NEURAL DIFFERENTIATION OF DENTAL PULP STEM CELLS

by

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Abstract

A variety of stem/progenitor populations have been isolated from human dental tissue over the past decade. Of these, dental pulp stem cells (DPSCs) are the best characterised. DPSCs reside in a perivascular niche within pulp tissue. Dental pulp originates from cranial neural crest (CNC) cells that migrate and differentiate into a number of cell types, including neurons, during embryonic development. Due to their CNC origin, DPSCs constrictively express certain neural markers, have neurosphere-forming abilities, and have been able to differentiate down the neural lineage *in vitro*.

In this study, we set out to differentiate rat DPSCs down the neural lineage using a variety of 2D monolayer differentiation protocols originally designed for human DPSCs. Previous studies have indicated that neurosphere formation is a prerequisite for the successful neural induction of rat DPSCs. However, neurosphere formation is labour intensive and is not amenable for robotic scale-up. Our results indicate poor neural induction across all medium formulations tested, as analysed by morphology and immunocytochemistry. Subpopulations of undifferentiated DPSCs expressed early neural markers, but these markers were not upregulated following neural induction. Further work is necessary to optimise the differentiation protocol to work efficiently with rat DPSCs as opposed to human cells.

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List of Abbreviations

ASC	Adult stem cell
BM	Bone marrow
CFU-F	Colony forming unit-fibroblastic
CNC	Cranial neural crest
dcAMP	Dibutryl cAMP
DCX	Doublecortin
DFPC	Dental follicle progenitor cells
DPSC	Dental pulp stem cell
ESC	Embryonic stem cell
FBS	Foetal bovine serum
GF	Growth factors
GFAP	Glial fibrillary acidic protein
ITTS	Insulin-transferrin-sodium selenite
LNGFR	Low-affinity nerve growth factor receptor
MSC	Mesenchymal stem cell
NC	Neural crest
NHS	Normal horse serum
NSC	Neural stem cell
P/S	Penicillin/streptomycin solution
PBS-T	PBS-Tween
PDL	Periodontal ligament
PDLSC	Periodontal ligament stem cells
PLO	Poly-L-ornithine
PSA-NCAM	Poly-sialated neural cell adhesion molecule
RA	Retinoic acid
SC	Stem cell
SCAP	Stem cells from apical papilla
SHED	Stem cells from human exfoliated deciduous teeth
SP	Side population

Chapter 1

Introduction

1.1 Stem Cells from Dental Tissue

Stem cells (SCs) have the capacity for self-renewal and multilineage differentiation at the clonal level (Weissman, 2000). They can be split into two groups: embryonic stem cells (ESC), which have the ability to differentiate into cells from all three germ layers, and adult stem cells (ASC), which are more restricted in their potency (Tarnok et al., 2010). Due to the safety and ethical issues surrounding ESC research, many groups have focused on identifying and charactering ASCs for future therapies (Watt and Driskell, 2010).

The best characterised ASC populations reside in the bone marrow (BM). Of these, BM-derived mesenchymal stem cells (MSCs) are considered as a potential cell source for stem cell therapies due to their plasticity and potent immunosuppressive capabilities (Nombela-Arrieta et al., 2011). Due to difficulties (e.g. pain, morbidity) in obtaining BM aspirates from patients, alternative sources of therapeutic MSCs have been sought. To this end, MSC-like populations have been identified in adipose tissue (Zuk et al., 2002), umbilical cord blood (Lee et al., 2004), tendons (Bi et al., 2007), amniotic fluid (Tsai et al., 2004) and dental tissues (Gronthos et al., 2000).

The isolation of MSC-like populations from dental tissue holds many advantages over more 'traditional' sources. Teeth are an easily accessible, non-essential organ that can be collected with minimal ethical issues after routine dental extractions or the exfoliation of deciduous teeth (Modino and Sharpe, 2005). Several populations of stem/progenitor cells have been identified in human teeth (Figure 1). These include dental pulp stem cells (DPSCs; Gronthos et al., 2000), stem cells from human exfoliated deciduous teeth (SHED; Miura et al., 2003), stem cells from apical papilla (SCAP; Sonoyama et al., 2008), periodontal ligament stem cells (PDLSC; Seo et al., 2004) and dental follicle progenitor cells (DFPC; Morsczeck et al., 2005) .

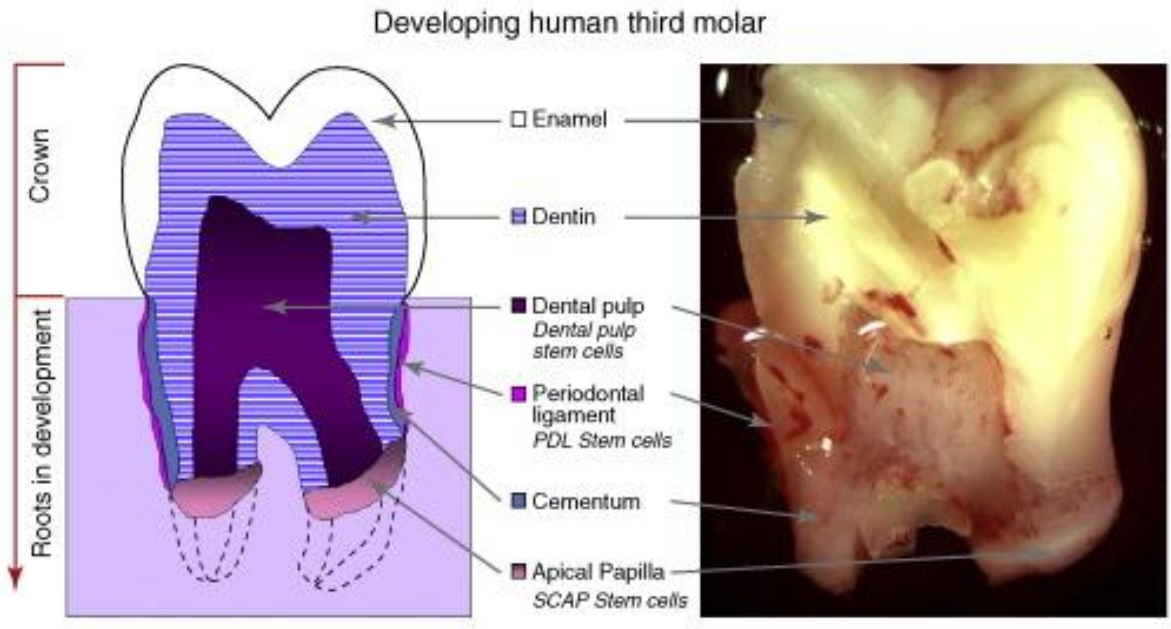

Figure 1 | **Anatomical locations of stem cells in human dental tissue**. The human third molar ('wisdom tooth') is commonly used for the isolation of dental stem cells. The basic anatomy of a hemisected tooth showing the locations of DPSCs/SHED, PLDSCs and SCAP is shown. Figure taken from Volponi *et al.*, 2010.

1.1.1 Dental Pulp Stem Cells

Seminal work by Gronthos *et al*. identified the presence of plastic-adherent cells within adult dental pulp that exhibited colony forming unit-fibroblastic (CFU-F) ability (Gronthos et al., 2000). Interestingly, DPSCs had significantly higher proliferation rates and CFU-F than BM-MSCs. DPSCs were negative for haematopoietic markers CD45, CD14 and CD34 and expressed MSC markers Stro-1 and CD146 (Gronthos et al., 2000). However, most markers were not uniformly expressed, suggesting that DPSC cultures contained heterogeneous populations of stromal cells, a common disadvantage of the plastic-adherence method of isolating MSC-like cells (Bianco et al., 2008). *In vitro* differentiation showed that DPSCs formed sparse nodules of calcification and failed to differentiate into adipocytes (Gronthos et al., 2000). Later studies revealed DPSCs could differentiate down the adipogenic and neural lineages when the protocols were lengthened (Gronthos et al., 2002). Papaccio *et al*. showed that cryopreserved DPSCs retained their marker profile and *in vitro* differentiation ability, suggesting that these cells can be 'banked' for future uses (Papaccio et al., 2006).

In vivo transplantation of DPSCs on an appropriate scaffold resulted in the creation of an ectopic pulp-dentin complex composed of vascularised pulp-like tissue surrounded by odontoblasts that secreted dentin (Gronthos et al., 2000). Injection of GFP^+ DPSCs in a rodent myocardial infarction model resulted in improved cardiac function when examined four weeks post-transplantation (Gandia et al., 2008). This improvement was mediated by the secretion of trophic factors, as no GFP^+ cells had engrafted. Similar clinical improvements have been seen in models of muscular dystrophy (Kerkis et al., 2008) and Parkinson's disease (Apel et al., 2009).

Parallel to events in the MSC field, many groups raced to find markers to prospectively isolate DPSCs from pulp tissue (Volponi et al., 2010). Initial studies utilised the STRO-1 antigen to isolate clonogenic DPSCs from pulp tissue (Shi and Gronthos, 2003). STRO-1^+ DPSCs co-expressed CD146 and the pericyte marker 3G5, and were found in a perivascular niche *in vivo*. BM-MSCs are also localised to a perivascular niche and some groups have suggested that pericytes are the *in vivo* 'MSC' (Meirelles et al., 2008). Iohara *et al*. isolated side population (SP) cells from dental pulp that displayed enhanced stem cell characteristics (Iohara et al., 2006). SP cells were also found in a perivascular niche, and were able to differentiate into chondrocytes *in vitro*, a characteristic previously not attributed to DPSCs.

