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CD271 Negative Human Dental Pulp Cells Yield Significantly More Adherent Colony Forming Cells than the Positive Phenotype

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Abstract

Cell surface markers for isolating proliferative human dental pulp stromal cells are currently lacking. Other tissues containing mesenchymal stromal cells have been studied in greater depth and candidate markers for cell isolation identified, one such marker being CD271. Previous reports suggest CD271 as a marker for isolating dental pulp stromal cells from rat incisors. We aimed to study the utility of CD271 as a marker for isolating human dental pulp stromal cells. CD271 positive cells from both third molar dental pulp and bone marrow mononuclear cells were isolated by magnetic separation followed by *in vitro* expansion. Phenotypic analysis was performed by flow cytometry. Our data showed that although CD271 is present in the adult molar dental pulp, significantly greater numbers of colonies are produced from the CD271 negative fraction. The opposite was seen with bone marrow mononuclear cells; all colony forming cells were derived from the CD271 positive fraction. Phenotypic analysis of expanded CD271 negative cells showed that these cells are identical to dental pulp stromal cells isolated by non-selective plastic adherence. We conclude that, in contrast to bone marrow, CD271 is not a positive selection marker for the predominant colony forming cell types from human molar dental pulp.

Keywords

Dental pulp, CD271, Magnetic activated cell sorting, MACS, Mesenchymal stem cells, Flow cytometry

Introduction

Human dental pulp stromal cells (hDPSCs) are plastic adherent cells capable of *in vitro* colony formation, mineralisation and demonstrable mesenchymal differentiation potential [1,2]. They are of interest for tissue engineering and regenerative medicine applications, in particular dental and orthopaedic procedures, due to the potential for banking cells from the deciduous dentition [2,3]. Within the dental pulp (DP), hDPSCs are present in comparatively low numbers, but undergo expansion *in vitro* and are phenotypically similar to mesenchymal stromal cells (MSCs) from other tissues [4,5]. *Ex vivo* expansion is important because native hDPSC numbers are insufficient for regenerative therapies [6]. However, a potential problem with this is heterogeneity, a recognised issue with MSCs and one exacerbated by non-selective plastic adherence isolation [7]. Cell sorting can reduce heterogeneity by selecting specific cell populations but, to achieve this, cell surface markers for hDPSCs are required. MSCs from other tissues, such as bone marrow (BM), have been studied in greater depth and candidate markers for MSC enrichment have been identified such as CD146, CD105 and mesenchymal stem cell antigen 1 (MSCA-1) also known as tissue non-specific alkaline phosphatase (TNAP) [8-10]. One interesting marker is the neutrophin receptor CD271 which has shown specificity for colony forming cells from BM [11-13].

CD271 (also known as (L) NGFR, NTR and p75) is important for neuronal cell survival but is also found on MSCs from BM and both trabecular and cortical bone [11,14]. These studies selected multipotent MSCs expressing CD271 and showed standard MSC phenotype and trilineage differentiation [5]. Studies of developing human teeth have shown that prior to matrix production, CD271 expression maps to the inner enamel epithelial and the dental follicle [15]. Human exfoliated deciduous teeth show continued expression [16], as do mature DPs, with increased expression associated with dental caries [17,18]. CD271 has been identified within the human third molar apical papillae and studies with cultured hDPSCs described a subpopulation of cells with neurogenic potential that express CD271 [19,20]. Further studies with cultured hDPSCs have also shown that expression of CD271 may be induced if the cells are grown in non-adhesive culture vessels, allowing for the formation of spheroid aggregates [21]. This may be because the aggregates more accurately mimic the 3D environment from which the hDPSCs come compared to the 2D conditions which are used in standard cell culture.

Studies with rat DP have shown CD271 expression in epithelial, mesenchymal and nerve structures of developing and adult molar pulps, with expression in incisors mapping to polarising odontoblasts [22,23]. Further studies using rat incisor DP found that CD271 can be used to isolate proliferative DPSCs before expansion [24]. It is for

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these reasons that we have chosen to investigate CD271 as a marker for hDPSC isolation.

The aim of this study was to investigate CD271 expression by adult human DP and to subsequently determine its utility for hDPSC isolation. We hypothesised that hDPSCs would likely be isolated using CD271 given that it is an established marker for BMSCs, with evidence of utility in isolating rat DPSCs.

