injury and (2) if they remain neuronally differentiated and will further differentiate in response to traumatic injury. In addition, *in vivo* studies are required to elucidate whether undifferentiated or predifferentiated hDPSC are more appropriate for transplantation into CNS tissue after injury.

In vitro and *in vivo* studies focusing on the directed differentiation of DPSC into specific neuronal cells, for instance dopaminergic neurons, their possible trophic support of degenerating neurons and their general neurosecretoric activity might result in new advances in treatment of central nervous disorders like for example Parkinson's disease.

Characterization of the glial cell differentiation potential of hDPSC

Injury to the inferior alveolar nerve is a severe complication which can occur after extraction of the lower third molars [89, 193]. Although it has been assumed that peripheral nerve injuries can recover, complete recovery is rather rare, misdirected, or associated with neuropathic pain [97]. During the last decade, several techniques have been developed as treatment for peripheral nerve injury such as SC being seeded into conduits, followed by transplantation into the lesion site [194-196]. However, the isolation of SC requires the sacrifice of another peripheral nerve and SC possess a slow and time consuming growing capacity [96, 97]. Recently, promising results were obtained in *in vitro* and *in vivo* studies when using MSC as treatment for peripheral nerve injury [94, 98-103, 197, 198]. Since DPSC are described as MSC-like stem cells, we explored in **chapter 4** whether human DPSC were able to undergo peripheral glial cell differentiation.

Since DPSC are describe to be derived from the neural crest, we first investigated the expression of pluripotency and neural crest markers in hDPSC cultures in order to gain insight into their origin. The results described in **chapter 4** demonstrate that hDPSC possessed transcripts of pluripotency and neural crest markers on the mRNA level confirming their pluripotency and neural crest origin as previously demonstrated [24, 79, 156]. However, it must be noted that the expression of these markers varied between hDPSC cultures due to patient variability and possibly due to the presence of different stem cells niches within dental pulp tissue as illustrated by Atari *et al.* [150]. Furthermore, as explained in chapter 2, the presence of serum in the culture media could have altered gene expression and cells in culture could have divided asymmetrically, resulting in a more differentiated daughter stem cell. Therefore, immunohistochemical stainings have to be performed on dental pulp tissue in order to elucidate the expression of pluripotency and neural crest markers in a model closer to the *in vivo* situation.

To investigate whether hDPSC were able to undergo glial cell differentiation, a mixture of cytokines and growth factors was added to the culture medium in order to induce Schwann cell differentiation. After the differentiation protocol, cells obtained a Schwann cell-like cell morphology and the expression of SC marker GFAP. Furthermore, a decreased expression of nestin and Stro-1 was observed in our experiments. It is commonly known that nestin and Stro-1 expression decrease during differentiation. However, it must be taken into account that also the expression of other (immature) SC markers, like cell surface glycoprotein AN2, myelin protein zero P0 and cell adhesion proteins L1 and N-CAM, have to be further explored to determine if the differentiation of hDPSC into Schwann cells succeeded. In addition, by performing direct co-culture experiments with DRG neurons, the myelination ability should be investigated in which both immunostaining for myelin basic protein (MBP) and electron microscopical analysis have to be performed. MBP is required for myelin compaction and is therefore frequently used as an indication of Schwann cell myelination [197, 199]. Ultrastructural analysis can give more detailed information whether compact myelin is formed by the differentiated cells.

