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Immunocytochemical characterisation of primary teeth pulp stem cells from resorbing teeth cultured in serum-free medium

Abstract

Background/Aims: Dental pulp stem cells from primary teeth cultured in serum-free conditions may have clinical use for the repair and regeneration of teeth as well as other complex tissues and organs. The aim of this study was to test the change in the stem cell markers expression/ stem cell population in human primary pulp cells at the different stages of root resorption.

Methods: Caries-free human primary canines at defined stages of physiological root resorption were included (n=9). *In vitro* cultures were established in xeno-free, serum-free Essential 8™ medium with human truncated vitronectin for cell attachment. An embryonic stem cell line (GENEA002) was used as a positive control. The expression of embryonic stem cell markers (Oct4, Nanog and Sox2), neural crest stem cell markers (nestin and Dlx2), and mesenchymal stem cell surface markers (CD90, CD73 and CD105) were investigated by immunocytochemistry. Mesenchymal stem cell markers CD105, CD73 and CD90 and haematopoietic markers: CD45, CD34, CD11b, CD19 and HLA-DR, were quantified with flow cytometry.

Results: The early neural progenitor markers nestin and Dlx2 were detected in most serum-free cultured dental pulp stem cells, regardless of the tooth resorption stage from which they were harvested. Only isolated cells were found that expressed the embryonic stem cell transcription factors Oct4A, Nanog and Sox2 and in the late stages of resorption no Oct4A was detected. The majority expressed the mesenchymal stem cell markers CD90, CD73 and

CD105. Flow cytometry found positive signals for CD90 >97.3%, CD73 >99.6% and CD105 >82.5%, with no detectable differences between resorption stages.

Conclusions: This study identified populations of dental pulp cells *in vitro* with markers characteristically associated with embryonic stem cells, neural crest derived cells and mesenchymal stem cells. Flow cytometry found CD105 expressed at lower levels than CD90 and CD73. The consistency of stem cell marker expression in cells cultured from teeth at different resorption stages suggests that pre-exfoliated primary teeth that are free of caries may provide a convenient source of multipotent stem cells for use in regenerative medicine.

Introduction

Mesenchymal stem cells (MSCs) from human exfoliated deciduous teeth (SHEDs) may constitute a readily available source of stem cells for regenerative medicine. Dental pulp MSCs could be bio-banked for future use in the repair and regeneration of teeth and alveolar bone. These cells will require amplification *in vitro* to produce sufficient stem cells for transplantation. To reduce the risk of disease transmission, the cells should be cultured in xeno- and serum-free media rather than foetal bovine serum (FBS), which could carry pathogens. Clinicians will also require clarity over whether the resorption stage of the teeth from which dental pulp MSCs are harvested impacts on the regenerative potential of the cultured stem cells. Tooth resorption is associated with cellular changes including an increased presence of odontoclasts. Whether the number of haematopoietic stem cells and resident MSCs changes during resorption is unknown. The hypothesis of this study was that dental pulp cells from three defined stages of tooth resorption would all possess neural crest and MSC markers but not embryonic stem cell markers.

The tooth pulp contains cells that are clonogenic and multipotent[1] with unique potential due to being neural crest (NC) derived.[2] These cells maintain their ability to differentiate into dental and non-dental cells such as odontoblasts, hair follicle cells, hepatocytes, neurones, islets of Langerhans, cardiomyocytes, osteoblasts, adipocytes and corneal epithelium.[3-8] In dentistry, an important objective of endodontic tissue engineering is to regenerate a functional dental pulp that produces new dentin upon demand, and that is capable of responding to harmful stimuli. This approach has received considerable attention over the last decade, particularly after the discovery of stem cells within the dental pulp.[1,9]

The collection and *in vitro* expansion of SHEDs for clinical applications requires careful consideration. Procedures to minimise the risk of contamination during harvesting of the pulp tissue are essential, and extraction of teeth prior to exfoliation may be optimal to reduce bacterial contamination of the pulp. In addition, avoidance of conditions that could result in virus or prion transmission to the cells has led to the development of FBS-free culture systems for cell amplification.[10] A number of serum-free culture systems have been investigated for the dental pulp which result in the retention of stemness consistent with multi-lineage differentiation capacity.[11-13] The use of a chemically defined serum-free system which maintains amplification efficiency while minimising the introduction of genetic or epigenetic changes is critical for future clinical translation of dental pulp stem cells (DPSCs).