Materials and Methods

Cell isolation

Dental pulp: Healthy third molars (n = 14, 10 donors (3 male, 7 female), 18-33 years), obtained with written informed patient consent through the Leeds Dental Institute Research Tissue Bank (ethical approval for this study was granted by the Dental Research Ethics Committee of the University of Leeds, study number 07/H1306/93+5), were washed with 70% (*v*/*v*) ethanol before removal of attached tissue and fracturing. DPs were excised, mechanically disrupted and digested in 3 mg/mL collagenase I (Sigma-Aldrich, UK) and 4 mg/mL dispase (Roche, Germany) for 45 minutes at 37°C with agitation. Cell suspensions were filtered (70 µm) (BD Biosciences, NJ) and centrifuged at 200 × *g* for 5 minutes. Cells were cultured, sorted or analysed by flow cytometry. Cultured cells were resuspended in α-MEM (Life Technologies, UK) supplemented with 15% FCS (Biosera, UK), 2 mM L-glutamine (Sigma-Aldrich) and 100 units/mL penicillin/100 µg/mL streptomycin (Sigma-Aldrich) before incubation at 37°C in 5% CO_2 for 10-14 days. Cells for sorting and flow cytometry were resuspended in 80 µL magnetic activated cell sorting (MACS) buffer (PBS, 2 mM EDTA (Alfa Aesar, UK) and 0.5% BSA (Sigma-Aldrich)).

Bone marrow: Primary posterior iliac crest BM (n = 4, 2 male, 2 female, 30-54 years), obtained with written informed consent following ethical approval for this study (NHS Health Research Authority, Leeds West Research Ethics Committee 06/Q1206/127) was diluted 1:1 with PBS, filtered at 70 µm and layered over 10 mL Biocoll Separating Solution (density 1.077 g/mL) (Biochrom, Germany). Cells were centrifuged at 445 × *g* for 35 minutes at 20°C. Mononuclear cells (MNCs) were aspirated and washed in MACS buffer before centrifugation at 200 × *g* for 10 minutes, twice. The resulting cell pellet was resuspended in 80 µL MACS buffer/1 mL of BM.

Cell culture

Subconfluent cells were passaged by treatment with 0.25% trypsin/0.02% EDTA (Sigma-Aldrich) and the resulting suspensions were transferred to T75 flasks, designated as passage 1 (p1). Passaged cells were subsequently sub-cultured in basal medium with 10% FCS until 90% confluency to p5.

CD271 sorting of dental pulp and bone marrow

Digested DP and isolated BM-MNCs, resuspended in 80 µL MACS buffer, were labelled with 10 µL anti-CD271-PE and 10 µL FcR blocking reagent (both Miltenyi Biotec, Germany) for 10 minutes at 4°C. Labelled cells were washed with 1 mL MACS buffer, centrifuged at 200 × *g* for 5 minutes and resuspended in 70 µL MACS buffer before incubation with 20 µL anti-PE microbeads (Miltenyi Biotec) and 10 µL FcR blocker at 4°C for 15 minutes. Cells were then washed, resuspended in 500 µL MACS buffer and added to a magnetised MS MACS column (1 column / DP or mL BM-MNCs), columns were then washed with 500 µL MACS buffer (x3). Eluted unlabelled cells were collected and designated the $CD271^-$ (negative) fraction. Labelled cells, retained on the column, were isolated by demagnetising the column and washing with 1 mL of MACS buffer, these cells were designated the $CD271^+$ (positive) fraction. Separation efficiency was confirmed by the use of BM as a positive control. Depletion efficiency from DP was found to be in excess of 90%, i.e., more than 90% of $CD271^+$ cells were removed from the $CD271^-$ fraction. All sorted cells were culture expanded in primary DP medium.

Colony forming unit fibroblast assay

Sorted cells from whole DP were plated into separate T25 flasks at the ratio of $CD271^+$ and $CD271^-$ cells present within the DP (1 sorted DP or 1 mL sorted BM-MNC per flask) with medium changes every 4 days. After 14 days, flasks were fixed with 10% (*v*/*v*) neutral buffered formalin (Sigma-Aldrich) and stained with 1% (*w*/*v*) methyl violet (VWR, UK) for 30 minutes before washing and colony counting.