When considering hDPSC as treatment for peripheral nerve injury, hDPSC must not only be able to differentiate into Schwann-like cells, but they must also be capable of producing and secreting neurotrophic factors that support neuronal cell survival and regulate axonal outgrowth and guidance. For that reason, we investigated the secretion of neurotrophic factors by undifferentiated and SC-differentiated hDPSC. Our results demonstrated that several growth factors were produced and secreted by both hDPSC and SC-hDPSC. A significant increased production of BDNF was observed after SC differentiation. BDNF is known to promote neuronal rescue and survival, and neurite outgrowth and guidance. In addition, other factors, such as GDNF and NCAM-1, were secreted by both naïve and differentiated hDPSC. Further experiments are necessary to quantify whether there is a difference in the secretion amount of these growth factors before and after differentiation. In addition, both hDPSC and SC-hDPSC were shown to promote survival and the neurite outgrowth of dorsal root ganglia neurons after the addition of conditioned medium. Furthermore, SC-hDPSC possess significantly more neuroprotective and regenerative capacities *in vitro* than naïve hDPSC. In order to gain more insight in which growth factors are responsible for these characteristics, further experiments need to be conducted by adding neutralizing antibodies against BDNF or other growth factors in the conditioned medium of undifferentiated and SC-hDPSC. In addition, animal studies need to be performed to investigate and compare the neuroprotective and regenerative capacities of hDPSC and SC-hDPSC *in vivo*. As our *in vitro* data clearly indicate that SC-hDPSC have a better neuroprotective and neurotrophic effect than naïve stem cells, we suggest that SC-hDPSC might provide a better treatment of PNS traumas than naïve hDPSC *in vivo*.

Characterization of stem cells from apical papilla

Dental stem cells are widely investigated during the last several years. Due to their ready accessibility, high proliferative capacity, ease of isolation and multilineage differentiation potential, dental stem cells are considered to be a potential cell source for tissue engineering or cell based replacement studies [37, 38, 175]. In **chapter 5**, SCAPs were investigated concerning their marker expression and multilineage differentiation potential into adipocytes,

chondrocytes and osteoblasts. Although, several publications already provide evidence of their multilineage differentiation potential, no adequate reports are available investigating the chondrogenic differentiation potential of SCAPs. Our results demonstrated that SCAPs were able to differentiate into cells of the chondrogenic lineage. Although *in vitro* cultures showed the expression of aggrecan, a structural glycoprotein of cartilage, no signs of cartilage fragments could be observed at the ultrastructural level compared to the hDPSC [52]. However, a high accumulation of vesicles, containing extracellular matrix components, and collagen fibers in the extracellular space suggest an incomplete chondrogenic differentiation of the cells. In addition, no electron microscopical analysis of the multilineage differentiation is previously reported [34, 35, 76, 78, 177, 178]. To our knowledge, we were the first to provide ultrastructural evidence of the morphological changes upon differentiation, which is essential to determine the success of differentiation.
Compared to DPSC, SCAPs possess a higher proliferation rate and an increased capacity for *in vivo* dentin regeneration. In addition, unlike DPSC and MSC, SCAPs possess telomerase activity which is also present in embryonic stem cells. This might indicate that SCAPs are a immature cell source, which have shown to have great potential in root development, apexogenesis, pulp/dentin regeneration, and bioroot engineering [34, 35, 37, 78, 177, 178]. Overall, SCAPs are derived from a developing tissue that may represent a population of early stem/progenitor cells. SCAPs may thus be a superior cell source for tissue regeneration compared to DPSC. However, whether SCAPs are more neurogenic than DPSC warrant further investigations.

In conclusion, the results presented in this thesis demonstrate that hDPSC already express neuronal and glial markers in the undifferentiated state. Therefore, careful consideration of marker expression after differentiation must be made. However, the success of 'real' differentiation can not only be based on neural and glial marker expression, but functionality experiments and ultrastructural analysis are indispensable. Our results showed that DPSC are able to differentiate into neuronal-like and glial cell-like cells, but further research is required to explore whether naïve or predifferentiated DPSC are more potent in the regeneration of CNS/PNS injury *in vivo*.