Little is known about whether haematopoietic and resident stem cell populations alter as a tooth resorbs and is exfoliated. Several studies have investigated the histological differences between resorbing and non-resorbing deciduous and permanent teeth.[14,15] However, the classification systems used to generate root resorption groups and examine changes over time in these studies were based on subjective evaluation rather than objective calculations. An equation developed by Monteiro et al.[6] based on the root length norms

published by Kramer and Ireland[16] has been reported to be a reproducible method for classifying root resorption.[17] It classifies roots into three resorption groups: Group A: Minimal resorption (root resorption < 33.3 %), Group B: Intermediate resorption (root resorption 33.3% - 66.6%) and Group C: Advanced resorption (root resorption > 66.6 %). Based on these root resorption groupings, any changes to the primary teeth pulp cells can be further examined during tooth exfoliation.

Cranial neural crest cells have been reported to contribute to the formation of the tooth.[18] A population of neural crest stem cells (NCSCs) have been shown to persist beyond the period of NC cell induction and migration, in various adult tissues.[2] SHEDs have also been defined as NCSCs as determined by their differentiation capacity, neurogenic potential, clonogenic capacity, expression of the neural crest-associated markers p75 and HNK-1, and a WNT induced epithelial-like phenotype.[19]

Pluripotent embryonic stem cells, NCSCs and MSCs are defined by a number of markers. In pluripotent cells the role of Oct3/4, Sox2, cMyc and Klf4 in the production of induced pluripotent stem cells (iPSC) from somatic cells is well accepted.[20] Of these, Oct4A,[21] Sox2,[22] along with the addition of Nanog[23] are highly expressed and form a transcriptional network that maintains cells in a pluripotent state.[24] These three protein markers are specific to embryonic stem cell physiology and are fundamental to maintaining the undifferentiated state.

Nestin is an intermediate filament protein which is expressed predominantly in stem cells of the central nervous system and has been used to identify NCSCs.[25] Distal-less homeobox 2 (Dlx2) is a transcription factor and ectomesenchymal marker critical to cranial NC cells,[26] and all Dlx proteins are expressed in dental

ectomesenchyme during normal development.[27] Thus, both nestin and Dlx2 are considered markers of NCSCs.

MSC surface markers CD105, CD73 and CD90 have been used in studies for the characterisation of dental pulp cells and to compare the properties of stem cells from different tissue origins.[28-30] The definition of the International Society for Cellular Therapy for MSCs includes expression of these three markers and lack of expression of haematopoietic stem cell markers.[31]

In this study primary DPSCs from three stages of root resorption were collected and cultured in serum-free medium. The aim of this study was to investigate the expression of specific embryonic, NCSC and MSC markers/ populations in primary pulp from teeth at different stages of tooth resorption.

Materials and Methods

Primary canine teeth were selected due to their greater root length and the size of the pulp cavity. Teeth at the three defined stages of physiological root resorption were extracted (n=18) from six to 12-year-old healthy children as part of their treatment plans. Included teeth were caries-free with no enamel defects or excessive tooth tissue loss, or root fracture during extraction. The procedure was performed under local or general anaesthetic at the University of Otago Dental School. Ethical approval was obtained from the Otago University Ethics Committee (No. H13/075). Information packs were provided and consent forms signed prior to tooth extraction. Nine teeth were included in the present study and nine teeth were excluded due to culture contamination.

The extracted teeth were cleaned with sterile PBS to remove any clotted blood. Attached gingivae and periodontal tissues were then scraped off using a sterile scalpel blade.

