Characterisation of proliferation, differentiation potential, and gene expression among clonal cultures of human dental pulp cells

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Research article

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Abstract

Background: Mesenchymal stem cells are a highly promising source of cells for regeneration therapy because of their multilineage differentiation potential. However, distinct markers for mesenchymal stem cells are not well-established. To identify new candidate marker genes for multipotent human dental pulp stem cells, we analysed the characteristics and gene expression profiles of cell clones obtained from a single dental pulp specimen.

Results: Fifty colony-forming single cell-derived clones were isolated from a single dental pulp specimen. These clones varied in their proliferation abilities and surface marker (STRO-1 and CD146) expression patterns, as well as their odontogenic, adipogenic, and chondrogenic differentiation potentials. Four clones maintained their original differentiation potentials during long-term culture. Gene expression profile analysis of five representative clones identified 1227 genes that were related to multipotency. Ninety of these 1227 genes overlapped with genes reportedly involved in 'stemness or differentiation'. Based on the predicted locations of expressed protein products and large changes in expression levels, 14 of the 90 genes were selected as candidate dental pulp stem cell markers, particularly in relation to their multipotency characteristics.

Conclusions: This characterisation of cell clones obtained from a single specimen of human dental pulp provided information regarding new candidate marker genes for multipotent dental pulp stem cells, which could facilitate efficient analysis or enrichment of multipotent stem cells.

Background

Because of their multilineage differentiation potential, mesenchymal stem cells (MSCs) are a highly promising source of cells for regeneration therapy to repair damaged tissues. Human postnatal MSCs have been identified in various tissues, including dental pulp; notably, dental pulp is an attractive cell source because dental pulp tissues can be obtained from extracted teeth in a noninvasive manner (these teeth are typically discarded). MSCs, including dental pulp stem cells, have been extensively characterised: they have the capacity for clonogenic self-renewal, potential for multilineage differentiation (multipotency, including odontogenic, adipogenic, and chondrogenic differentiation) [1] and potential for rapid proliferation in vitro [2, 3]; moreover, they exhibit tissue regeneration potential in vivo [4]. Isolated human dental pulp stem cells can form dentin/pulp-like complex tissues following subcutaneous transplantation into immunocompromised mice [5].

However, populations of mesenchymal cells are often heterogeneous, such that they are composed of both genuine multipotent stem cells and committed progenitor cells with restricted differentiation potentials. Growth capacity [6], differentiation potential [7], regeneration potential [8], and surface marker expression characteristics [7] exhibit considerable variation among mesenchymal cell populations; this heterogeneity is a source of complexity that interferes with understanding of the stem cell mechanism.

Because of the heterogeneity of MSCs, it remains controversial whether the multipotency of mesenchymal cell populations arises from genuine multipotent stem cells or the coexistence of distinct, committed progenitor cells. In previous attempts to address this issue, mesenchymal stem cell experiments have been performed with single cell-derived populations, which are regarded as clonal populations; thus, they are considered to be homogeneous, given that all cells originated from the same parent cell and replicated under very similar culture conditions [9]. Multiple investigations have been performed to analyse human mesenchymal cell clones derived from tissues such as dental pulp [6, 8, 10–12], bone marrow [12–19], hair follicle [20], endometrium [21], and umbilical cord [22]. However, in studies that involved small numbers of clones obtained from multiple donors, differences in gene expression among clones obtained from multiple donors might have reflected the different genetic backgrounds of the donors, rather than phenotypic differences between multipotent stem cells and committed progenitor cells [23]. Analysis of gene expression profiles among clones obtained from a single donor may allow researchers to eliminate the differences in genetic backgrounds that are associated with the use of multiple donors [19].