1.1.2 Stem Cells from Human Exfoliated Deciduous Teeth

Three years after the isolation of DPSCs, Miura and Gronthos repeated their DPSC isolation protocol on exfoliated deciduous teeth and were able to isolate a population of proliferative cells with CFU-F potential (Miura et al., 2003). SHED were more proliferative than DPSCs, and were also capable of multi-lineage differentiation. SHED shared a similar antigen profile to DPSCs, and were also found in a perivascular niche. They were able to differentiate into functional odontoblasts *in vitro*, but were unable to recreate a pulp-dentin complex *in vivo* (Miura et al., 2003).

Interestingly, SHED also expressed certain neural markers (Nestin, GFAP, NeuN and βIII-tubulin) and were able to form sphere-like clusters *in vitro*. When cultured under neurogenic conditions, SHED developed long, multicytoplasmic processes reminiscent of neurons. Neural-primed SHED transplanted into the dentate gyrus of immunocompromised mice

were shown to survive for 10 days (Miura et al., 2003). A more recent study transplanted SHED spheres into the striatum of parkinsonian rats (Wang et al., 2010). They reported improved behavioural outcomes in treated animals, but suggested that the improvements seen were due to the release of trophic factors. These findings, coupled with the increased proliferative potential and differential gene expression profile (Nakamura et al., 2009), suggest that SHED represent a distinct, more immature population of stem cells than DPSCs.

1.1.3 Stem Cells from Apical Papilla

The apical papilla is a neural crest-derived tissue that appears during root development prior to tooth eruption (Volponi et al., 2010). Sonoyama and colleagues identified a population of STRO-1^+ cells on the root apical papilla that were able to form CFU-F (Sonoyama et al., 2006). SCAP were able to differentiate *in vitro* into odontoblasts and adipocytes, and formed a pulp-dentin complex when transplanted *in vivo* (Sonoyama et al., 2008). SCAP shared a similar antigenic profile to DPSCs, but also expressed various neural markers such as nestin, βIII-tubulin, neurofilament, and NeuN after stimulation in neurogenic medium (Sonoyama et al., 2008, Abe et al., 2007). In contrast to DPSCs, SCAP exhibited improved proliferation, migration and telomerase activity, suggesting that SCAP and DPSCs identify two discrete stem cell populations (Huang et al., 2008).

1.1.4 Periodontal Ligament Stem Cells

The periodontal ligament (PDL) is a specialised connective tissue originating from neural crest cells. Its main function is to support the tooth in the alveolar bone ('tooth socket') and to act as a shock absorber during mastication (Petrovic and Stefanovic, 2009). Miura and Gronthos again repeated their isolation technique for DPSCS/SHED on human PDL and isolated a population of PDLSCs that were clonogenic and highly proliferative (Seo et al., 2004). Immunohistochemical analysis showed that PDLSCs again resided in the perivascular region, as reported for other dental SC subsets (Chen et al., 2006). These cells were positive for STRO-1/CD146 and were able to form calcium rich deposits and adipocytes *in vitro*. Their isolation technique was also successful in isolating PDLSCs from 3-year old cryopreserved PDLs (Seo et al., 2005). When transplanted *in vivo*, PDLSCs formed a cementum-PDL complex similar in structure to native PDL and were able to repair a surgical PDL defect in rodent models (Seo et al., 2004).

A more recent study isolated and cultured rat PDLSCs as neurospheres in suspension (Techawattanawisal et al., 2007). Early spheres expressed the neural markers nestin, Sox2, Sox9 and GFAP. When removed form suspension and plated down, PDLCs differentiated into $MyoD^+$ muscle fibres, neurofilament-positive neurons, $GFAP^+$ astrocytes and CNPase-positive oligodendrocytes (Techawattanawisal et al., 2007).

1.1.5 Dental Follicle Progenitor Cells

The dental follicle is another neural crest-derived tissue that is responsible for the development of PDL, cementum and alveolar bone (i.e. all supporting tissues of a tooth; Huang et al., 2009). Stem/progenitor cells were isolated from enzymatically digested human dental follicles based on plastic-adherence (Morsczeck et al., 2005). These cells had CFU-F capabilities, expressed STRO-1 and nestin, and were able to differentiate into cementoblasts and adipocytes *in vitro* (Morsczeck et al., 2010). Yao *et al*. demonstrated that rat DFPCs could also differentiate into neurofilament-positive neurons *in vitro* (Yao et al., 2008). A recent study compared the neurogenic potential of DFPCs and SHED (Morsczeck et al., 2010). They conclude that both sets of cells have neural differentiation potential, but SHED consistently expressed more late-stage markers such as MAP2 when cultured in the same conditions. Finally, Dai and co-workers recently showed that DFPCs cultured in hypoxic conditions exhibited enhanced proliferation and differentiation down the osteogenic and adipogenic lineages (Dai et al., 2011).

1.2 Dental Stem Cells and Neural Repair

1.2.1 Clinical Need

There is an urgent clinical need for novel therapies to combat neural damage and degeneration in many human conditions, such as Alzheimer's disease, Parkinson's disease, and spinal cord injury (Lindvall and Kokaia, 2010). For most neurodegenerative disorders, currently available therapies range from surgery to rehabilitative care, with many patients still suffering a poor quality of life (Coutts and Keirstead, 2008). It is hoped that novel SC therapies could potentially replace lost neural tissue or facilitate endogenous regeneration.

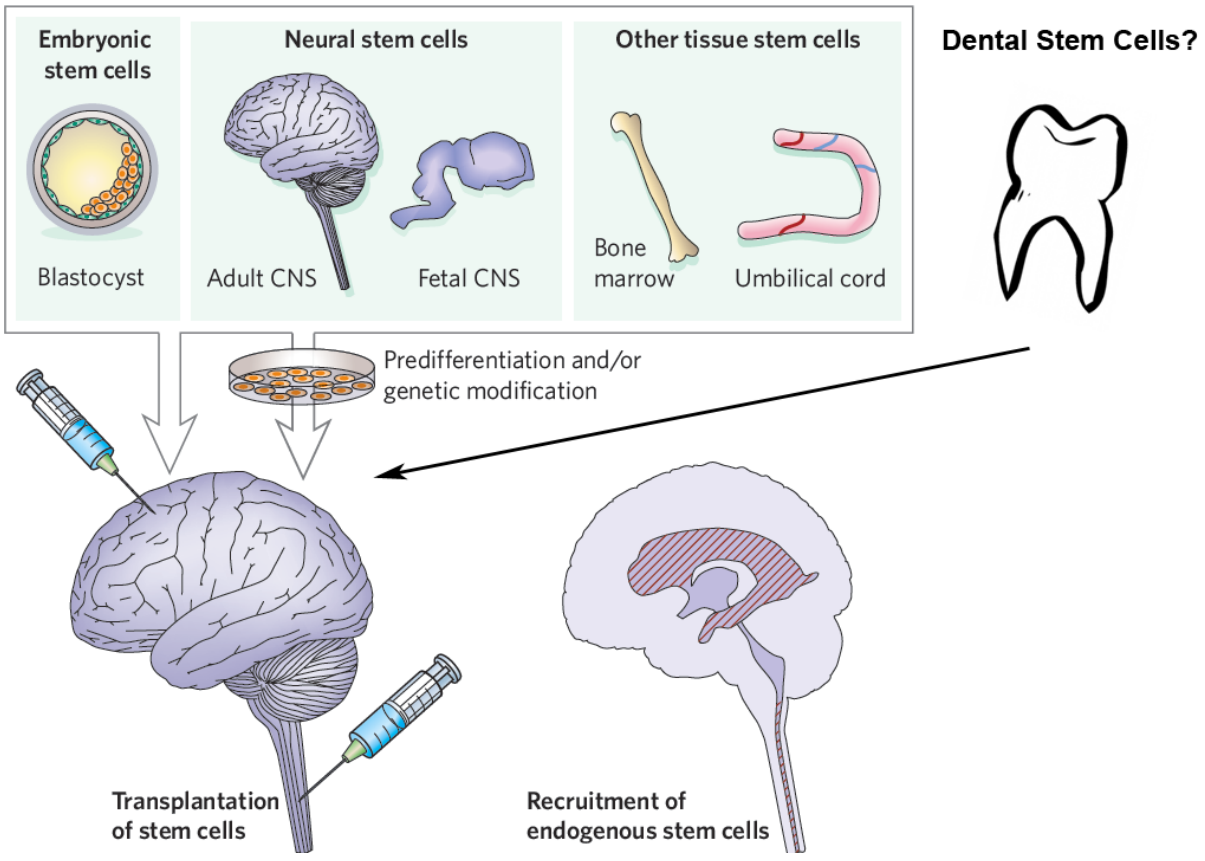

Figure 2 | **Stem cell sources for neuroregeneration therapies**. Immature or pre-differentiated ESCs, NSCs and MSCs have been studied in detail for potential clinical uses. By contrast, dental SCs have not gained as much publicity, but their inherent potential to differentiate down the neural lineage can lead to the creation of novel therapies. Picture edited from Lindvall and Kokaia, 2006.