Flow Cytometry

Primary DP cells and cultured hDPSCs (unsorted and CD271-sorted) were resuspended in 80 µL MACS buffer and 10 µL FcR blocking buffer before antibody incubation (10 µL/1 × 10^6 cells) for 20 minutes at room temperature in the dark. MACS buffer (900 µL) was added before centrifugation and resuspension in 500 µL MACS buffer. Samples were analysed using a BD LSR Fortessa running BD Diva 7 software and data analysis was performed using Kaluza 1.3 software (Beckman Coulter, Pasadena, CA). Antibodies used were CD271-PE (mouse IgG1 κ monoclonal, clone ME20.4, WM245 melanoma cells as antigen), CD29-Alexa Fluor 488 (mouse IgG1 κ monoclonal, clone TS2/16), CD44-FITC (mouse IgG1 κ monoclonal, clone VI A034, normal human PBL as antigen), CD56-PE (mouse IgG2a κ monoclonal, clone VI NK26, KG-1 cell line as antigen), CD73-PE (mouse IgG1 κ monoclonal, clone AD2), CD90-APC (mouse IgG1 κ monoclonal, clone 5E10, HEL cells as antigen), CD105-FITC (mouse IgG1 κ monoclonal, clone 43A3, L-cells transfected with human CD105 as antigen), CD146-Alexa Fluor 488 (mouse IgG2a κ monoclonal, clone SHM-57, HEL cells as antigen) and CD166-PE (mouse IgG1 κ monoclonal, clone 3A6, cultured human thymic epithelial cells as antigen) (all Biolegend, San Diego, CA). Threshold values were obtained by measuring non-labelled cell autofluorescence and isotype matched control reactivity, colour compensation was performed to reduce channel crossover.

Statistical Analysis

All measurements were performed in at least triplicate and all graphs are displayed as the mean ± SEM. The data were subsequently analysed using a two sample *t* test. P values of less than 0.05 (*) were considered significant and 0.01 (**) very significant.

Results

CD271 expression in DP and hDPSCs

Analysis of uncultured DP by flow cytometry confirmed $CD271^+$ cells were present at 1.24% ± 0.46 of total cells (Figure 1a). However, analysis of cultured hDPSCs, derived from plastic adherence, revealed that CD271 expression decreased to very low levels upon induction into 2 dimensional culture with an average expression of 0.2% across passages 2 to 4 (Figure 1b).

Sorting hDPSCs using the CD271 surface marker

Cells from whole digested DP were sorted for expression of CD271, isolated cells were used in colony formation unit fibroblast (CFU-F) assays, $CD271^+$ and $CD271^-$ cells were plated at the ratio of cells found within the DP. The results showed that the mean number of colonies forming from the $CD271^+$ fraction was 5 ± 2 per pulp compared to 55 ± 17 colonies per pulp from the $CD271^-$ fraction (Figure 2). These results show that significantly more colony forming cells were present in the $CD271^-$ fraction compared to the $CD271^+$ cells (p = 0.027). Positive control experiments with BM showed the opposite relationship between CD271 expression and colony formation, with an average of 18 ± 5 colonies per mL of $CD271^+$ sorted BM and 1 ± 1 colonies in the corresponding $CD271^-$ fraction, which was statistically significant (p = 0.039). These data are summarised in figure 2.

Having shown the relative colony forming capacities of $CD271^+$ and $CD271^-$ DP cells, we next determined whether cells derived from the $CD271^-$ fraction were phenotypically similar to cells expanded from unsorted DP. To achieve this hDPSCs from both unsorted and $CD271^-$ sorted DP were compared for the expression of various hDPSC and MSC markers [5,25]. The results of these analyses failed to show any significant differences in the expression patterns of the