Distinct markers that define genuine MSCs are not yet well-established [3, 24]. Various surface molecules have been used as putative mesenchymal stem cell markers, both alone and in combination (e.g., STRO-1 [25–27], melanoma cell adhesion molecule [MCAM, better known as CD146] [28, 29], endoglin [ENG, better known as CD105] [30], Thy-1 cell surface antigen [THY1, better known as CD90] [31, 32], and KIT proto-oncogene, receptor tyrosine kinase [KIT, better known as CD117] [33, 34]. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has proposed minimum criteria to define mesenchymal stromal (stem) cells [1]: mesenchymal stromal cells must express ENG (CD105), 5'-nucleotidase ecto (NT5E, better known as CD73), and THY1 (CD90); additionally, mesenchymal stromal cells must lack expression of haemocyte-associated markers [1]. However, these markers were originally used to identify other tissues, such as endothelium or haematopoietic stem cells [24]; this overlap may cause confusion regarding the proper identification of mesenchymal stromal cells. The identification of unique mesenchymal stem cell markers will increase the efficiency of analysis and facilitate the enrichment of multipotent MSCs.

In the present study, we analysed the proliferation and differentiation characteristics of 50 single cell-derived clones that were obtained from a single specimen of human dental pulp, then characterised the gene expression profiles of five representative clones. We analysed genes that demonstrated altered expression among clones with variations in differentiation potential, then selected a subset of these as candidate markers for human dental pulp stem cells.

Results

Differentiation potentials and tissue regeneration potentials of dental pulp cell populations

We first investigated the multipotency of heterogeneous human dental pulp cell (DPC) populations *in vitro* and *in vivo*. DPC populations obtained from a single specimen of human dental pulp expressed

CD105, CD73, CD90, CD146, and (weakly) STRO-1 (Fig. 1a), whereas they lacked expression of haemocyte-associated markers (CD45, CD34, CD14, CD79a, and HLA-DR) (Fig. 1a). The human DPC populations exhibited fibroblast-like morphology *in vitro* (Fig. 1b). The differentiation potentials of human DPC populations were analysed by histochemical staining and quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays. Differentiation-induced primary cultures of DPC populations were positively stained with Alizarin Red S (odontogenic differentiation) (Fig. 1c), Oil Red O (adipogenic differentiation) (Fig. 1d), and Alcian blue (chondrogenic differentiation) (Fig. 1e, f). The expression levels of integrin binding sialoprotein (IBSP), lipoprotein lipase (LPL), and collagen type X alpha 1 chain (COL10A1) (respective odontogenic, adipogenic, and chondrogenic differentiation markers) were considerably greater in differentiated cell populations than in undifferentiated control populations (Fig. 1g–i). The tissue regeneration potential was analysed *in vivo* by transplantation of human DPC populations into immunocompromised mice; dentin/pulp-like complex tissues were formed after transplantation (Fig. 1j). Furthermore, odontoblast-like cells were observed in connective tissue adjacent to the surface of the dentin-like structures (Fig. 1j). These findings demonstrated that heterogeneous human DPC populations exhibit multipotency *in vitro* and tissue regeneration potential *in vivo*.

Colony-picking and proliferation of isolated clones

Colony-forming single cell-derived clones were isolated from heterogeneous multipotent human DPC populations. The colony formation rate was $64.3 \pm 3.01\%$. Fifty colonies (clones) were isolated and separately cultured until growth cessation. Each clone was identified by clone (CL) number (CL 1 to CL 50). The population doubling level (PDL) at growth cessation varied among clones, from 30.1 PDL to 67.3 PDL (Additional file 1: Table S1).

Expression of surface markers by each clone

The expression of two well-known mesenchymal stem cell surface markers (STRO-1 and CD146) by each clone was examined by immunocytochemical analysis (Fig. 2a–c; Table 1; Additional file 2: Table S2). Forty-five (90%) of the 50 clones were positive for both STRO-1 and CD146 expression at 17.6 PDL. Thirty-six of the 50 clones were examined at both 17.6 PDL and >40 PDL. Twenty-three of these 36 clones (64%) were positive for STRO-1 and CD146 expression at both 17.6 PDL and >40 PDL, demonstrating that the majority of clones maintained expression of both mesenchymal stem cell surface markers throughout long-term culture (Table 1; Additional file 2: Table S2).