To date, the majority of studies have utilised ESCs, neural stem cells (NSCs) or MSCs as a stem cell source (Figure 2; Lindvall and Kokaia, 2006). ESC-based therapy holds great promise, but the ethical and safety issues surrounding ESC research still needs to be overcome. Other groups have studied NSCs as they are already neurally-committed (Coutts and Keirstead, 2008). However, harvesting NSCs from humans remains a major hurdle. MSCs isolated from BM or other sources have also been studied for their neurogenic potential, due to their ability to differentiate into non-mesenchymal tissue *in vitro* (Sensebe et al., 2010). However, protocols for the neural differentiation of MSCs are relatively inefficient, and the clinical improvements seen in rodent models were due to the secretion of trophic factors rather than engraftment and differentiation (Meyer et al., 2010).

1.2.2 Potential of Dental Stem Cells

As described previously, the neurogenicity of SCs from dental tissues appears to be greater than that of BM-MSCs (Huang et al., 2009). This is widely attributed to the extensive contribution of neural crest (NC) cells in tooth development (Chai et al., 2000). The vertebrate NC is a transient, multipotent, migratory population of cells that gives rise to both ectodermal and mesenchymal tissues throughout the embryo (Figure 3; Knecht and Bronner-Fraser, 2002). The cranial neural crest (CNC) cells contribute extensively to craniofacial development (Chai et al., 2000). Most of the mature tooth has a CNC origin, including dental pulp, apical papilla, PDL and dental follicle mesenchyme – all places where dental stem cells have been identified and isolated (Figure 4; Miletich and Sharpe, 2004). This close relationship between dental and neural tissues has led many researchers to utilise dental SCs for future neuroregeneration strategies.

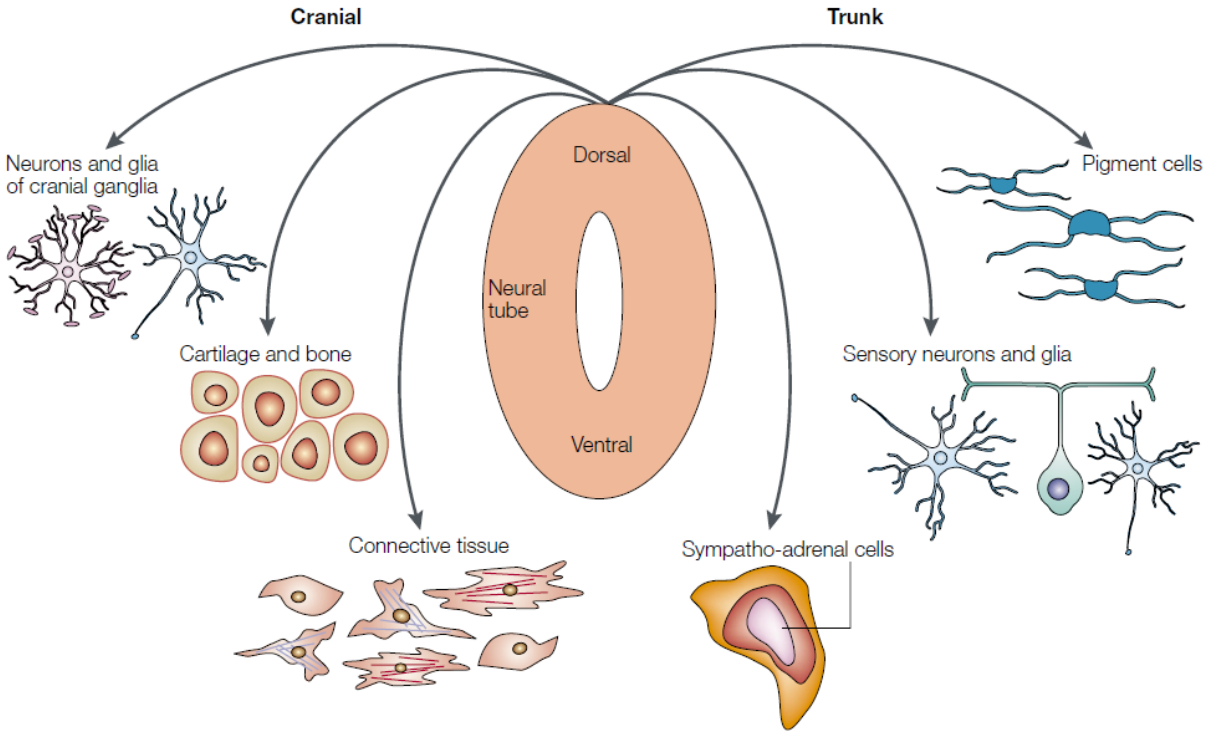

Figure 3 | **Fate of neural crest cells during development**. Vertebrate NC cells can give rise to many lineages during embryonic development. The fate of NC cells depends on where they migrate to. Cranial NC cells contribute heavily to craniofacial development, giving rise to most mesenchymal and neural structures in the head and neck. Dorso-laterally migrating trunk NC cells give rise to the melanocytes, while ventral trunk NC cells make up the sensory nervous system. Figure taken from Knecht and Bronner-Fraser, 2002.

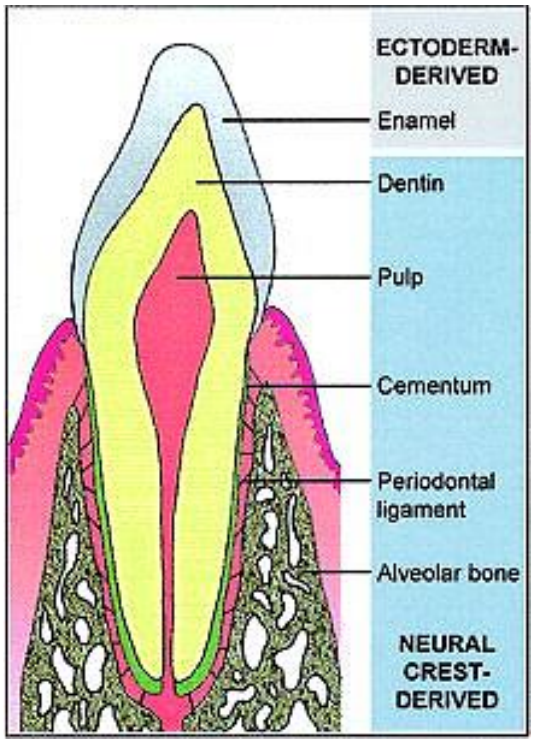

Figure 4 | **Contribution of CNC cells to mammalian teeth**. CNC-derived cells make up most of the living part of teeth, as indicated above. Only enamel (secreted by ameloblasts) has an ectodermal origin. Figure taken from Miletich and Sharpe, 2004.

1.2.3 *In vitro* Neural Differentiation of Dental Stem Cells

Initial studies for the *in vitro* directed differentiation of DPSCs towards the neural lineage mirrored previous work in the MSC and NSC fields (Morsczeck et al., 2010). NSCs can be propagated in specialised serum-free medium as free-floating neurospheres in the presence of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF; Reynolds and Weiss, 1992). To induce differentiation, these spheres are plated down onto coated substrates in the absence of growth factors (GFs). Neural progenitors begin to migrate out and differentiate into neurons or glia in a random manner (Figure 5; Vescovi et al., 2006). By stimulating certain signalling pathways, researchers are able to direct differentiation down specific neural lineages (Rajan and Snyder, 2006).

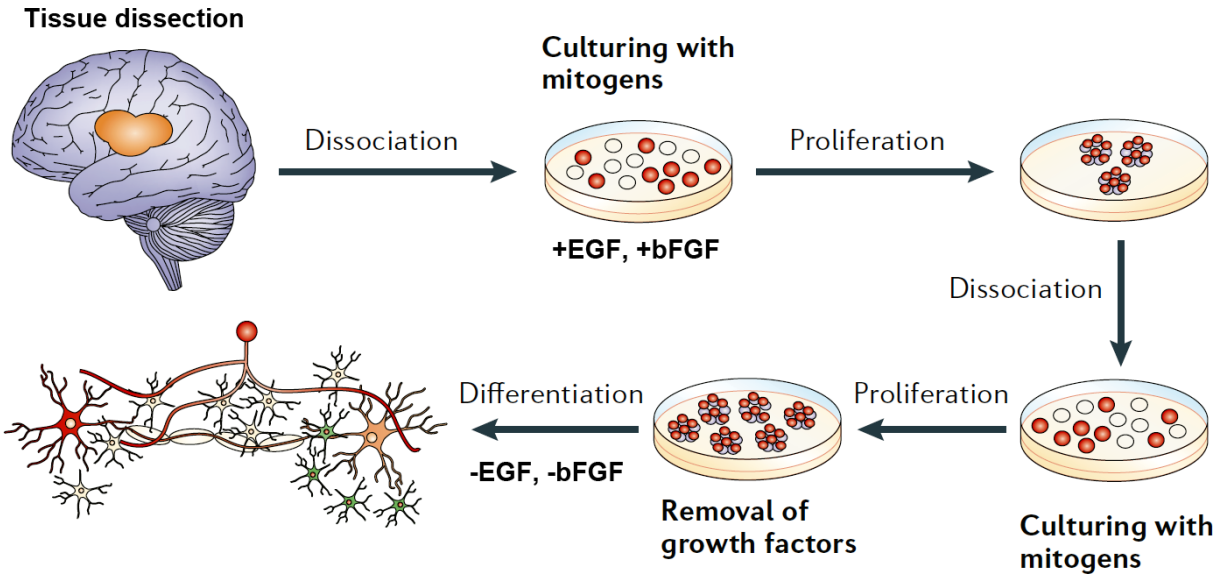

Figure 5 | **The neurosphere assay**. NSCs are isolated and cultured in specialised serum-free medium in the presence of EGF and bFGF. The lack of serum results in the death of most cells, but potential progenitors respond to mitogenic stimuli and form free floating neurospheres. These can be dissociated as single cell suspensions and re-plated numerous times. Removal of GFs from culture medium results in the random differentiation towards neurons, astrocytes and oligodendrocytes. Figure edited from Vescovi *et al*. (2006).