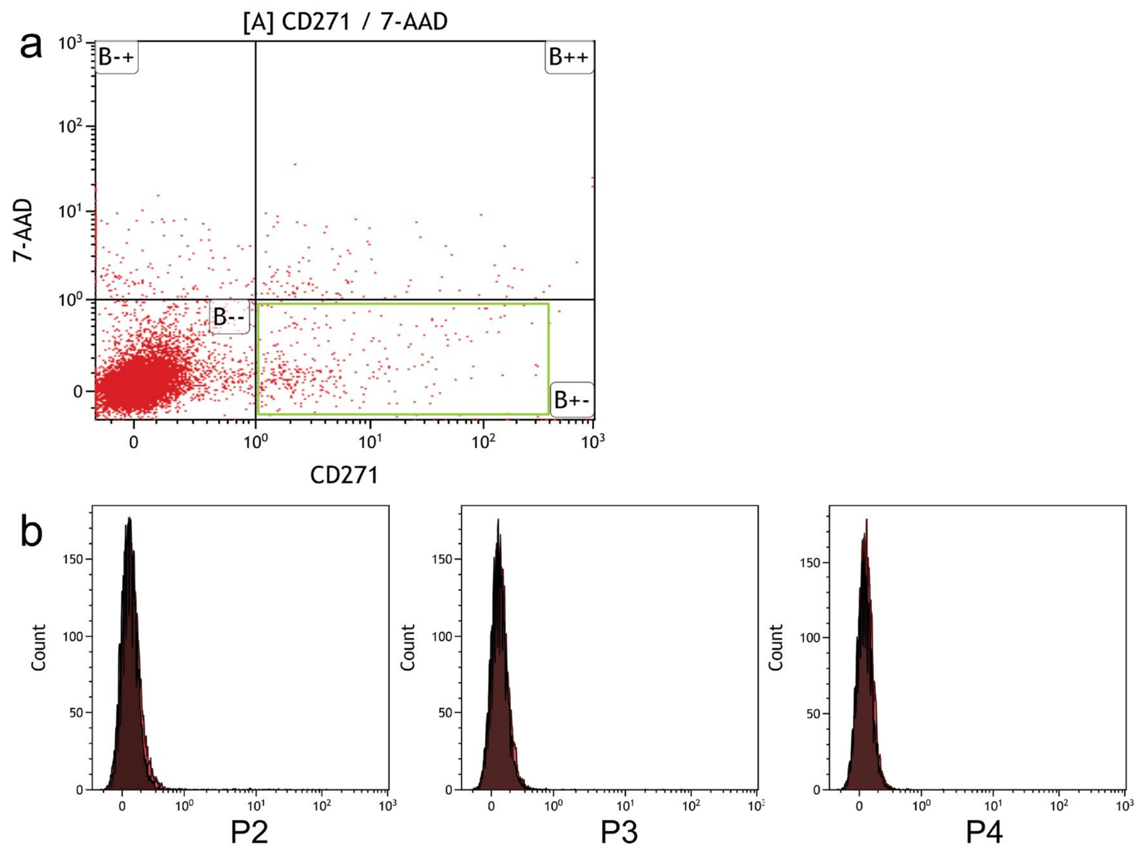

Figure 1: Expression of CD271 by primary DP cells. (**a**) Flow cytometry plot demonstrating low-level CD271 expression by a small population of cells from digested DP (quadrant B+- for viable CD271$^+$ cells); (**b**) CD271 expression by adherent hDPSCs: Histograms of flow cytometric analyses of CD271 expression by cultured hDPSCs (red) and isotype controls (black) showing that this marker is not expressed by early (p2) to mid-passage (p4) cells.

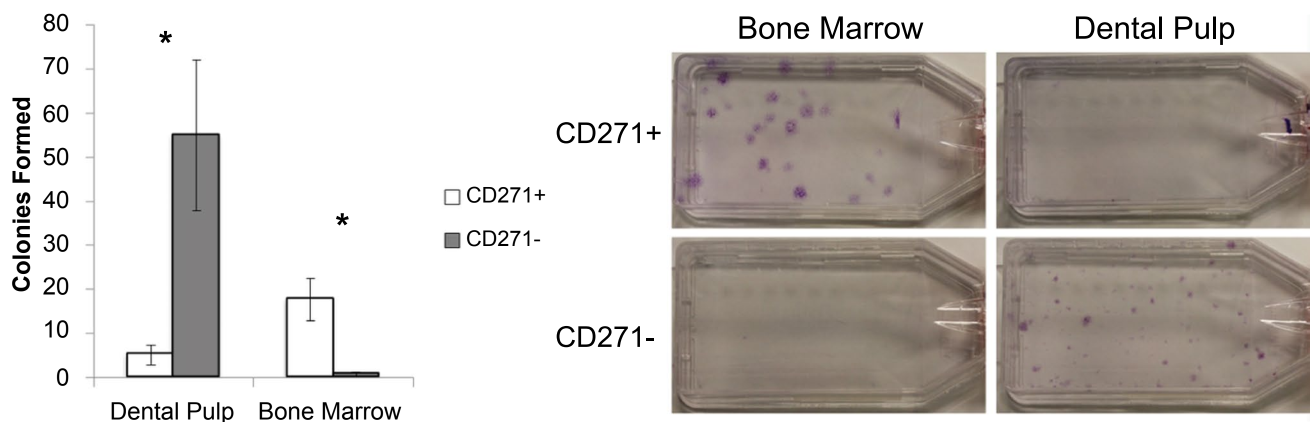

Figure 2: Averaged results of CFU-F assays performed with CD271$^+$ and CD271$^-$ sorted digested DP compared with BM-MNCs isolated by density gradient centrifugation, white = CD271$^+$ and dark grey = CD271$^-$; photographs show representative colony counts for each condition, * = $p < 0.05$, n=10 for dental pulp and n = 4 for bone marrow, data shown ± SEM.

Table 1: Comparative flow cytometry data showing average percentage expression of hDPSC markers expressed by expanded hDPSCs from both unsorted and CD271- cultures. No statistically significant differences were observed in the marker expression profiles, n = 3.