Differentiation potentials of each clone

We examined the odontogenic and adipogenic differentiation potentials of each clone at early (24.1 PDL) and late (>40 PDL) stages of culture. Odontogenic and adipogenic differentiation potentials at 24.1 PDL were analysed in 28 clones; eight of these 28 clones (29%) were both odontogenic and adipogenic, four clones (14%) were odontogenic only, 10 clones (36%) were adipogenic only, and six clones (21%) did not demonstrate either differentiation potential (Fig. 2d, e; Table 2; Additional file 3: Table S3). Odontogenic and adipogenic differentiation potentials were assayed at >40 PDL in 12 clones; eight of these 12 clones (67%) were both odontogenic and adipogenic, while four clones (33%) were odontogenic only (Table 2; Additional file 3: Table S3).

In total, eight clones were assayed for odontogenic and adipogenic differentiation potentials at both 24.1 PDL and >40 PDL (Additional file 3: Table S3). Four of these eight clones exhibited similar differentiation potentials at 24.1 PDL and >40 PDL, suggesting that their differentiation potentials were maintained throughout long-term culture (CL 4 and CL 35, both odontogenic and adipogenic; CL29 and CL 38, odontogenic only) (Additional file 3: Table S3). Notably, the odontogenic differentiation potential of CL 21 was not assayed at 24.1 PDL, because these cells detached during the differentiation period; however, this clone maintained adipogenic differentiation potential and exhibited odontogenic differentiation at 60.7 PDL. Furthermore, CL 21 demonstrated the highest proliferation ability (Additional file 1: Table S1). We identified five representative clones and tested their chondrogenic differentiation potentials (Fig. 2f; Additional file 3: Table S3) at >40 PDL: CL 4, CL 21, CL 29, CL 35, and CL 38. All of these clones, with the exception of CL 38, exhibited chondrogenic differentiation potential (Additional file 3: Table S3).

A summary of the differentiation potentials among representative clones is shown in Table 3. CL 4, CL 21, and CL 35 exhibited tri-lineage differentiation potential (tripotent). CL 29 and CL 38 exhibited bi-lineage and uni-lineage differentiation potentials, respectively (bipotent and unipotent, respectively). All representative clones maintained the expression of STRO-1. However, CL 4 had lost expression of CD146 by 43.7 PDL, while CL 38 did not exhibit expression of CD146 throughout the experiment (Additional file 2: Table S2; Additional file 4: Figure S1). Cell morphology was similar among all five clones. Each clone eventually senesced, demonstrating senescence-associated β -galactosidase (SA- β -Gal) activity at the end of the culture period (Additional file 5: Figure S2).

Gene expression profiles of representative clones

Gene expression analyses were performed on the above-selected five representative clones to detect genes related to multipotency. We compared seven data sets of gene expression profiles among the five clones: tripotent compared with bipotent (CL 4 compared with CL 29, CL 21 compared with CL 29, and CL 35 compared with CL 29), tripotent compared with unipotent (CL 4 compared with CL 38, CL 21 compared with CL 38, and CL 35 compared with CL 38), and bipotent compared with unipotent (CL 29 compared with CL 38). The criteria for identification as a gene with altered expression were that the probeset signal

intensity value was >100 and the ratio of the signal intensity value for each comparison was >1.5 or <0.67 in at least one comparison. In total, 1950 probesets met these criteria. Unsupervised hierarchical clustering analyses of the 1950 probesets, utilising genes with altered expression between clones (represented by heat mapping), revealed that the clusters were not aligned on the basis of multipotency (Fig. 3a). Functional analysis was performed on genes that demonstrated altered expression among the clones. Gene ontology analyses demonstrated that the main functions of genes with altered expression were related to processes such as cell cycle, cell division, and mitosis (Additional file 6: Table S4). Subsequently, we performed pathway analysis of cell cycle pathways (Additional file 7: Figure S3), which demonstrated that more cell cycle-related genes were upregulated in tripotent clones (CL 4, CL 21, CL 35) than in bipotent (CL 29) or unipotent (CL 38) clones. In contrast, more cell cycle-related genes (e.g., cyclin A2 [CCNA2], cyclin B1 [CCNB1], cyclin B2 [CCNB2], cyclin E2 [CCNE2], cell division cycle 2 [CDC2], and cyclin dependent kinase 2 [CDK2]) were upregulated in the unipotent clone (CL 38) than in the bipotent clone (CL 29) (Additional file 7: Figure S3). This analysis demonstrated that major genes with altered expression were not directly related to multipotency.