7

Nederlandse Samenvatting

Therapieën die gebaseerd zijn op het gebruik van cellen, worden de laatste jaren steeds vaker beschouwd als een mogelijke toepassing in de behandeling van neurologische aandoeningen zoals Alzheimer en Parkinson. Deze neurodegeneratieve aandoeningen zijn gekarakteriseerd door het verlies en/of afsterven van neuronen waardoor functionele tekortkomingen ontstaan. Het vertragen van deze schadelijke effecten, veroorzaakt door het verlies van neuronen of andere celtypen, is een mogelijke therapie die als behandeling ontwikkeld kan worden.
Neurale stamcellen worden beschouwd als de meest ideale bron voor stamceltherapie in neurologische aandoeningen aangezien zij een zelf-vernieuwende capaciteit bezitten en kunnen differentiëren tot neurale cellen zoals neuronen, astrocyten en oligodendrocyten. Echter, neurale stamcellen zijn moeilijk te isoleren vanuit een volwassen hersenstel waardoor er een nood is aan andere stamcelbronnen. Het volwassen menselijk lichaam bevat verschillende bronnen van stamcellen verspreid over het hele lichaam waaronder de mesenchymale stamcellen (MSC). Deze multipotente stamcelpopulatie is in staat om te differentiëren naar cellen van adipogene, chondrogene, osteogene, myogene en neurogene cellijnen. Bovendien is er geweten dat MSC groeifactoren, cytokinen and chemokines secreteren die een belangrijke rol spelen tijdens weefsel- of celschade. Recent is er bewezen dat de dentale pulpa een MSC-achtige celpopulatie bevat, de dentale pulpa stamcellen (DPSC), die door hun afkomst van de neurale lijst, neurale eigenschappen bezitten.

In de meeste studies wordt het succes van neuronale differentiatie gebaseerd op morfologische veranderingen tijdens differentiatie en op de expressie van specifieke neurale makers na differentiatie zoals neurofilament en β-III-tubulin. Echter, de expressie van deze specifieke markers in culturen van ongedifferentieerde DPSC moet worden uitgeklaard alvorens de neuronale differentiatie bestudeerd kan worden. Op deze manier kan het verschil in de expressie van neuronale markers voor en na differentiatie een indicatie zijn voor het succes van de differentiatie. De resultaten weergegeven in **hoofdstuk 2** tonen aan dat ongedifferentieerde humane DPSC neurale markers tot expressie brengen zoals β-III-tubulin, synaptophysin, neurofilament en S100. Een subpopulatie van deze cellen brengt ook GalC en NGFRp75 tot expressie. De

expressie van deze neurale markers in niet-gedifferentieerde humane DPSC kan verklaard worden door hun afkomst van de neurale lijst. Echter, studies waarbij medium met een lage concentratie serum (2% in plaats van 10%) gebruikt wordt, verdwijnt de expressie van deze neurale markers. Het serum bevat verschillende groeifactoren waardoor het gebruik van hoge serumconcentraties in het medium een andere mogelijke verklaring kan zijn voor de expressie van neurale markers in onze controle culturen. Hoewel hoge serumconcentraties (10%) nodig zijn voor celadhesie in de initiële fase van de cultuur, is er aangetoond dat DPSC hun *in vitro* differentiatie capaciteit behouden richting osteogene, chondrogene, endotheliale en neurale cellijnen wanneer deze in cultuur gebracht worden in media met lage serumconcentraties (2%). Daarentegen is er aangetoond dat medium met 2% serum leidde tot een onvolledige neuronale differentiatie waarbij DPSC zowel immature als mature neurale markers tot expressie brachten. Verder onderzoek is dan ook noodzakelijk om te bepalen of DPSC in lage serumconcentraties kunnen differentiëren naar volwassen en functionele neuronen *in vitro*.

Aangezien de samenstelling van het medium en de omstandigheden waarin DPSC in cultuur gehouden worden, een invloed heeft op de differentiatie, moet men goed overwegen welke celkweek condities er gebruikt worden alvorens de neuronale differentiatie te bestuderen. Bovendien mag het succes van differentiatie niet alleen gebaseerd zijn op de morfologische veranderingen en de marker expressie na differentiatie, maar moeten er ook functionele studies uitgevoerd worden om zo een succesvolle differentiatie *in vitro* te bepalen.

Immunohistochemische kleuringen van neurale markers op dentale pulpaweefsels kunnen uitgevoerd worden om een beeld te krijgen van de neurale marker expressie in een model dat dichter bij de *in vivo* situatie aanleunt.