Miura *et al*. used the neurosphere formation method to randomly differentiate SHED down the neural lineage (Miura et al., 2003). They report the production of neuron-like cells expressing βIII-tubulin, neurofilament and nestin, as well as the post-mitotic microtubule protein MAP2. However, differentiation efficiencies were not reported, and no functional examinations were performed (Miura et al., 2003).

A more detailed study by Sasaki *et al*. investigated whether adult rat teeth had neurosphere-forming ability (Sasaki et al., 2008). They report that, unlike SHED, rat DPSCs were dependent on bFGF alone for neurosphere formation. However, these spheres were unable to be serially passaged. When plated down onto poly-L-ornithine (PLO)/fibronectin coated slides, DPSC neurospheres formed small populations of $MAP2^{+}$/$\beta III\text{-tubulin}^{+}$ neurons. Once again, no functional assays were performed to characterise the differentiated progeny.

The Gronthos group published one of the more 'famous' neural differentiation protocols for DPSCs (Arthur et al., 2008). They bypass the neurosphere-culture step of previous protocols and directly differentiate DPSCs as a 2D monolayer. Two differentiation regimes were tested: (1) three weeks' culture in serum-free medium supplemented with EGF and FGF; and (2) a multi-step protocol involving sequential changes of media supplemented with retinoic acid (RA). Both regimes were similarly effective in differentiating DPSCs into $\beta III\text{-tubulin}^{+}$/$\text{neurofilament}^{+}$/$\text{PSA-NCAM}^{+}$ neurons at efficiencies approaching 80%. The electrophysiology of DPSC-derived neurons was recorded using patch-clamp analysis, which revealed the presence of functional voltage-gated sodium (but not potassium) channels.

More recent studies attempted to refine the protocol published by Arthur *et al*. (2008). Kiraly and colleagues described a complex 3-step monolayer differentiation protocol involving the 24 hour pre-treatment of DPSCs with 5-azacytidine, a DNA methyltransferase inhibitor, to revert DPSCs back to a more multipotent state (Király et al., 2009). This was followed by three days' culture in a cocktail of GFs with simultaneous activation of protein kinase C and cAMP pathways to induce neural differentiation. Finally, putative neural progenitors were matured under increased cAMP and neurotrophin levels for three days prior to characterisation. They report impressive differentiation when starting with human DPSCs or PDLSCs, demonstrating the reproducibility of their protocol. They were also able to show a stepwise decrease in the expression of mesenchymal and early neural markers (Vimentin, nestin) and an increase in the expression of post-mitotic markers (NeuN, neurofilament-M) as differentiation progressed. Over 50% of cells were $NeuN^{+}$, and path-clamp analysis proved the function of both voltage-gated sodium and potassium channels (Király et al., 2009). In a follow-up study, Kiraly and co-workers injected their differentiated cells into a rodent model of traumatic brain injury (Király et al., 2011). They showed robust engraftment of labelled cells around the lesion site. This was an important advancement as no *in vivo* study prior to this had successfully transplanted pre-differentiated DPSCs in rodent models of neural damage. These two papers by the Kiraly group represent the current state of the art regarding the directed differentiation of DPSCs into functional neurons *in vitro*.

1.3 Project Aims and Objectives

Dental stem cells have the potential to replace BM-MSCs as the first-choice cell source for stem cell therapies tackling neurodegenerative diseases. The tooth is an easily accessible organ that can be harvested with minimal clinical or ethical issues. DPSCs display a greater proliferative potential than MSCs while having an intrinsic capacity to differentiate easily down the neural lineage due to their CNC origins. Additionally, there has been rapid progress in developing novel differentiation strategies to push DPSCs down the neural lineage.

In this study, we set out to differentiate rat DPSCs down the neural lineage using a combination of the Arthur *et al*. and Kiraly *et al*. protocols (Király et al., 2009, Arthur et al., 2008). It would be interesting to see if rat DPSCs can differentiate into functionally active neurons when cultured in a monolayer, as previous studies have used neurosphere formation to induce differentiation (Sasaki et al., 2008). 2D culture systems are more amenable to automated scale-up, which is a prerequisite for the clinical uses of these cells (Thomas et al., 2009). Differentiated cultures will then be examined for the expression of early, intermediate and late neural markers by immunocytochemistry.

The successful completion of this project should give further evidence for the neurogenic potential of rat dental pulp. Rodent models have traditionally bridged the gap between scientific research and clinical uses. The creation of a reproducible, cost-effective neural differentiation protocol in our laboratory would enable future work assessing the ability of these cells in various rodent models of neural disease (Jay et al., 2011).

Chapter 2

Methods

2.1 Isolation of Rat DPSCs

Male Wistar rats (250-280g; Aston University, Birmingham, UK) were sacrificed by cervical dislocation. The upper and lower incisors were extracted and stored in α-MEM (Biosera, Ringmer, UK) supplemented with 1% penicillin/streptomycin (P/S) solution (Sigma-Aldrich, Dorset, UK). Further dissection was performed in a class II biosafety cabinet using strict sterile technique. The dental pulp was teased out of extracted teeth and mechanically minced until pieces of tissue were $<1mm^3$. Minced pulp was further digested in 0.25% trypsin-EDTA (Sigma) in a rotating incubator for 30 minutes at 37°C. The reaction was stopped using an equal volume of α-MEM supplemented with 20% foetal bovine serum (FBS; Biosera). The cell suspension was then filtered through a 70µm cell strainer (BD Biosciences, Oxford, UK) and pelletted at 1200rpm for 3 minutes. Cell pellets were resuspended in α-MEM supplemented with 20% FBS, 2mM L-glutamine (Sigma) and 1% P/S solution and seeded in a T25 flask (Corning, Amsterdam, NL).

2.2 Cell Culture

Primary DPSC cultures were maintained at 37°C, 5% CO_2 with medium changed every 2-3 days. When confluent, cultures were washed once in PBS and incubated with 0.25% trypsin-EDTA for 5-10 minutes at 37°C. The reaction was stopped in an equal volume of serum-containing medium and pelleted at 1200rpm for 3 minutes. The pellet was resuspended in standard α-MEM maintenance medium containing 10% FBS and expanded as necessary.

2.3 Neural Differentiation of DPSCs

Neural differentiation was induced according to the protocols described by Arthur *et al*. (2008) and Kiraly *et al*. (2009). 24-well tissue culture plates (Corning) were coated with 10µg/ml PLO (Sigma) overnight at room temperature. Following multiple washes in PBS, coated wells were incubated in 5µg/ml laminin (Sigma) for 30 minutes at room temperature. Each well was washed once with PBS prior to use.

Cultured DPSCs at passage 1 (P1) or P5 were re-seeded at a concentration of 25,000 cells/well in coated 24-well plates. Cultures were allowed to expand in standard α-MEM maintenance medium for three days prior to neural induction. Cells were then cultured in medium A, B, C or D for three weeks, as outlined in Table 1. Medium A consisted of rat NSC expansion medium (Millipore, Watford, UK) supplemented with 20ng/ml bFGF (Peprotech EC, London, UK), 20ng/ml EGF (Peprotech) and 1% P/S solution. Medium B was the same as medium A, but without the addition of EGF. Medium C consisted of three separate conditions over the course of the three week period: the first week consisted of culturing

DPSCs in medium A, followed by another seven days in DMEM:F12 (Sigma) supplemented with 1x insulin-transferrin-sodium selenite solution (ITTS; Roche, Burgess Hill, UK), 20ng/ml bFGF and 1% P/S solution. The final week consisted of culture in DMEM:F12 supplemented with 1x ITTS, 20ng/ml bFGF, 1% P/S solution, 1µM all-*trans* retinoic acid (RA; Sigma) and 0.5mM dibutryl cAMP (dcAMP; Sigma). Medium D was the same as medium C, but without the addition of EGF during the first week. Control cultures were kept in α-MEM maintenance medium throughout the procedure. The medium for all five conditions was refreshed twice weekly.

Table 1 | **Overview of neural induction protocol**.