Finally, we used an alternative analysis method for detection of genes related to multipotency. We identified probesets of genes that were correlated either positively or negatively with multipotency. Genes that were correlated positively with multipotency were tri- > bi- > uni-potent (CL 4 > CL 29 > CL 38; CL 21 > CL 29 > CL 38; or CL 35 > CL 29 > CL 38) (808 probesets, corresponding to 754 genes). In contrast, genes that were correlated negatively with multipotency were uni- > bi- > tri-potent (CL 38 > CL 29 > CL 4; CL 38 > CL 29 > CL 21; or CL 38 > CL 29 > CL 35) (611 probesets, corresponding to 473 genes). Hence, 1419 (808 + 611) probesets were correlated with multipotency, corresponding to 1227 (754 + 473) genes. Unsupervised hierarchical clustering analyses of genes with altered expression that were correlated positively or negatively with multipotency, as represented by heat mapping, are shown in Fig. 3b.

Additionally, a list of genes related to 'stemness or differentiation' was constructed using information from gene databases (Ingenuity Pathway Analysis [IPA] and Gene Ontology) and the scientific literature; it consisted of 1314 probesets, corresponding to 1246 genes (Additional files 8, 9: Tables S5, S6). We reduced the number of candidate dental pulp stem cell marker genes based on overlap between genes related to multipotency (1419 probesets of 1227 genes from DNA microarray data) and 'stemness or differentiation' (1314 probesets of 1246 genes from databases and the literature) (Fig. 3c). The list of genes that were correlated with multipotency in present experiment partially overlapped with the list of genes related to 'stemness or differentiation' constructed using information from databases and the literature, suggesting that the genes correlated with multipotency were also related to 'stemness or differentiation'. There were 95 overlapping probesets, which corresponded to 90 distinct genes (Fig. 3d; Additional file 10: Table S7). From among these 90 genes, we selected 14 representative genes, shown in Table 4, based on large changes in expression levels and a predicted location (either plasma membrane or extracellular space) for the expressed protein product that would facilitate its detection by flow cytometry or immunocytochemical analysis. Nine of these genes were positively correlated with multipotency, while five of these genes were negatively correlated with multipotency. Thus, these 14

genes are related to both multipotency and 'stemness or differentiation', and are candidates for use as markers of multipotent mesenchymal stem cells.

Discussion

The present investigation demonstrated that colony-forming single cell-derived clones, which are obtained from single dental pulp, varied in proliferation ability, surface marker expression, differentiation potential, and gene expression. Importantly, a single specimen of dental pulp contained both multipotent stem cell-like clones and progenitor-like clones with restricted differentiation potentials. These results support the findings of previous reports regarding variation in single cell-derived clones [11, 13–15, 18–20, 22].

The clonogenic cells in this study expressed both STRO-1 and CD146 at a high frequency at 17.6 PDL (Table 1; Additional file 2: Table S2). Gronthos et al. reported that isolation of cells from colony-forming units of fibroblasts led to enrichment of mesenchymal stem cell populations in vitro [4]. Another report confirmed the presence of six-fold more colony-forming cells in the STRO-1-positive fraction, compared with an unfractionated population of DPCs; in addition, those authors reported seven-fold more colony-forming cells in the CD146-positive fraction than in an unfractionated population of DPCs [25]. However, in our present study, some isolated clonogenic clones positive for STRO-1 or CD146 exhibited restricted differentiation potentials. Gharibi and Hughes analysed the expression of stem cell surface markers by flow cytometry; they showed that the expression of CD146 and other stem cell markers persisted despite the loss of differentiation potentials during long-term culture [35]. Therefore, cells expressing stem cell markers may include cells with restricted differentiation potentials. Furthermore, we found that the ratio of clonogenic cells expressing both STRO-1 and CD146 decreased at > 40 PDL (Table 1; Additional file 2: Table S2). These reductions in the expression ratio of STRO-1 and CD146 were due to the increased number of passages during long-term culture [36, 37].