Door middel van deze immunohistochemische kleuringen, aangevuld met markers voor mesenchymale stamcellen, kan de locatie van DPSC in het weefsel, de dentale pulpa, onderzocht worden. Voor deze experimenten werd jong pulpaweefsel geïsoleerd uit tanden waarbij minder dan 50% van de wortel gevormd was. In dit stadium wordt het pulpaweefsel nog als immatuur weefsel beschouwd. Immunohistochemische kleuringen toonden aan dat er mogelijk twee stamcelbronnen aanwezig zijn in het immature pulpaweefsel: perivasculair

en aan de cementum-enamel grens. Stamcellen, aanwezig in deze laatst genoemde stamcelbron, leken een mogelijk migratiepatroon te vertonen richting de apex van het weefsel. Dit kan duiden op de migratie van de dentale papilla die plaatsvindt tijdens de wortelvorming. Bovendien verdwijnt deze stamcelbron tijdens de maturatie van de tand, aangezien enkel een perivasculaire stamcelbron teruggevonden kon worden in pulpaweefsel van volwassen tanden. Andere onderzoeksgroepen hebben eveneens de aanwezigheid van een perivasculaire stamcelbron aangetoond, hetzij door gebruik van andere markers (stamcel marker Stro-1, perivasculaire celmarker CD146, alpha smoot muscle actin en pericyte associated antigen 3G5). Recent werd er gesuggereerd dat er meerdere stamcelbronnen aanwezig zijn in het pulpaweefsel in plaats van één enkele perivasculaire stamcelbron. Verder onderzoek is nodig om de aanwezigheid van één of meerdere stamcelbronnen in het pulpaweefsel aan te tonen.

In **hoofdstuk 3** hebben we gefocust op de neuronale differentiatie van humane DPSC. Verschillende protocols zijn beschikbaar voor de neuronale differentiatie van neurale en mesenchymale stamcellen en kunnen onderverdeeld worden in twee categorieën: (1) differentiatie via het genereren van neurosferen en (2) neuronale inductie en maturatie via het toevoegen van verschillende groeifactoren aan de celculturen.

Beide protocols werden toegepast in onze experimenten en een vergelijkende studie werd gemaakt op basis van celmorfologie, de expressie van specifieke neuronale markers en ultrastructurele kenmerken na differentiatie.
De neurosfeer en neuronal inductive media assay leidden tot verschillende resultaten. Cellen afkomstig van de neurosfeer assay vertoonden een bipolaire celmorfologie gelijkaardig aan neuronale progenitor cellen. Deze celmorfologie kan eveneens vergeleken worden met mature bipolaire neuronen doordat een subpopulatie van de cellen de mature neuronale marker NeuN tot expressie bracht. Verder werd een polaire verdeling waargenomen van vesikels die positief aankleurden voor synaptophysin. Dit fenomeen kan duiden op een synaps vorming tussen twee naburige cellen. Ultrastructurele analyse bevestigde de aanwezigheid van vesikels. Immuno-elektronen microscopie kan uitgevoerd

worden om de inhoud van deze vesikels te ontdekken waardoor het type neuron, dat gevormd wordt na neuronale differentiatie, geïdentificeerd kan worden. Eveneens kunnen er elektrofysiologische metingen uitgevoerd worden om de functionaliteit van de cellen te testen. Whole cell patch clamp metingen kunnen de mogelijke aanwezigheid van Na en K voltage gevoelige kanalen detecteren die nodig zijn voor het genereren van actiepotentialen.

Via de tweede differentiatie methode, de neuronal inductive media assay, verkreeg een deel van de celpopulatie een typische neuronale celmorfologie: cellen vertoonden een cellichaam met een axon en meerdere dendrieten. De overige cellen behielden hun vlakke morfologie en functioneerden mogelijk als anker voor de neuronale gedifferentieerde cellen. Aangezien geen NeuN expressie werd waargenomen, kunnen we besluiten dat de gedifferentieerde cellen met neuronachtige celmorfologie geen mature neuronen zijn. Echter, een daling in de expressie van Stro-1 en een verhoogde expressie van neurofilament duidt erop dat de cellen wel differentieerden richting neurale progenitor cellen. Ultrastructurele analyse bevestigde de immature neuronale differentiatie van de humane DPSC. Met behulp van elektrofysiologische metingen werden Na en K voltage gevoelige kanalen waargenomen waarvan de stromen geblokt kon worden door respectievelijk TTX en TEA. Echter geen actiepotentialen kon gegenereerd worden. Er is beschreven dat neurale progenitor cellen voltage gevoelige Na en K kanalen bezitten, hetgeen overeenstemt met onze resultaten. We kunnen hieruit besluiten dat neuronale differentiatie via de neuronal inductive media assay resulteerde in neurale progenitor cellen in plaats van mature neuronen. Verdere experimenten met aangepaste media, zullen uitgevoerd worden om DPSC te differentiëren naar meer mature neuronen.