	Medium A	Medium B	Medium C	Medium D	Control
Week 1	NSC Medium 20ng/ml bFGF 20ng/ml EGF 1% P/S	NSC Medium 20ng/ml bFGF 1% P/S	NSC Medium 20ng/ml bFGF 20ng/ml EGF 1% P/S	NSC Medium 20ng/ml bFGF 1% P/S	α-MEM 10% FBS 1% P/S
Week 2			DMEM:F12 ITSS 20ng/ml bFGF 1% P/S	DMEM:F12 ITSS 20ng/ml bFGF 1% P/S	α-MEM 10% FBS 1% P/S
Week 3			DMEM:F12 ITSS 20ng/ml bFGF 1µM RA 0.5mM dcAMP 1% P/S	DMEM:F12 ITSS 20ng/ml bFGF 1µM RA 0.5mM dcAMP 1% P/S	α-MEM 10% FBS 1% P/S

2.4 Immunocytochemistry

Immunofluorescence staining was performed to assess the expression of neural markers from all five culture conditions. Differentiated cultures were fixed in ice-cold 10% neutral buffered formalin for 20 minutes. Cells were permeabilised in 0.2% Triton X-100 in PBS for 5 minutes, and blocked with 5% normal horse serum (NHS) in PBS for 60 minutes at room temperature. Cultures were then incubated overnight at 4°C with the relevant primary antibody diluted in 2% NHS in 0.1% PBS-Tween (PBS-T). A list of primary antibodies, dilutions and suppliers is shown in Table 2.

Table 2 | **Primary antibodies for immunocytochemistry**.

Marker	Stage	Dilution	Species	Supplier
Thy-1 (CD90)	MSCs/DPSCs	1:500	Mouse	Santa Cruz
CD133	NSC	1:400	Rabbit	Abcam
Sox2	NSC	1:200	Mouse	Sigma
Nestin	Neural progenitor	1:600	Mouse	BD Biosciences
PSA-NCAM	Neural progenitor	1:200	Mouse	Developmental Studies Hybridoma Bank
DCX	Neural progenitor	1:200	Goat	Santa Cruz
LNGFR (CD271)	Progenitors and mature cells	1:400	Rabbit	Sigma
GFAP	Astrocytes	1:400	Rabbit	Sigma
βIII-tubulin	Early neuron	1:800	Mouse	Sigma

Abbreviations (in addition to those found in text): PSA-NCAM, Poly-Sialated Neural Cell Adhesion Molecule; DCX, Doublecortin; LNGFR, Low-Affinity Nerve Growth Factor Receptor; GFAP, Glial fibrillary acidic protein.

Wells were washed three times with 0.1% PBS-T prior to incubation with the relevant secondary antibodies diluted in 2% NHS in PBS-T for 60 minutes at room temperature. A list of secondary antibodies, dilutions and suppliers is shown in Table 3. Negative controls were set up by omitting incubation with primary antibodies. Wells were again washed three times with 0.1% TBS-T to remove any unbound secondary antibody. Finally, wells were mounted in Vectorshield mounting medium containing DAPI (Vector Labs, Peterborough, UK) prior to imaging.

Table 3 | **Secondary antibodies for immunocytochemistry**.

Fluorochrome	Dilution	Specificity	Supplier
Alexa 594	1:1000	Goat anti-Rabbit	Molecular Probes
Alexa 594	1:1000	Goat anti-Mouse	Molecular Probes
Alexa 488	1:2000	Donkey anti-Goat	Molecular Probes

Chapter 3

Results

3.1 Isolation of Rat DPSCs

Putative DPSCs were liberated by enzymatic digestion of rat dental pulp. 24 hours after plating, cultures were composed mainly of non-adherent haematopoietic cells, with a few fibroblast-like adherent cells. Clonal expansion of the adherent population resulted in primary cultures of DPSCs reaching confluence within 10-12 days. Interestingly, they exhibited cobblestone morphology reminiscent of endothelium rather than a characteristic MSC-like spindle shapes (Figure 5A). However, this morphology was retained after extended culture (Figure 5B), and other members of our group have been able to differentiate rat DPSCs down the osteogenic and adipogenic lineages, suggesting that rat DPSCs display a flatter, cobblestone-like morphology than human DPSCs *in vitro*.

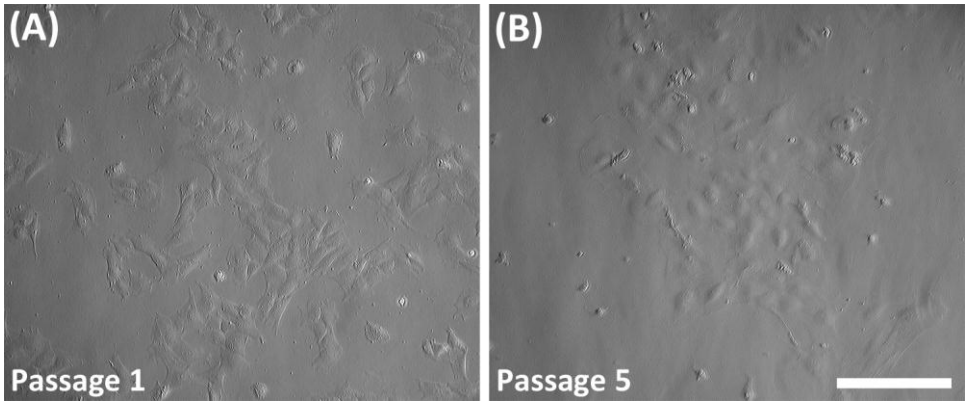

Figure 5 | **Morphology of rat DPSCs**. Representative images of rat DPSCs grown in α-MEM+10% FBS at (A) Passage 1 and (B) Passage 5. All images were taken at 100x magnification. Bar, 25µm.

3.2 Neural Induction of Rat DPSCs

In this study, we tested the effectiveness of multiple medium formulations (*n*=1 for each) in pushing early (P1) and late (P5) passage DPSCs down the neural lineage. Medium A consisted of culturing DPSCs in serum free medium containing bFGF and EGF for three weeks. Previous papers have utilised this approach to increase the expression of neural markers in human DPSCs (Arthur et al., 2008, Gronthos et al., 2002), SHED (Miura et al., 2003), and SCAP (Sonoyama et al., 2008). Medium B was the same as medium A without the addition of EGF, as an earlier paper showed that EGF was not required for the neural induction of rat DPSCs (Sasaki et al., 2008).

Medium C was a more complex protocol for the stepwise induction of neural fate. For the first week, DPSCs were cultured in a NSC specific media with bFGF and EGF to expand the numbers of NSCs/neural progenitors in culture. The second week consisted of DMEM:F12 basal medium supplemented with bFGF and ITTS, a supplement that provides purified factors normally found in serum. The final week consisted of DMEM:F12 supplemented with RA, a factor commonly used to prime ESCs down the neural lineage (Kim et al., 2009), and dcAMP, a non-hydrolysable derivative of cAMP that promotes and sustains second messenger signalling (Király et al., 2009). Medium D was the same as medium C without the addition of EGF during the first week.

Representative images of the morphological changes during differentiation can be seen in Figure 6. At P1 and P5, medium A and B cultures failed to show any neural-like morphology. Instead, cultures reached confluence and retained their cobblestone morphology. A

reduction in cell density could be seen during the later stages, and this might be due to cells lifting off the plastic due to overcrowding. Interestingly, at P1 medium B cells began to round-up by day 15 (indicated by arrow) and eventually died out by day 21. This phenomenon was not seen with P5 cultures.

The use of multi-step induction media C and D seemed more effective. Both P1 and P5 cells began to proliferate during the early stages, but a reduction in cell density could be seen at the midway point. Once exposed to RA, there was a dramatic change in morphology from a cobblestone monolayer to spiky, elongated cells (as indicated by arrows). These cells were found in areas of low cell density, suggesting that space to spread might be crucial for efficient differentiation. However, no complex neurite outgrowths or bipolar neurons could be observed in culture, suggesting that these cells failed to efficiently differentiate into neurons *in vitro*.