Tooth measurements were carried out from the most resorbed part to the cemento-enamel junction (CEJ) on both the buccal and the lingual sides, using sterile callipers. The percentage of root resorption was calculated according to an equation adopted from Monteiro et al.[6] The resultant measurement was then divided by the Kramer and Ireland published norms.[16] The percentage of root resorption was calculated as follows:

$$\%\,\boldsymbol{root\ resorption} = 100 - \left(\frac{\text{distance from CEJ to point of greatest resorption in mm x } 100}{\text{Expected preresorption root length (Kramer and Ireland's norms)}}\right)$$

The teeth were divided into three groups according to the degree of root resorption: Group A (minimal resorption < 33.3 %), Group B (intermediate resorption 33.3% - 66.6%) and Group C (highly resorbed > 66.6 %).

To prepare the teeth for pulp harvesting, a longitudinal groove was cut into the enamel and dentine, using a sterile high-speed hand piece with a No. 33 bur (single use), with coolant (water). The groove started one mm from the root apex without reaching the pulp cavity and moved up towards the crown. The tooth was then transferred to the laboratory in a sterile falcon tube containing ice cold sterile PBS for further processing in a Class-II laminar flow hood.

To allow the extraction of pulp for *in vitro* explant culture, teeth were split open longitudinally using side-cutting bone scissors. Pulp was transferred with minimal dissection into 6-well culture plates (Greiner Bio-one, Cat# 657160). The pulp was cultured using an outgrowth methodology in xeno-free, serum-free Essential 8™ (E8™) medium (Invitrogen™, Cat# A1517001) supplemented with 1% antibiotic-antimycotic (Invitrogen™, Cat# 15240), on a human recombinant vitronectin (VTN-N, Cat# A14700).[32] Relatively little is known about the serum-free harvesting of primary tooth stem cells and the advantages of an outgrowth

methodology over enzymatic digestion, however studies investigating both methodologies in serum-containing environments have found differences in the MSCs they yield.[33,34]After 2 weeks of culture, cells were dissociated in 0.5 mM EDTA solution and collected as single cell suspensions, washed twice in PBS, and seeded at 10^4 cells into VTN-N pre-coated T25 culture flasks (P2; passage 2). Cells were frozen in cryo-vials (Cryo.S, PP, Greiner Bio-One, Cat# 82050-154) with 90% E8™ medium and 10% Dimethyl-Sulphoxide (Sigma Cat# D8418) and stored in liquid nitrogen.

The human embryonic cell line GENEA002 was cultured and maintained in Genea Biocells M2™ stem cell medium; a fully defined serum-free medium for the maintenance and expansion of pluripotent stem cells. Cryopreserved GENEA002 cells ($5x10^5$ cells/vial) were recovered using M2™ medium in collagen I coated 25 cm^2 culture flasks (Corning™ BioCoat™ Cat# 354484). Further expansion of GENEA002 cultures into T75 culture flasks coated with rat-tail collagen I (Falcon®, FAL354236) was performed. Cells were frozen at P3.

Immunofluorescent labelling of GENEA002 cells was conducted on cells recovered from cryopreservation and cultured on collagen I (FAL354236) coated, 8-well Lab-Tek™ II Chamber Slides™ (Thermo Scientific™ Nunc™, Cat# 154534). GENEA002 embryonic stem cells were included as positive controls for comparison and validation of the immuno-staining technique. Primary teeth pulp cells from the three stages of root resorption (4^{th} passage) were seeded into VTN-coated chamber slides ($5x10^4$ cells/well) and cultured in E8™ medium. The experiments were performed for each resorption group in biological triplicate. All cultures were incubated at 37°C in 5% CO_2 with a high humidity environment and immunofluorescence conducted when cells reached 50-70% confluence. Expression of embryonic stem cell markers (Oct4A, Nanog and Sox2), neural crest cell markers (Nestin and Dlx2), and mesenchymal stromal markers (CD90, CD73 and CD105) was conducted using the antibodies in Table 1. All