De groep van Varga *et al.* heeft een protocol ontwikkeld voor de differentiatie van humane DPSC naar mature en functionele neuronen. Ze hebben echter geen ultrastructurele karakterisatie uitgevoerd door middel van transmissie elektronen microscopie. Op deze manier kunnen de morfologische veranderingen tijdens differentiatie waargenomen worden, net als de vorming van synapsen tussen de cellen.

Verschillende *in vivo* studies hebben aangetoond dat DPSC na transplantatie, de proliferatie en neuronale differentiatie van endogene cellen promoten door het secreteren van neurotrofe factoren. Er is echter weinig of geen bewijs of de

getransplanteerd DPSC ook differentieerden in neurale cellen *in vivo*. Daarom is verder onderzoek noodzakelijk om na te gaan of (1) neuronale pre-gedifferentieerde cellen als transplantaat gebruikt kunnen worden in hersenweefsel na schade en (2) of deze hun differentiatie behouden en mogelijk verder differentiëren in respons op weefselschade. Verdere *in vivo* studies zijn dan ook nodig om op te helderen of niet-gedifferentieerde DPSC of neuronale pre-gedifferentieerde DPSC beter geschikt zijn als transplantaat in weefsel van het centrale zenuwstelsel na schade.

In **hoofdstuk 4** werd de gliale differentiatie van DPSC onderzocht. Bij perifere zenuwschade, zoals schade aan de nervus alveolaris inferior, is een volledig herstel uitzonderlijk of geassocieerd met neuropatische pijn. Verschillende technieken zijn ontwikkeld in de behandeling van perifere zenuwschade waarbij Schwann cellen uitgezaaid worden op een drager en vervolgens getransplanteerd worden op de plaats van de laesie. Echter het gebruik van Schwann cellen heeft ook zijn nadelen: voor de isolatie van Schwann cellen moet een andere perifere zenuw opgeofferd worden en Schwann cellen hebben een trage groeicapaciteit. Hierdoor is er nood aan een andere bron van cellen. Recent zijn er veelbelovende resultaten behaald in *in vitro* en *in vivo* studies waarbij mesenchymale stamcellen gebruikt werden in de behandeling van perifere zenuwschade. Aangezien DPSC mesenchymale stamcel eigenschappen bezitten, kunnen ook DPSC een alternatieve bron zijn.
De gliale differentiatie van DPSC werd geïnduceerd door het toevoegen van cytokinen en groeifactoren aan het cultuurmedium. Cellen verkregen een celmorfologie gelijkend op Schwann cellen en brachten na differentiatie GFAP tot expressie. Ook was er een daling in de expressie van nestine en Stro-1 na differentiatie waargenomen hetgeen dat tijdens differentiatie ook moet plaatsvinden. Nochtans, moet ook de expressie van andere (immature) Schwann cell markers geanalyseerd worden zoals myelin protein zero P0 en celadhesie proteïnen L1 en N-CAM. Verder kan de myelinisatie capaciteit van de gedifferentieerde cellen nagegaan worden door middel van cocultuur experimenten met DRG neuronen. Zowel via immunokleuringen voor MBP als via een ultrastructurele analyse kan meer informatie verworven worden of er al dan niet (compact) myeline gevormd wordt door de gedifferentieerde cellen.