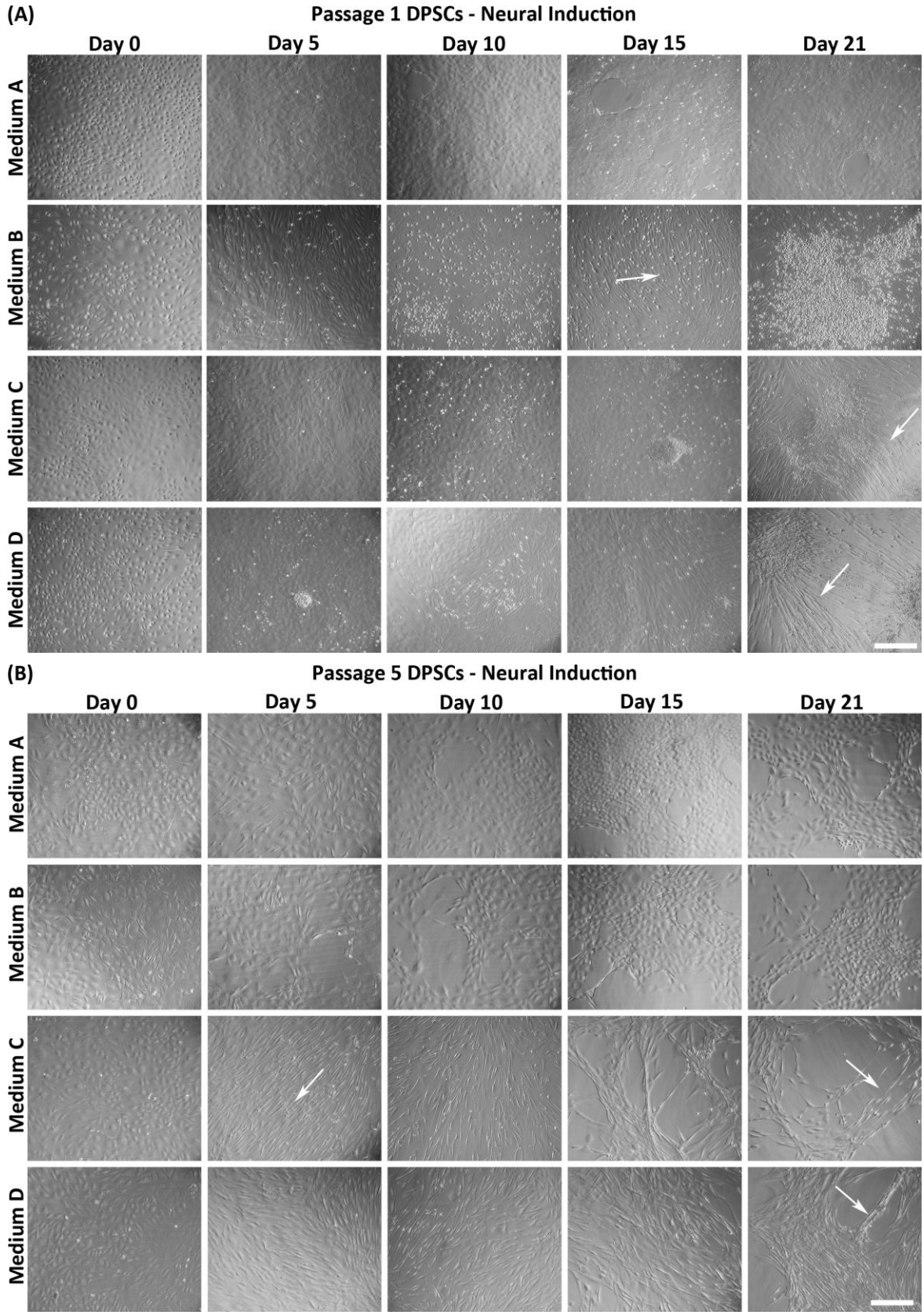

Figure 6 | **Morphological changes during neural differentiation**. Snapshots of (A) P1 DPSCs and (B) P5 DPSCs differentiating down the neural lineage in our four medium formulations. Arrows point at regions of interest described in text. All images were taken at 100x magnification. Bar, 25µm.

3.3 Analysis of Neural Marker Expression

The expression of several markers indicative of the differentiation state was analysed using immunocytochemistry (Figures 7 and 8). The results obtained painted a mixed picture, with many contradictory results and the persistent expression of MSC markers, suggesting that neural induction was incomplete. A typical immunoreactivity profile for both populations of cells can be seen in Table 4, and secondary antibody only controls are displayed in Figure 9.

Thy-1 (CD90) is a cell surface protein that is a typical marker of DPSCs and MSCs (Dominici et al., 2006). However, Thy-1 expression can also be detected in mature neurons (Tokugawa et al., 1997) and fibroblasts (Koumas et al., 2003). As expected, undifferentiated DPSCs expressed high levels of Thy-1 at P1 and P5. However, this expression was maintained in the differentiated cultures on cells that did not display neuron-like morphology. This suggests that the induction protocol has produced fibroblast-like cells from DPSCs, a phenomenon commonly reported in other papers (Király et al., 2009, Arthur et al., 2008).

We then analysed the expression of NSC markers CD133 and Sox2 (Sun et al., 2009), as well as neural progenitor markers nestin (Hendrickson et al., 2011), PSA-NCAM (Varea et al., 2007), DCX (Brown et al., 2003), and LNGFR (CD271; Pruszak et al., 2007). Sox2 expression was not detected in any sample tested here. However, Liu *et al*. identified a subpopulation of human DPSCs that did express Sox2 at earlier passages (Liu et al., 2011). CD133 expression was largely negative, although P1 medium A cultures did express CD133 at a low level. CD133 expression has previously been detected on human DPSCs (d'Aquino et al., 2007), and has been used to enrich for MSC populations from various tissues (Bakondi et al., 2009).

Nestin expression was seen at P1 in undifferentiated DPSCs, but this was lost by P5. No upregulation of nestin was seen when samples (barring P5 medium C and D) were placed in neural induction media, which is surprising as nestin upregulation has been commonly reported in previous literature (Huang et al., 2009). PSA-NCAM expression was not detected in all samples, but we were not confident about the specificity of this antibody and future studies using positive controls will be required to address this issue. Low intensity staining of the microtubule-associated protein DCX was seen in some samples, and there seemed to be an increase in staining at P5. A similar pattern of staining could be seen with LNGFR (CD271), with P5 cultures seeming more intense.

Finally, we analysed the expression of more 'mature' neural markers, GFAP and βIII-tubulin. GFAP expression could be detected in P1 samples under neural induction medium C and D, while P5 samples readily expressed GFAP in all four differentiation conditions. βIII-tubulin expression was seen in P1 undifferentiated DPSCs, but was lost following neural induction. P5 cells readily expressed βIII-tubulin when cultured in media A, B or C. Surprisingly, medium D gave negative results for βIII-tubulin and more repeats are necessary to ensure that this was not an anomalous result.

It is difficult to draw any firm conclusions from this small dataset. So far, our neural induction protocols have been ineffective, although the multi-step formulations (C and D) seem to have greater expression of more mature markers. Additionally, it also seems like P5 cells expressed more late-stage markers than P1 cells. However, more repeats and further experiments are required to back up these statements.

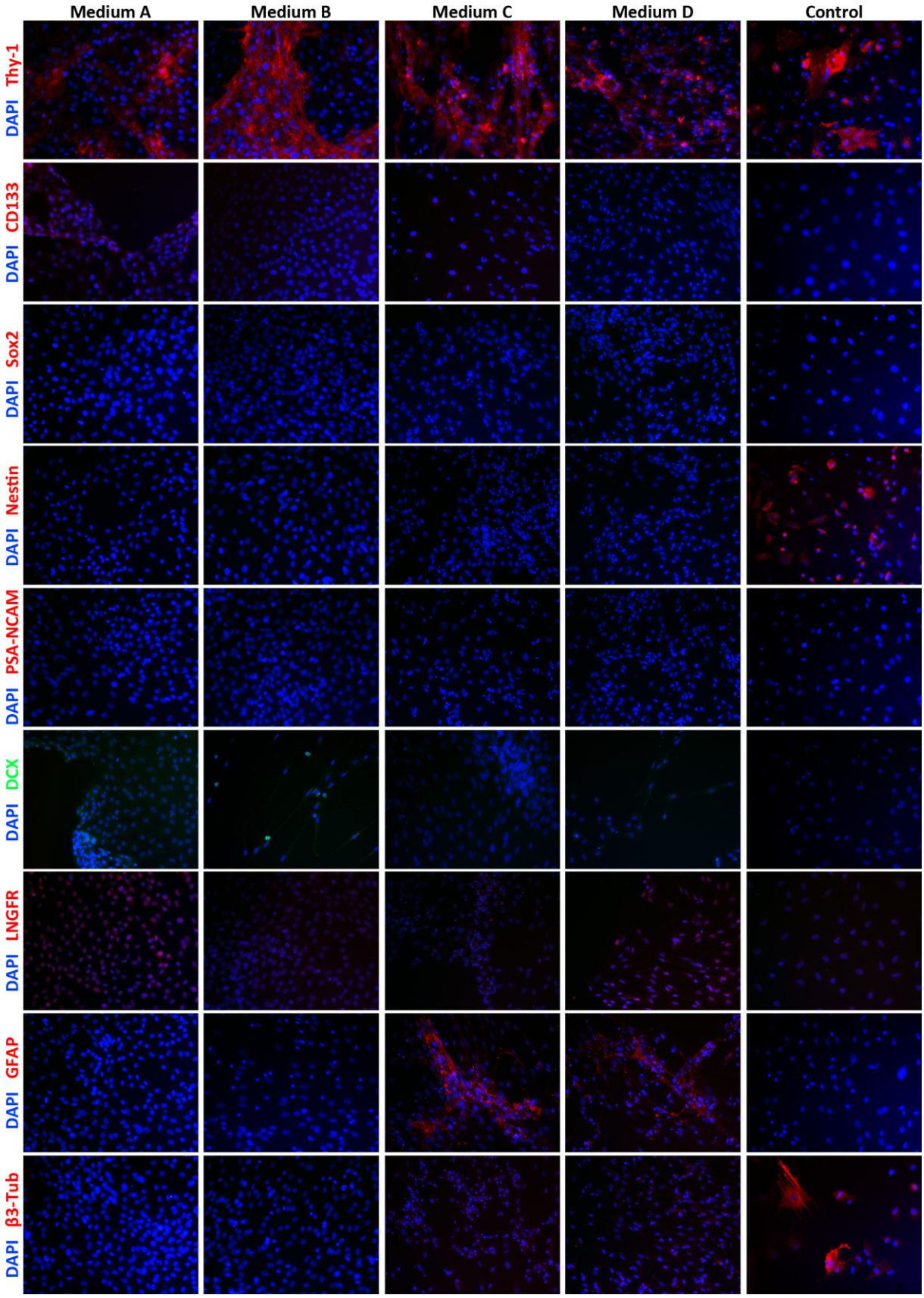

Figure 7 | **Neural Induction of P1 DPSCs**. Day 21 differentiation cultures were stained for the expression of various neural lineage markers as depicted. All images were taken at 200x magnification.