cells were fixed with ice-cold acetone for 10 minutes, dried and stored. Following washing, cells were blocked with 20% heat-treated goat serum in PBS with 1% BSA (Sigma, BSA fraction-V, A-9306-5G) and 0.3% Triton™ X-100 (Sigma-Aldrich Cat# T8787). This was followed by overnight incubation in primary antibody or isotype matched control. The slides were then washed and incubated with secondary antibody for one hour in the dark (Table 1). Nuclei were counterstained with DAPI using Vectashield® mounting medium (Vector Laboratories, Cat# H-1200) and a coverslip was applied. Imaging was conducted using a Nikon Eclipse Ti-U inverted microscope. The results are presented as high-resolution pictures representative of the expression pattern observed over the whole sample using ImageJ software.[35]

Flow cytometry experiments investigating the MSC marker expression levels in primary teeth pulp cells were performed. The methodology followed that supplied with the BD Stemflow™ hMSC Analysis Kit (BD Stemflow™, Cat# 562245) and was performed at the Flow Cytometry Facility, University of Otago. The following labelled antibodies were used for MSCs: CD90-FITC, CD105-PerCP-Cy5.5, CD73-APC; and haematopoietic cells: CD34-PE, CD11b-PE, CD19-PE, CD45-PE, HLA-DR-PE. Controls included: mIgG1-FITC, mIgG1-PerCP-Cy5.5, mIgG1-APC, mIgG1-PE and mIgG2a-PE. Briefly, cultured cells were washed in PBS at RT and harvested by addition of BD™ Accutase™ cell detachment solution (Cat# 561527). Dissociated cells were triturated by adding additional cell culture medium to obtain a single-cell suspension. A small subset of the cell suspension was then examined under a microscope to confirm the presence of single cells. Cells were centrifuged at 300 g for 4 minutes and re-suspended in FACS buffer to achieve a density of $1x10^7$ cells/mL. A live/dead assay was performed on 100 µL of cell suspension using Zombie NIR™ fixable viability dye (BioLegend, Cat# 423105), with incubation for 15-30 min at RT in the dark. Labelled tubes containing 100 µL of cell suspension from the two cell lines were washed with cold PBS, and incubated on ice

with the respective monoclonal antibodies conjugated with either fluorescein isothiocyanate (FITC), Allophycocyanin (APC), PerCP-Cy5.5 or P-phycoerythrin (PE) for 30 min in the dark. The isotype controls were used to determine background non-specific binding. Labelled cells were then fixed with 2% formaldehyde/PBS and analysed using a BD™ LSR Fortessa™ cell analyser, acquiring 10,000 events per tube. Data were analysed with FlowJo™ software (Tree Star; Ashland, OR). Gates for cell-surface markers were set using isotype controls (≤ 1.5 % positive). Compensation and background controls were calculated to subtract any spectral overlap between the fluorophores used in the multicolour flow cytometric assay.

Results

Eighteen caries-free primary canine teeth were extracted from six to 12-year-old healthy children as part of their treatment plans. Teeth were measured (Table 2) and their resorption group established. The groups were: minimal resorption (root resorption < 33.3 %; 3 excluded), intermediate resorption (root resorption 33.3% - 66.6%; 2 excluded) and highly resorbed (roots resorption > 66.6 %; 4 excluded) with n=3 teeth per group. Exclusions were due to infection.

Spindle shaped cells were observed migrating from dental pulp explant tissue after 5 days of culture in E8™ medium and VTN-N coated wells. The same piece of dental pulp without enzymatic treatment was transferred sequentially to another well for further expansion. Following two transfers, and after 14 days, approximately 2 x 10^6 cells were observed in each well of a 6-well plate. Isolated primary DPSCs were initially small cells with a reduced cytoplasm and high proliferative capacity. The morphology of primary pulp cells at 17 days in E8™ medium from resorption groups A, B and C were similar. They appeared as heterogeneous populations of cells with both neuronal-like long spindle shaped cells and

fusiform cells which lacked contact inhibition (Figure 1A-C). Cultured human embryonic stem cells (GENEA002) were small compact cells displaying a high nuclear to cytoplasmic ratio and prominent nucleoli, which formed characteristic well-defined colonies (Figure 1D).