In de behandeling van perifere zenuwschade is niet enkel de gliale differentiatie van de cellen belangrijk, maar ook de mogelijkheid tot het secreteren van neurotrofe factoren die helpen in de neuronale overleving en bij het reguleren van de axonale uitgroei en geleiding.
Onze resultaten toonden aan dat zowel niet-gedifferentieerde als gedifferentieerde DPSC verschillende groeifactoren produceren en secreteren zoals BDNF, GDNF, NCAM-1. De secretie van BDNF was significant verhoogd na gliale differentiatie. BDNF speelt een belangrijke rol in het promoten van neuronale overleving en neuriet uitgroei. Verdere experimenten zijn nodig om uit te klaren of ook de andere factoren na gliale differentiatie meer geproduceerd en gesecreteerd worden. Bovendien had het geconditioneerd medium van zowel niet-gedifferentieerde als gedifferentieerde DPSC een positieve invloed op de overleving en op de axonale uitgroei van DRG neuronen. Gedifferentieerde DPSC hadden daarenboven significant verhoogde neuroprotectieve en regeneratieve capaciteiten ten opzichte van de niet gedifferentieerde DPSC. Om meer inzicht te verkrijgen in welke groeifactoren verantwoordelijk zijn voor deze eigenschappen, kunnen experimenten uitgevoerd worden waarbij neutraliserende antilichamen toegevoegd worden aan het geconditioneerd medium van beide celtypen. De regeneratieve en neuroprotectieve eigenschappen van zowel niet-gedifferentieerde als gedifferentieerde cellen zullen vervolgens in dierexperimenten onderzocht en vergeleken worden.

Naast de DPSC, behoren ook de SCAPs tot de dentale stamcellen. Deze stamcelpopulatie is eveneens gemakkelijk te isoleren en bezit een hoge proliferatie capaciteit. In **hoofdstuk 5** werd de marker expressie en de differentiatie potentiaal richting adipocyte, chondrocyte en osteoblast onderzocht. Hoewel verschillende publicaties reeds bewijs gaven van hun differentiatie mogelijkheden, zijn er geen geschikte publicaties beschikbaar betreffende de chondrogene differentiatie. Onze resultaten demonstreerden dat SCAPs kunnen differentiëren tot chondrogene cellen. Hoewel de *in vitro* culturen de expressie van aggrecan vertoonden, een structureel glycoproteïne van kraakbeen, werden er geen kraakbeen fragmenten waargenomen op ultrastructureel niveau. Daarentegen was een verhoogde hoeveelheid vesikels

met extracellulaire matrix componenten en collageenvezels in de extracellulaire ruimte aanwezig wat duidt op een onvolledige chondrogene differentiatie. Zover geweten, zijn wij de eerste die een ultrastructurele analyse voorzien van de morfologische veranderingen die plaatsvinden tijdens differentiatie. Dit is noodzakelijk in het bepalen van het succes van differentiatie.
Vergeleken met de DPSC bezitten SCAPs een hogere proliferatie snelheid een verhoogde capaciteit voor *in vivo* dentine regeneratie en telomerase activiteit. Verschillende studies hebben reeds aangetoond dat SCAPs een belangrijke rol spelen in wortel ontwikkeling, apexogenese, pulpa/dentine regeneratie en bioroot engineering. SCAPs zijn afkomstig van een weefsel in ontwikkeling, de apical papilla, en vertegenwoordigen een populatie van meer vroege stamcellen. SCAPs kunnen dus een betere celbron zijn voor weefselregeneratie in vergelijking met DPSC. Of SCAPs ook een betere neurogene differentiatie capaciteit en eigenschappen bezitten dan DPSC, vergt verder onderzoek.

De resultaten weergegeven in deze thesis geven aan dat humane DPSC neuronale en gliale markers tot expressie brengen vóór differentiatie. Daarom moet er een goede keuze gemaakt worden welke neuronale of gliale markers na differentiatie onderzocht worden. Bovendien mag het succes van differentiatie niet alleen bepaald worden door morfologische veranderingen en specifieke marker expressie, maar moeten er ook functionele studies en ultrastructurele analyses toegevoegd worden aan de experimenten. Onze resultaten toonden aan dat DPSC kunnen differentiëren tot neuronachtige en Schwann cell-achtige cellen, maar verder *(in vivo)* onderzoek is noodzakelijk om uit te klaren of niet-gedifferentieerde of gedifferentieerde DPSC meer potentieel bezitten in de regeneratie van CZS/PZS trauma's.

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Curriculum Vitae

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