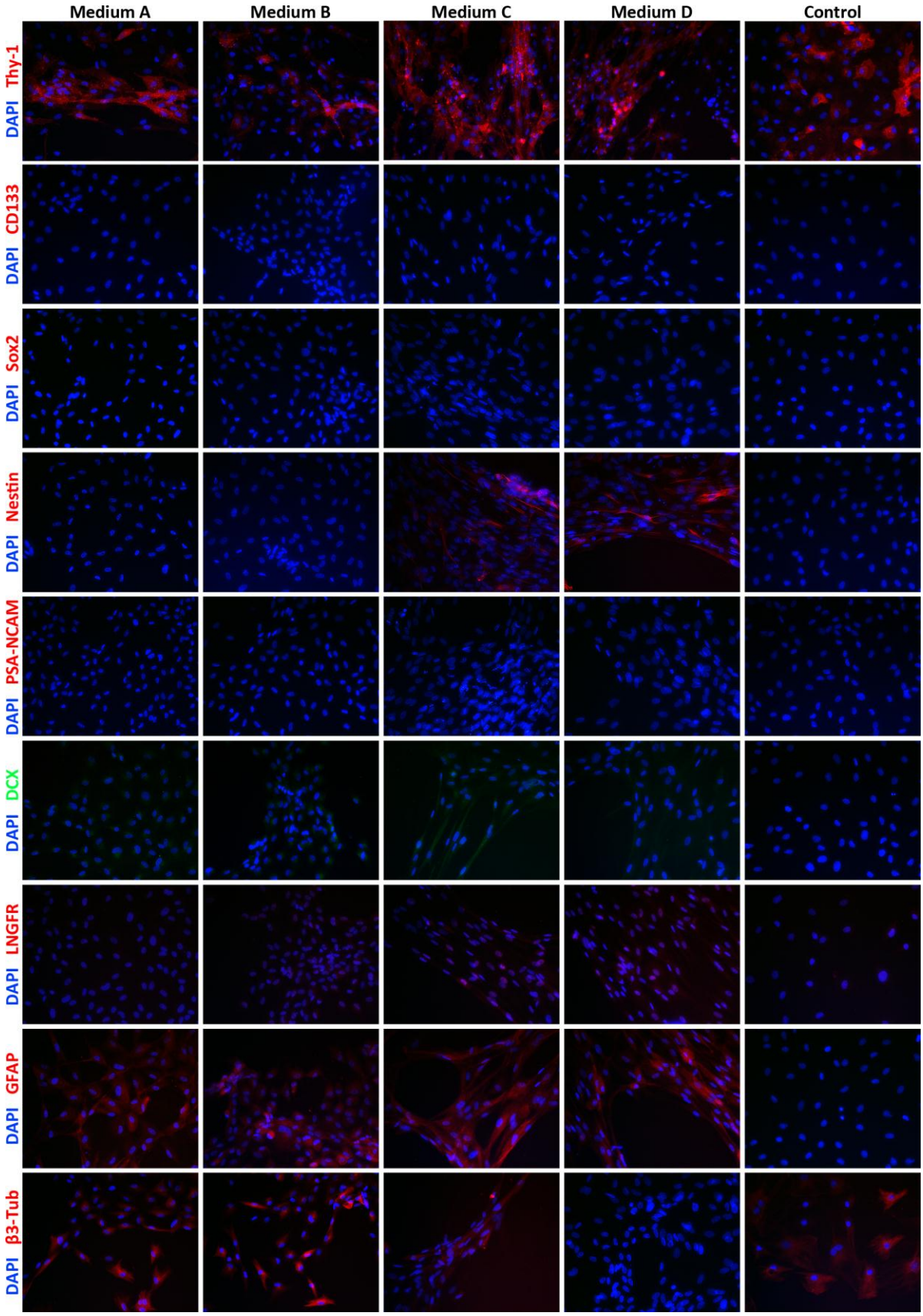

Figure 8 | **Neural Induction of P5 DPSCs**. Day 21 differentiation cultures were stained for the expression of various neural lineage markers as depicted. All images were taken at 200x magnification.

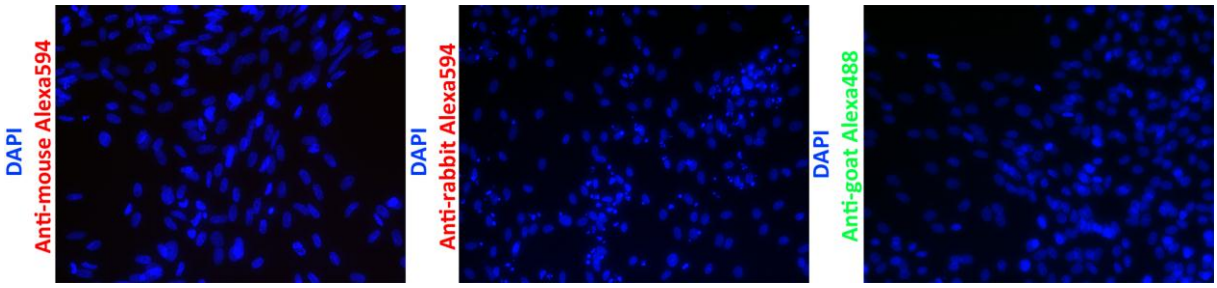

Figure 9 | **Negative controls for Immunocytochemistry**. Negative controls were set up by omitting incubation with the primary antibody. No staining could be observed. All images were taken at 200x magnification.

Table 4 | **Summary of Immunocytochemical Analysis**

Marker	Passage	Medium A	Medium B	Medium C	Medium D	Control
Thy-1	Passage 1	++	+++	+++	+++	+++
	Passage 5	++	++	+++	+++	+++
CD133	Passage 1	+	-	-	-	-
	Passage 5	-	-	-	-	-
Sox2	Passage 1	-	-	-	-	-
	Passage 5	-	-	-	-	-
Nestin	Passage 1	-	-	-	-	++
	Passage 5	-	-	++	++	-
PSA-NCAM	Passage 1	-	-	-	-	-
	Passage 5	-	-	-	-	-
DCX	Passage 1	+	+	-	-	-
	Passage 5	+	-	+	+	-
LNGFR	Passage 1	+	-	-	+	-
	Passage 5	-	-	+	+	-
GFAP	Passage 1	-	-	++	++	-
	Passage 5	++	++	++	++	-
βIII-tubulin	Passage 1	-	-	-	-	++
	Passage 5	++	++	++	-	++

Key: +, weak staining; ++ moderate staining; +++, strong staining; - negative result.

Chapter 4

Discussion

4.1 Isolation of Rat DPSCs

In this study, rat DPSCs were isolated using enzymatic digestion of dental pulp followed by extended culture on tissue culture plastic. Over many passages, any differentiated or non-adherent cells would be lost, leaving behind an enriched population of progenitor cells. This 'plastic-adherence' isolation method was first used to isolate MSCs from BM. However, the adherent population was 80% positive for CD11b and CD45, markers of leukocytes and haematopoietic cells (Phinney et al., 1999). The heterogeneity of primary DPSC cultures has been mentioned in previous publications, but the functional consequences of this have not been addressed (Miura et al., 2003, Gronthos et al., 2000). This could be the reason behind differing DPSC immunoreactivity profiles and differentiation capacities reported in previous literature. In this study, putative DPSCs exhibited uncharacteristic cobblestone morphology rather than the spindle-shaped cells reminiscent of human DPSCs. No further characterisation was performed on these cells, so it remains to be seen whether these were contaminating cells or DPSCs. This could explain the disappointing neural differentiation seen here. However, the cultures did consistently express Thy-1 and nestin, two well-known markers of DPSCs. Future studies should perform a more robust surface marker analysis and *in vitro* differentiation studies to conclusively prove the presence of DPSCs in culture.

As an alternative to plastic-adherence, some groups have used Stro-1 (Shi and Gronthos, 2003) or the side population phenotype (Iohara et al., 2006) to enrich for DPSCs with enhanced CFU-F and differentiation abilities. However, these markers are not specific to DPSCs and can isolate a variety of adherent stromal cells (Huang et al., 2009). Thus, extended culture is still a prerequisite to end up with a more homogenous population of DPSCs.

To date, there have been no publications detailing the prospective isolation of DPSCs. The biology and functional abilities of DPSCs have been deduced from the study of *in vitro* cultured cells, which may exhibit traits specific to culture-manipulated cells alone. Technically speaking, these heterogeneous DPSC cultures do not exhibit the two qualities required to be labelled as stem cells: self-renewal and multipotency *in vivo* (Vats et al., 2005). By comparison, a single, prospectively-identified haematopoietic stem cell has the capability to serially reconstitute haematopoietic niche in irradiated mice (Krause et al., 2001). Similar experiments have recently been performed with prospectively-identified murine MSCs as well (Mendez-Ferrer et al., 2010, Morikawa et al., 2009). Future developments in the dental SC field should work towards addressing these issues and identifying DPSC-specific markers to study these cells *in situ*. The development of novel, dental-specific assays to comprehensively establish SC function would also be of benefit in sorting through the different dental SC subsets identified (DPSCs, SHED, SCAP, PDLSCs, DFPCs). Due to the close relationship between DPSCs and MSCs, key findings in the more mature MSC field could directly impact on dental SCs, and help standardise this emerging branch of stem cell biology.