Expression of mesenchymal stromal markers was investigated in primary DPSCs and a human pluripotent embryonic stem cell line. Only for CD105 could a small population of unlabelled cells be seen (Figure 2 CD105:A-C). There was no noticeable difference between resorption groups in their expression of CD73 or CD90 proteins (Figure 2 CD73:A-C and CD90:A-C, Table 3), with high numbers of positive cell observed. Primary DPSC cultures were heterogeneous with some cells lacking contact inhibition. Cells appeared as a number of morphologies including bipolar fibroblastic-like cells, polarised cells and long spindle shaped cells more neuronal in shape. The smaller phenotype and colony forming ability of GENEA002 cells were evident along with high expression of CD105, CD73 and CD90.

Human embryonic stem cells (GENEA002) were purchased and phenotyping confirmed the presence of defined pluripotency markers: Nanog, Oct4, Tra1-60, and SSEA4. The presence of Oct4A protein was confirmed in this study (Figure 3 Oct4A:D; Table 3). Expression of Oct4A protein in primary DPSCs cultured in E8™ medium from each resorption group was very minimal (Figure 3 Oct4A:A-C; Table 3). Oct4A only appeared within small clusters of cells within the whole population and in the advanced resorption group no Oct4A was detected. The positive cells were notable for their high cytoplasmic to nuclear ratio. Oct4A protein was intra-nuclear in location, as expected for a transcription factor, and also detected within the cytoplasm of pulp cells.

Nanog protein was present within some cultured primary pulp cells from all resorption groups (Figure 3 Nanog:A-C). The pattern of expression was similar in all cells and found within

the nuclei as well as the cytoplasm of a small population of cells. Nanog staining in the embryonic stem cells was highly evident (Figure 3 Nanog:D).

The pluripotent stem cells marker Sox2 was evident in all resorption groups (Figure 3 Sox2:A-C). The expression followed the same pattern as Oct4A and Nanog and appeared within cell clusters and located within the nuclei/cytoplasm of the pulp cells. Sox2 was highly expressed within the human embryonic stem cells (Figure 3 Sox2:D).

The presence of the intermediate filament protein nestin did not differ among the primary DPSC resorption groups. High levels of nestin protein were found localised within the cytoplasm and extending into the cell processes (Figure 4 Nestin:A-C, Table 3). All observed primary DPSCs were positive for this neural crest stem cell marker. Although less evident, due to the smaller cytoplasm of the embryonic stem cells, nestin was detected in most but not all GENEA002 cells (Figure 4 Nestin:D).

The expression of Dlx2 protein was evident in all resorption groups of cultured primary pulp cells (Figure 4 Dlx2:A-C), however only a subpopulation of cells were positive. Nucleocytoplasmic localisation was evident in all the three-resorption groups. Cells showing strong labelling for Dlx2 were dispersed among the pulp cells but tended to be in clusters (Figure 4 Dlx2:A-C). Embryonic stem cells were also only sometimes positive for Dlx2 protein (Figure 4 Dlx2:D, Table 3). Isotype negative IgG controls in primary teeth pulp cells and the embryonic stem cells are presented in Figure 5.

Primary DPSCs were cultured in E8™ medium/VTN coating and were detached using BD™ Accutase™ cell solution and then stained with the positive and negative cocktails or the positive and negative isotype control cocktails (Figures 6 & 7; Table 4). The plots were derived from gated events based on the light scattering characteristics of the MSCs. The levels of CD90 and CD73 in primary DPSCs were an average of 98.4% and 99.75% respectively. Detection of

CD105 staining was lower with 96.2% and 82.5% of cells positive. The presence of haematopoietic cells was determined with a cocktail of antibodies (CD45-PE, CD34-PE, CD11b-PE, CD19-PE and HLA-DR-PE). Less than 2.45% of cells were positive for haematopoietic markers. Primary DPSCs culture in serum-free E8™ medium were thus found to be positive for the MSC markers CD90 and CD73, and to a lesser extent to CD105. Only very small numbers of cells were positive for the haematopoietic cell markers.