Somoza et al. analysed 38 human bone marrow-derived cell clones and found that 10 (26%) were both osteogenic and adipogenic, two (5%) were osteogenic only, 21 (55%) were adipogenic only, and five (13%) did not demonstrate either differentiation potential [15]; notably, these results in bone marrow-derived cell clones were similar to our results in dental pulp-derived cell clones (Table 2 at 24.1 PDL). Many studies have shown that MSCs lose their differentiation capacity during long-term culture [13, 38–42]. In the present study, the ratio of adipogenic clones decreased in later passages, compared with early passages, whereas the ratio of osteogenic clones increased in later passages (Table 2; Additional file 3: Table S3). The findings reported by Wagner et al. support our results that long-term cultured stem cells derived from bone marrow exhibited a rapid reduction in adipogenic differentiation potential, whereas they exhibited an increased propensity for osteogenic differentiation, even in later passages [40]. There is general agreement that the adipogenesis potential of MSCs tends to decline with consecutive passages under standard culture conditions [43]. During the aging process, ectopic ossification occurs in dental pulp;

specifically, increases are observed in the thicknesses of dentine and cementum [44]. The increased ratio of osteogenic clones in our study might be a result of the aging process. Some clones were positive for Alizarin Red S and/or Oil Red O stainings under the culture conditions with control medium at 40.1–60.7 PDL. We excluded these spontaneously differentiated clones from the analysis (a portion of clones denoted as 'ND' [i.e., not determined] in Additional file 3: Table S3) because we could not distinguish whether the positive staining results were due to the induction of differentiation or were the product of denaturation related to senescence [45].

In an additional study, Muraglia et al. analysed the hierarchy of multipotency (osteogenic, chondrogenic, and adipogenic differentiation) in human bone marrow-derived cell clones. They reported that clones progressively lost adipogenic differentiation potential, then lost chondrogenic differentiation potential with an increasing number of cell doublings [14]. This hierarchy was also present in our results (Table 3).

In the present study, gene expression profile analyses were performed on four clones which maintained their differentiation potentials, as well as the most highly proliferative clone (CL 21); notably, the odontogenic potential of CL 21 at early PDL was not determined. The cells detached from the culture surface and formed aggregates during odontogenic induction periods because these cells proliferated actively at early PDL. The gene expression levels of CL 21 were highest for many genes related to differentiation potential and 'stemness or differentiation' (Fig. 3d). Therefore, CL 21 was regarded as a stem cell-like clone.

In the present study, we found that the analysis of major functions of genes with altered expression was not sufficient to make conclusions regarding their relationships with multipotency, because the results of gene ontology analysis and pathway analysis did not directly indicate multipotency (Fig. 3a; Additional file 6: Table S4; Additional file 7: Figure S3). Gene functions such as cell cycle, cell division, and mitosis were the most prominent categories identified in gene ontology analysis of the clones (Additional file 6: Table S4). Hence, we explored an alternative analysis method to identify genes correlated with multipotency, then reduced the number of candidate dental pulp stem cell marker genes using a list of genes related to 'stemness or differentiation'. Accordingly, we identified 90 genes that were related to both multipotency and 'stemness or differentiation' (Fig. 3c).

A variety of well-known stem cell marker genes were correlated with multipotency in our study. KIT (CD117) [33, 34]; MYC proto-oncogene, bHLH transcription factor (MYC) [46, 47]; and transferrin receptor (TFRC, better known as CD71) [31] were included in our list of 90 genes that were related to both multipotency and 'stemness or differentiation' (Additional file 10: Table S7). Our findings support the use of KIT (CD117), MYC, and TFRC (CD71) as stem cell markers.