	CD271- Sorted	Unsorted	p value
CD29	99.40%	99.00%	0.43
CD44	99.50%	98.70%	0.38
CD56	88.20%	87.20%	0.9
CD73	99.40%	98.80%	0.29
CD90	99.20%	97.30%	0.15
CD105	36.40%	27.40%	0.34
CD146	53.70%	45.40%	0.57
CD166	99.40%	98.70%	0.21

markers analysed, CD29 (p = 0.43), CD44 (p = 0.38), CD56 (p = 0.9), CD73 (p = 0.29), CD90 (p = 0.15), CD105 (p = 0.34), CD146 (p = 0.57) and CD166 (p = 0.21) (Table 1). These data showed that based upon the markers examined, the CD271$^-$ cells derived from primary DP are phenotypically identical to hDPSCs isolated by plastic adherence.

Discussion

We have studied the presence of the human '*in vivo*' BMSC marker CD271 by adult human DP and *in vitro* expanded hDPSCs. We showed that CD271 is weakly expressed in DP and cultured hDPSCs show no upregulation in expression. These results confirm previous studies whereby CD271 was found within adult human DP and also showed similar *in vitro* expression to BM [12,18].

Following confirmation of CD271 expression in adult DP, and

given that CD271 has been used to isolate BMSCs [11,13,26], we attempted to use it to selectively isolate hDPSCs. These studies showed that while CD271 was expressed in DP, the CD271$^+$ fraction contained significantly fewer colony forming cells than the CD271$^-$ fraction ($p = 0.027$). To validate these findings, we utilised the same experimental regimen to isolate BMSCs from BM-MNCs. The results of these experiments were in stark contrast to those for DP with significantly more colony forming cells found in the CD271$^+$ fraction ($p = 0.039$) and are consistent with previously published BM data [11,13,14,26]. These previous studies also showed that collagenase digestion does not impair CD271 based selection [11,14].

Finally, we compared the expression of a range of common hDPSC markers by CD271$^-$ hDPSCs and unsorted hDPSCs, isolated by plastic adherence. These results showed near identical expression profiles, confirming that CD271$^-$ derived cells are the same as those isolated by plastic adherence and are in keeping with previously published data [27-29]. We can therefore conclude that cells from the CD271$^-$ fraction are indeed the cells commonly described as hDPSCs. This does not preclude the possibility that CD271$^+$ cells also form colonies with similar profiles, but in smaller numbers. In this respect, it is important to mention that previous reports have shown that multiple proliferative cell populations exist within the human DP, including Oct-4 and Nanog expressing immature DP stem cells [24,30,31]. Recent reports have shown that cultured hDPSCs contain some cells of neural crest origin and it has been proposed in rats that CD271 is a marker for neural crest derived populations [24,32]. From our data, it is possible to say that CD271 identifies a small subpopulation of cells within dental pulp and these may be the neural crest derived cells. However, in our experimental conditions, selection based on its expression produces significantly fewer colonies than the CD271$^-$ fraction.

It is interesting to note that previous studies have identified CD271 in the apical papilla as well as the continuously erupting rat incisor [19,24]. It can therefore be postulated that CD271 may mark for early stage proliferating DP cells, and potentially prevents differentiation in deciduous teeth, but upon tissue maturation its expression is lost [16].

Our data also provide further evidence for differences in stromal cell biology between hDPSCs and BMSCs. These cell types are ostensibly similar with many commonalities [25,33]; however it is clear from our data that a key '*in vivo*' marker of BMSCs –CD271– is lacking in the majority, if not all of colony forming hDPSCs. The rationale for this finding is likely related to the developmental origin of these tissues, with DP being a neural crest ectomesenchymal derivative and BM originating from, in the most part, the mesodermal germ layer [34-36]. It is therefore likely that despite the many phenotypic similarities following culture, the differences in embryonic origin mean that each cell type is likely to have fundamental differences in terms of '*in vivo*' marker expression and inherent differentiation potentials [33]. Indeed this difference in differentiation potential is well known with hDPSCs producing globular dentin following *in vivo* transplantation whereas BMSCs produce lamellar bone [1]. We also note from our data that colonies formed from DP appear smaller in size than those from BM.

Conclusion

We conclude that CD271 is expressed within adult DP but that a significant majority of colony forming DP cells do not show CD271 expression. Following *in vitro* expansion these cells possess sufficiently similar expression profiles to conventionally isolated hDPSCs so as to be indistinguishable. CD271$^+$ cells showed limited colony forming potential and may be a distinct neural crest subpopulation within the DP. Overall, we conclude that CD271 is not a specific marker for hDPSCs and that it should not be used to isolate these cells from mature DP.

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