Discussion

The collection of DPSCs at the time of primary tooth resorption provides a convenient source of MSCs for clinical applications in relation to both dental trauma and regenerative medicine. *In vitro* amplification of DPSCs in a defined serum-free medium is critical for safe clinical application.[10] This study investigated the feasibility of collecting human teeth pre-exfoliation and the differentiation status of primary DPSCs collected from minimal, intermediate or advanced root resorption groups. Collection of pulp tissue from teeth with advanced root resorption resulted in the highest contamination rates possibly due to microbial infiltration into the root, thus the collection of teeth from early resorption stages may be a promising approach for *in vitro* primary DPSC amplification. Explant cultures were successfully established and cells attached to VTN-treated plastic in a serum-free environment. There were no detectable morphological differences in the cells from the three resorption stages when grown in E8™ medium. This study investigated the *in vitro* cell expression of three classes of markers associated with the characterisation of embryonic, neural crest derived and MSCs. Expression levels were compared to human embryonic stem cells (GENEA002). The aim was to characterise primary DPSCs grown from the three stages of resorption in a serum-free medium.

In this study, primary DPSCs variably expressed the pluripotency markers Nanog and Sox2. Oct4A was only present in a limited number of cells. ESCs derived from mice, rats and humans share a common subset of transcription factors specifying stemness, among which Oct4A, Sox2 and Nanog constitute the core pluripotency circuitry as described by Boyer et al.[36] Oct4A expression has been described in a variety of somatic stem cell compartments and in cultured somatic progenitor cells[37-39] where it has been suggested to function in a manner similar to its role in embryonic stem cells.[40] In this study, only a low number of cells were found to be positive for Oct4A in the minimal and intermediate resorption groups, while no positive cells were detected in the advance resorption group. Oct 3/4 has also been previously reported in association with CD117 purified DPSC and SHEDs *in vitro*,[41] while others have reported relatively low levels.[13] The low expression of Oct4A protein and its presence in both the nucleus and cytoplasm in this study were attributes consistent with postnatal somatic cells. Two isoforms of human Oct4A have been identified in embryonic stem cells and in DPSCs.[42] Oct4A protein, localised to the nucleus of ESCs, is critical for maintaining the pluripotency of ESCs and promoting tumorigenesis in human tissues. Little is known about the properties of Oct4B which is cytoplasmic and cannot maintain the undifferentiated state of cells.[43] Immunofluorescent studies by Liu et al.[39] proposed that Oct4 was maintained within the nucleus in early passages (from P0 to P2), then it is translocated to the cytoplasm, implying the potential loss of pluripotency with extended passaging. They supported this observation with alternations in colony-forming efficiency, cell cycle and multilineage differentiation capability of rat dental pulp cells with prolonged *in vitro* culture.[44] Whether the loss of Oct4A in the advanced resorption group is associated with tissue remodelling and loss of pluripotency will require further research.

Moderate expression of Nanog and Sox2 were found in this study. The gene regulatory network between Oct4A which is critical for maintaining pluripotency,[45-47] Nanog's dynamic expression,[24,48,49] and Sox2 as a regulator,[22,39,46] is crucial for deciding cell self-renewal and differentiation ability. Fluctuating expression of Nanog in adult and primary teeth dental pulp cells has been previously reported to reflect the differentiation status of the cells.[50] Sox2 is a marker of uncommitted neuroepithelium and has previously been found in association with dental pulp *in vivo*[51] and DPSCs *in vitro*.[42] The presence of a population of Oct-4A, Nanog and Sox2 positive cells in serum-free cultured primary DPSCs suggests some cells can retain stemness and have a unique somatic cell signature. A limitation in the application of DPSCs over embryonic cells is the much lower expression of Oct-4A, Nanog and Sox2, suggesting more limited differentiation potential.