A portion of our 90 selected genes overlapped with those described in other reports of gene expression profiles in MSCs. Mareddy et al. compared fast-growing and slow-growing bone marrow-derived clones from three donors; they identified 17 upregulated and eight downregulated genes in fast-growing clones, compared with slow-growing clones [18]. Two of the selected 90 genes in our study were consistent with their findings (bone morphogenetic protein 2 [BMP2] and delta-like canonical Notch ligand 3 [DLL3]). In

another study that utilised cells derived from bone marrow, dental pulp, and periodontal ligament, Menicanin et al. compared clones that exhibited high growth/multi-differentiation potentials with clones that exhibited low growth potentials; they identified 24 genes that were upregulated in clones that exhibited high growth/multi-differentiation potentials [6]. Notably, replication protein A3 (RPA3) was identified in both their study and our study. In yet another investigation, Sworder et al. measured tissue regeneration potentials in bone marrow-derived clones obtained from a single donor and identified 19 genes that were differentially expressed in multipotent clones [19], including two genes (BMP2 and intercellular adhesion molecule 1 [ICAM1]) that were also identified in our study. In all three of these prior studies, the investigators used bone marrow-derived mesenchymal cells. Bone marrow tissue originates from the mesoderm and neural crest, whereas dental pulp tissue is presumably derived from migrating neural crest cells [48]. Accordingly, we found some overlap in the data, but observed multiple differences that may be related to the human dental pulp origin of our cells. Additional, detailed experiments are thus required to elucidate differences between mesenchymal cells derived from bone marrow and those derived from dental pulp.

We selected 14 genes (Table 4) for further analysis from among the 90 genes that were related to both multipotency and 'stemness or differentiation'. One of these, ATPase phospholipid transporting 8B (ATP8B1) encodes a member of the subfamily of aminophospholipid-transporting ATPases (NCBI Gene ID: 5205). Desmoplakin (DSP) encodes a protein that forms an obligate component of desmosomes (NCBI Gene ID: 1832); it is also expressed in odontoblasts and cultured dental pulp fibroblasts [49]. ICAM1 is a cell adhesion molecule that is typically expressed on endothelial cells and cells of the immune system (NCBI Gene ID: 3383); it is reportedly indispensable for MSC-mediated immunosuppression [50, 51]. Inhibin beta A subunit (INHBA) encodes a member of the transforming growth factor-beta superfamily of proteins. The encoded preproprotein is proteolytically processed to generate a subunit of the dimeric activin and inhibin protein complexes (NCBI Gene ID: 3624). INHBA is regarded as a component of the cell differentiation pathway in the Gene Ontology database. Neuronatin (NNAT) encodes a proteolipid that may be involved in the regulation of ion channels during brain development. The encoded protein may also play a role in the formation and maintenance of nervous system structure (NCBI Gene ID: 4826). NNAT is regarded as a component of the neuron differentiation pathway in the Gene Ontology database. Oxytocin receptor (OXTR) encodes a protein that belongs to the G-protein coupled receptor family and acts as a receptor for oxytocin (NCBI Gene ID: 5021). Serpin family E member 1 (SERPINE1) encodes a member of the serine proteinase inhibitor (serpin) superfamily that inhibits fibrinolysis (NCBI Gene ID: 5054). SERPINE1 has been reported as an adipogenesis-related gene [52]. Another gene, sortilin 1 (SORT1) is known as a neurotensin receptor, which plays a role in the trafficking of different proteins to the cell surface or subcellular compartments (e.g., lysosomes and endosomes) (NCBI Gene ID: 6272). SORT1 has been reported as an adipogenesis- and osteogenesis-related gene [52]. Serglycin (SRGN) encodes a protein known as a haematopoietic cell granule proteoglycan; this protein has been associated with the macromolecular complex of granzymes and perforin, which may serve as a mediator of granule-mediated apoptosis (NCBI Gene ID: 5552). Adhesion G protein-coupled receptor A (ADGRA2) (NCBI Gene ID: 25960) is related to signal transduction. Anthrax