4.2 Neural Differentiation of DPSCs

There is a clear clinical need for novel therapies to treat millions of patients worldwide who are suffering from neural trauma or neurodegenerative conditions (Lindvall and Kokaia, 2010). Stem cells have the potential to meet this clinical need, as we can expand and differentiate various SC subsets into neuron-like cells *in vitro*. In particular, the CNC origin of many dental SC subsets have given them an almost intrinsic capacity to differentiate down the neural lineage (Miletich and Sharpe, 2004). Several groups have attempted to take advantage of this unique property of DPSCs in rodent models of neural damage, with most reporting favourable outcomes (Petrovic and Stefanovic, 2009).

Therapeutic uses of DPSCs in neural repair would require the creation of simple, scalable and reproducible neural differentiation protocols. Most protocols have been directly transferred over from the ESC, NSC or MSC fields. In this study, we investigated the 2D monolayer neural induction protocols of two papers that were successful in differentiating human DPSCs into functionally active neurons (Király et al., 2009, Arthur et al., 2008). To date, protocols for the neural induction of rat DPSCs require neurosphere formation, which is relatively more labour intensive than standard monolayer cultures (Sasaki et al., 2008). Additionally, 2D monolayers are more amenable to automated scale-up, which is essential to meet the supply required for future clinical or industrial uses (Thomas et al., 2009).

In this study, we tested four medium formulations for the neural induction of rat DPSCs. Medium A and B essentially consisted of three weeks' culture in serum-free medium with the addition of EGF and FGF (medium A) or FGF alone (medium B). Initial ESC differentiation

protocols used serum-supplemented medium to induce differentiation, but the undefined nature of serum influenced the differentiation process and introduced unwanted variability between batches (Kim et al., 2009). More recent protocols have gone the serum-free route and have reported improved yields of neural progenitors and mature neurons (Abranches et al., 2009). However, these studies have utilised proprietary basal medium and supplements (e.g. B27 and N2 supplements), which has made it difficult to ascertain the relative importance of exogenous factors on neural differentiation. In our hands, medium A and B failed to differentiate rat DPSCs into neurons, as analysed by morphology (Figure 6) and immunocytochemistry (Figure 7). Potential reasons for this could include the initial seeding densities, as DPSCs readily expanded and reached confluence in GF-supplemented serum-free medium. Patches of cells that looked more spindly and elongated were found in areas of lower cell density, while confluent patches still retained cobblestone morphology. It is difficult to draw firm conclusions from an experiment that has not been repeated, and we have yet to optimise these medium formulations to work with rat DPSCs instead of human cells. Interestingly, we did not see any observable differences when EGF was added to the differentiation medium, backing up the report by Sasaki *et al*. (2008).

Medium C and D were more complex formulations that attempted to induce DPSCs down the neural lineage in a stepwise manner. FGF signalling was maintained throughout the three week protocol due to the importance of this pathway in inducing a neural fate in ESCs and NSCs (Stavridis et al., 2010). Importantly, the final week of culture exposed differentiating cells to RA and dcAMP. RA is a key molecule involved in the specification and induction of neural differentiation during embryonic development, and for the maintenance

of mature neurons in the adult (Maden, 2007). RA acts as a ligand for the retinoic acid receptor, which, upon activation, forms a heterodimer with the retinoid X receptor and binds to response elements on DNA (Balmer and Blomhoff, 2002). Kim *et al*. identified that RA treatment increased the expression of mature neural genes, such as synaptic molecules, neurotransmitters and receptors, compared to ESCs differentiated in RA-free medium (Kim et al., 2009). Dibutryl cAMP is a non-hydrolysable, cell permeable analog of cAMP that maintains elevated cAMP levels (Király et al., 2009). Increased cAMP levels have previously been shown to induce the expression of certain neural proteins (βIII-tubulin, nestin, NSE and neurofilament) in MSCs (Kim et al., 2005, Deng et al., 2001) and DPSCs (Király et al., 2009). In our hands, rat DPSCs seemed to respond better to medium C and D compared to A and B. The expression of nestin, GFAP and βIII-tubulin was increased, suggesting that the addition of RA and dcAMP has a beneficial effect (Table 4). Additionally, there was a greater number of long, spindly cells in medium C and D cultures compared to A and B.

Finally, our results also suggest that P5 cells expressed more late stage neural markers than P1 cells, irrespective of the differentiation medium used (Table 4). This could be due to the plastic-adherence isolation technique used in this study. At P1, the cultures may have contained a high percentage of progenitors or fibroblastic cells with limited potencies, as seen previously with BM MSCs (Peister et al., 2004). When placed in induction medium, the majority of cells did not respond, resulting in poor differentiation. Conversely, by P5, the majority of contaminating cells would have been lost by serial passaging, resulting in a relatively ‘pure’ population of DPSCs. These cells would be able to respond to neural induction, resulting in an upregulation of characteristic neural markers, as seen here.

4.3 Future Prospects

4.3.1 Optimisation of Neural Induction Protocol

This project has failed to meet the initial aims of differentiating rat DPSCs down a neural lineage. This could be due to species-specific differences, as the protocols we followed were tested on human DPSCs. It still remains to be seen whether rat DPSCs can form functioning neurons without neurosphere formation.

The first stage of optimisation concerns the isolation and characterisation of DPSCs. Future studies could use magnetic sorting to isolate Stro-1^+ cells from dental pulp that exhibit greater neurogenic potential (Shi and Gronthos, 2003). Once in culture, these cells would need to be characterised using growth curves, surface marker expression ($CD146^+$, $CD44^+$, $CD90^+$, $CD45^-$, $CD34^-$, $CD14^-$) and *in vitro* differentiation analysis to ensure our starting population has self-renewal and multi-lineage potential.

Secondly, the initial seeding densities could also be optimised for rat DPSCs. Our cultures were >90% confluent when swapped over to neural induction medium, and our results show a more neural-like morphology in areas of low cell density. We could control this issue by varying the seeding density, or by initiating differentiation earlier. Cell proliferation has an impact on neural differentiation, as neural precursors need to exit the cell cycle and become postmitotic to fully mature (Ohnuma and Harris, 2003). Previous studies have shown that RA induces cell cycle arrest in mouse ESCs by increasing the expression of cyclin dependent kinase inhibitors (Lin et al., 2005). Future studies could address this issue by quantifying the

number of proliferating cells during the induction process using bromodeoxyuridine incorporation or the cell proliferation marker Ki67.

Thirdly, the duration of the neural induction protocol can be optimised to work with rat DPSCs. The final concentrations of most factors used in this study have previously been used in literature, but the duration of time that cells are exposed to certain factors varies. For example, Arthur *et al*. (2008) suggests a three-week protocol, while Kiraly *et al*. (2009) only required 10 days' induction. One method to optimise this would be to look at gene expression changes (DPSC/mesenchymal markers → neural progenitor markers → mature, postmitotic neural markers) at different time points during the process. This would allow us to judge the optimal time to change medium during all stages of commitment.

We could also look into adding other factors to promote differentiation. Synthetic retinoids (EC23) have recently been developed that are more photostable and potent than their natural derivatives (Christie et al., 2008). The addition of EC23 significantly improved neural differentiation of human neuroprogenitor cells as well as human ESCs (Christie et al., 2010). Intracellular levels of cAMP can be elevated using forskolin, a factor which activates adenylyl cyclase (Kim et al., 2005), or IBMX (3-isobutyl-1-methylxanthine), a non-selective phosphodiesterase inhibitor (Tio et al., 2010). The use of small molecules to increase the homogeneity, functionality and yield of differentiated cells is another option (Chambers et al., 2009, Xu et al., 2008). Finally, the use of additional end-point analysis such as transcriptional profiling (RT-PCR, qPCR, microarrays) and electrophysiological assessments would allow us to characterise the differentiated cells in more detail.

4.3.2 Immunomodulatory Phenotype of DPSCs

One area of dental SC biology that has not received much attention so far is their immunomodulatory properties. It is well known that DPSCs share many functions with BM MSCs, such as marker expression and differentiation ability (Huang et al., 2009). One of the most clinically relevant properties associated with MSCs is their immunosuppressive functions (Uccelli et al., 2008). MSCs can inhibit the proliferation of cells from both the innate and adaptive immune systems via the secretion of soluble factors and cell-cell contact (Ben-Ami et al., 2011). If DPSCs could also exhibit an immunoregulatory phenotype, they could easily be harvested and used in autologous treatment regimens to combat a variety of autoimmune disorders (Ankrum and Karp, 2010).

To date, there have been two publications looking at the immunosuppressive activities of dental SCs. A very basic study by Pierdomenico *et al*. showed that DPSCs inhibited the proliferation of phytohaemagglutinin-stimulated $CD2^+$ T cells by 91%, compared to only 75% for BM-MSCs (Pierdomenico et al., 2005). A more recent study by the Gronthos group showed that the immunosuppressive properties of DPSCS and PDLSCs were mediated by transforming growth factor-β1, hepatocyte growth factor, and IDO (Wada et al., 2009).

Future studies in this area could attempt to identify further factors responsible for DPSC-mediated immunosuppression by using inhibitors of known factors from MSC literature. Comparisons can be made between MSCs and DPSCs in models of graft-versus-host disease, diabetes and multiple sclerosis. DPSCs could also be used to dampen the tissue damage caused by activated macrophages following spinal cord injury (David and Kroner, 2011).

Chapter 5

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