NCSCs may have higher differentiation capability for regenerative medicine and postnatally DPSC provide an accessible source of these cells.[2] DPSCs have been shown to express proteins consistent with being NCSCs and decreasing serum levels *in vitro* has been found to increase the number of cells expressing the neural crest markers p75 and HNK-1.[19] Nestin is a class VI intermediate protein also used as a marker for NCSCs[2,52] and all cell lines from the three groups of root resorption expressed abundant nestin within their cell bodies as well as the long processes of the polarised spindle shaped cells. This finding was at variance with a report that SHEDs had a reduced ability to express nestin when compared to DPSC.[53] Others however have reported that odontoblasts from younger permanent human teeth and odontoblasts around an injury site have been found to express nestin, while older permanent teeth show no expression.[54] Nestin expression may thus be associated with the NCSC status of the cells and its expression influenced by harvesting and culture conditions. Cranial specific Dlx2 is an ectomesencyhmal marker of NCSCs and may play an important role in bone and

cartilage formation.[26] The expression of Dlx2 homeobox proteins involved in early craniofacial development has been used to distinguish odontoblast precursor cells from other mesenchymal progenitors.[55] Cultured primary pulp cells in this study variably expressed Dlx2 protein in a clustered pattern, possibly associated with its role in the formation of cell condensations which form cartilage and bone *in vitro*.[56] These findings not only suggest that these cells are from an odontoblastic lineage but also support the view that in serum-free culture nestin and Dlx2 positive primary pulp cells have retained the overall features of active odontoblasts *in vitro*. The high expression of NCSC markers under non-neuronal inductive conditions was a significant finding that supports the neurogenic potential of primary teeth pulp cells**.** This also makes them a valuable source of postnatal stem cells for neuronal differentiation, supported by the previous finding of increased cognitive functioning in rats injected with DPSCs after global ischaemia.[57]

MSCs according to the ISCT minimal criteria are CD73, CD90 and CD105 positive but lack expression of haematopoietic markers.[31] The current study showed positive immunocytochemical MSC marker expression in the primary DPSCs. Flow cytometry analyses confirm these findings with CD90 >97.3% positive, CD73 >99.6% positive and CD105 >82.5% positive. The effects of serum-free culture on MSC marker expression are a current topic of debate. MSCs cultured in serum-free media and with multi-lineage differentiation capability have been reported to down-regulate CD105, SSEA4, CD146 and Stro-1 expression and that this down-regulation is more marked with passage number. They however have been reported to maintain high levels of CD73 and CD90 expression.[11,12,58] There is emerging evidence that serum-free culture of primary DPSCs results in down-regulation of CD105, while retaining their NCSC phenotype and multi-lineage differentiation capability.[59]

In conclusion, this study provides evidence that primary teeth pulp cells comprise a heterogeneous population of cells that retain the unique characteristics of NCSCs. Most cells were found to express the NCSC markers nestin and Dlx2. Isolated cells expressed the embryonic stem cell transcription factors Oct4A, Nanog and Sox2, while the majority expressed the mesenchymal stem cell surface markers CD90 and CD73, with slightly lower levels of CD105. The serum- and xeno-free amplification of dental pulp cells, while retaining neural crest and MSC marker expression, is a critical step in the process of providing a safe stem cell source for tooth repair and regenerative medicine.

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FIGURE 1 A - C, Primary pulp cells at day 17 of culture in Essential 8™ medium with human truncated vitronectin for cell attachment. A, Group A - minimal resorption. B, Group B - intermediate resorption. C, Group C - highly resorbed. Cells were observed as spindle shaped with very long processes and fusiform shaped. Cells were at times seen to be overlapping each other. D, Embryonic stem cells (GENEA002) cells grown on a collagen I matrix and maintained in M2™ medium. Scale bar = 100 µm.