toxin receptor 1 (ANTXR1) (NCBI Gene ID: 84168) is a tumour-specific endothelial marker that has been implicated in colorectal cancer. Collagen type I alpha 2 chain (COL1A2) is a fibril-forming collagen found in most connective tissues and is abundant in bone, cornea, dermis, and tendon (NCBI Gene ID: 1278); it has been reported as an osteogenesis-related gene [52]. Collagen type III alpha 1 chain (COL3A1) encodes a fibrillar collagen that is found in a variety of connective tissues such as skin, lung, uterus, intestine, and the vascular system (NCBI Gene ID: 1281). Moreover, COL3A1 is expressed various oral mesenchymal stem cell populations in vitro [5], and has been reported as a marker for odontoblast differentiation [53]. Integrin subunit alpha 8 (ITGA8) (NCBI Gene ID: 8516) regulates the recruitment of mesenchymal cells into epithelial structures, mediates cell–cell interactions, and regulates neurite outgrowth of sensory and motor neurons; ITGA8 plays an important role in wound-healing and organogenesis. ATP8B1, ICAM1, ADGRA2, and ANTXR1 were reported as stemness-related genes that were downregulated during differentiation but upregulated during dedifferentiation in MSCs [54]. In addition, OXTR and SRGN are upregulated during osteoblast differentiation [52].

A key criterion was that the selected 14 gene products were expressed on the plasma membrane or in the extracellular space, which are sites of expression that simplify protein detection by immunocytochemical analysis or flow cytometry. Moreover, the signal intensity values of the 14 selected genes were high and were altered appreciably among clones with disparate differentiation potentials. Although most of the selected genes have not previously been used as markers of MSCs, they might serve as promising candidate markers for dental pulp stem cells. Further investigations are needed to determine whether these 14 candidate markers are reliable indicators of multipotency.

Variations in differentiation potential and gene expression among clones obtained from a single specimen of dental pulp were analysed in this study. The advantage of this strategy was that the underlying genetic variance was minimised among clones. Thus, our strategy allowed clearer detection of differences in gene expression among clones that exhibit disparate differentiation potentials. However, Sworder et al. demonstrated that the expression level of one of their candidate marker genes might vary among donors [19]. Our planned future studies include the analysis of a large series of cells from multiple donors to confirm the most reliable markers among our candidate genes for the identification of genuine multipotent dental pulp stem cells.

Conclusions

Fifty clones were isolated from a single specimen of human dental pulp. We assessed their proliferation abilities, surface cell marker expression patterns, and differentiation potentials. Analysis of the gene expression profiles of five representative clones enabled identification of 14 genes related to multipotency and ‘stemness or differentiation,’ as candidate markers for dental pulp stem cells. These candidate genes could be used to isolate and manipulate multipotent dental pulp stem cells for regeneration therapies.

Methods

Cells and culture medium

A normal, impacted third molar was obtained from an 11-year-old female patient at the Nippon Dental University Hospital in Tokyo, Japan. The informed consent was taken from parent/LAR of the patient with the approval of the Committee of Ethics at the Nippon Dental University School of Life Dentistry at Tokyo. Dental pulp tissue was separated from the tooth and DPC populations were enzymatically released from the tissue, then passed through a 70- μ m cell strainer (Corning, NY, USA) [4, 55]. The cells were plated into 75-cm² flasks (Corning) and incubated at 37°C in a humidified atmosphere of 5% CO₂/95% room air. The culture medium used for cellular growth was minimum essential medium alpha (MEM α) (Thermo Fisher Scientific, MA, USA), supplemented with 20% fetal bovine serum (FBS) (SAFC Biosciences, KS, USA), 100 μ M L-ascorbic acid phosphate magnesium salt n-hydrate (ascorbic acid) (Wako Pure Chemical, Osaka, Japan), 2 mM L-glutamine (Thermo Fisher Scientific), 100 units/ml penicillin and 100 μ g/ml streptomycin (Thermo Fisher Scientific). When DPCs reached semi-confluence, they were passaged at a ratio of 1:2 or 1:4. The PDL number was calculated by the formula, $2^n = N$, where n = PDL and N = number of cells per flask. Mycoplasma tests of this DPC population by nested-PCR and by fluorescence staining using VERO cells were both negative.