FIGURE 2 Representative images of mesenchymal stromal cell markers CD105, CD73 and CD90 detected by immunocytochemistry (green) in primary dental pulp cells and pluripotent human embryonic stem cells (GENEA002). A, Group A - minimal resorption; B, Group B - intermediate resorption. C, Group C - highly resorbed. D, Embryonic stem cells (GENEA002). Protein was detected within the cytoplasm of most cells (green). DAPI stained nuclei (blue). Scale bar = 100 µm.

FIGURE 3 Representative images of embryonic markers detection in primary teeth pulp cells and GENEA002 cells. A, Group A - minimal resorption; B, Group B - intermediate resorption. C, Group C - highly resorbed. D, Embryonic stem cells (GENEA002). Oct4A protein (green) was detected in isolated groups of cells in cultured primary teeth pulp cells from resorption groups A and B, while no expression of Oct4A was observed in resorption group C cells. GENEA002 cells showed defined colonies of compact cells with different levels of Oct4A expression. Nanog protein (green) was expressed in some cells from all groups within the nuclei as well

as peri-nuclear cytoplasm and strongly detected in GENEA002 cells. Sox2 was expressed in all resorption groups within the nuclei and cytoplasm of some triangular and elongated cells and in GENEA002 cells. DAPI stained nuclei (blue). Scale bar = 100 µm.

FIGURE 4 Representative images of neural crest markers nestin and Dlx2 in primary teeth pulp cells and GENEA002 cells. A, Group A - minimal resorption; B, Group B - intermediate resorption. C, Group C - highly resorbed. D, Embryonic stem cells (GENEA002). Nestin was highly expressed in cultured primary pulp cells from all resorption groups (red). The intermediate filament protein was clearly visible forming the cytoskeleton of spindle shaped cells and extending into their processes. GENEA002 colonies showed nestin expression. Dlx2 protein was detected within the cytoplasm of some cells (red) as well as in cultured GENEA002 cells (H). DAPI stained nuclei (blue). Scale bar = 100 µm.

FIGURE 5 Isotype matched negative IgG controls in primary dental pulp cells and GENEA002 cells. A, Rabbit IgG (green) as control for Oct4A, Nanog, Sox2 and CD90 antibodies on cells from Group A (minimal resorption). B, Mouse IgG1 (red) as a control for nestin, Dlx2, CD105 and CD73 antibodies, on cells from Group A (minimal resorption). C, Rabbit IgG (green) on pluripotent stem cells (GENEA002). D, Mouse IgG1 (red) on pluripotent stem cells (GENEA002). DAPI stained nuclei (blue). Scale bar = 100 µm.

FIGURE 6 Flow cytometry analyses of mesenchymal stem cell marker expression (CD90-FITC, CD105-PerCP-Cy5.5, CD73-APC) in primary dental pulp stem cells for two different cultures (A and B).

FIGURE 7 Flow cytometry analyses of haematopoietic marker expression: CD34-PE, CD11b-PE, CD19-PE, CD45-PE, HLA-DR-PE. Two primary dental pulp stem cells for two different cultures were investigated (A and B).

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TABLE 1 Antibodies used for immunocytochemical characterisation

TABLE 2 Root length measurements and root resorption staging. Group A: Minimal resorption (root resorption < 33.3 %), Group B: Intermediate resorption (root resorption 33.3% - 66.6%) and Group C: Advanced resorption (root resorption > 66.6 %).

TABLE 3 Expression of stem cell markers as assessed from each resorption group (n=3; biological replication) and GENEA002 cells (n=3; technical triplicate). '-' = no positive cells observed; '+' = less than 10% of cells positive; '++' = 10 - 80% positive cells; '+++' = Greater than 80% positive cells.

TABLE 4 Flow cytometry assessment of mesenchymal stem cell and hematopoietic markers in primary teeth pulp cells from groups A and B.