Isolation and culture of cell clones

Single cell-derived clones were isolated from DPC populations that were obtained from a single specimen of human dental pulp. DPC populations in primary culture (passage 0) were plated on 100-mm dishes (Corning) at a density of 200 cells/dish and incubated for 10 days to form colonies. The single cell ratio of the cell suspension at the time of plating was >97%. Colonies with >50 cells obviously distinct from other colonies were isolated separately, using cloning cylinders with an inside diameter of 6 mm. Each clone was identified by clone (CL) number (CL 1 to CL 50). All harvested clones were separately passaged when they reached semi-confluence, then expanded until cells were spread in 25-cm² flasks (Corning). For subsequent serial cultivation, cells were passaged at a ratio of 1:2 or 1:4.

Flow cytometry

DPC populations were incubated with antibodies for 10 min at 4°C after incubation with FcR blocking reagent (Miltenyi Biotec, North Rhine-Westphalia, Germany). The following antibodies were used: phycoerythrin (PE)-conjugated anti-human CD105 (clone: 43A4E1) (Miltenyi Biotec), PE-conjugated anti-human CD73 (clone: AD2) (Miltenyi Biotec), PE-conjugated anti-human CD90 (clone: DG3) (Miltenyi Biotec), PE-conjugated anti-human CD146 (clone: 541-10B2) (Miltenyi Biotec), PE-conjugated anti-human CD45 (clone: 5B1) (Miltenyi Biotec), PE-conjugated anti-human CD34 (clone: AC136) (Miltenyi Biotec), PE-conjugated anti-human CD14 (clone: REA599) (Miltenyi Biotec), PE-conjugated anti-human CD79 α (clone: HM47) (Miltenyi Biotec), and PE-conjugated anti-human HLA-DR (clone: IS5-20C4) (Miltenyi Biotec). PE-conjugated mouse IgG1 isotype control antibody (clone: IS5-21F5) (Miltenyi Biotec) was used for anti-CD105, CD73, CD90, CD79 α and CD146 antibodies. PE-conjugated mouse IgG2a isotype control antibody (clone: S43.10) (Miltenyi Biotec) was used for anti-CD45, CD34, CD14, and HLA-DR antibodies. Anti-human STRO-1 (clone: STRO-1) (R&D Systems, MN, USA) was labelled with PE-conjugated anti-mouse IgM (clone: REA979) (Miltenyi Biotec). PE-conjugated mouse IgM isotype control antibody (clone: IS5-20C4) (Miltenyi Biotec) was used for the anti-STRO-1 antibody. After cells had been washed with

autoMACS Running Buffer (Miltenyi Biotec), they were analysed using a Guava® easyCyte flow cytometer (Luminex, TX, USA) and FlowJo software (version 10.4.2) (Becton, Dickinson and Company, NJ, USA).

Histochemical staining

Multiple aspects of cellular differentiation potential were examined by histochemical staining. DPC populations and clonal cells were both incubated in standard growth medium until they reached confluence. Then, cells were incubated in differentiation induction media as follows. To assess odontogenic differentiation, cells were incubated with MEM that was supplemented with 10% FBS, 100 μ M ascorbic acid, 2 mM L-glutamine, 10 mM sodium α -glycerophosphate n-hydrate (Wako Pure Chemical), and 10 nM dexamethasone (Wako Pure Chemical) [55]. To assess adipogenic differentiation, cells were incubated with MEM that was supplemented with 20% FBS, 0.5 mM 3-isobutyl 1-methylxanthine (Merck, Darmstadt, Germany), 0.5 μ M hydrocortisone (Merck), 60 μ M indomethacin (Merck), 100 μ M ascorbic acid, and 2 mM L-glutamine [56]. To assess chondrogenic differentiation, cells were incubated in Dulbecco's modified Eagle medium (Thermo Fisher Scientific), supplemented with 10% FBS, 10 μ g/ml insulin-transferrin-selenium-X (Thermo Fisher Scientific), 5.35 μ g/ml linoleic acid (Merck), 1.25 μ g/ml bovine serum albumin (Merck), 2.6 μ M dexamethasone, 35 μ M ascorbic acid, and 10 ng/ml transforming growth factor beta-3 (R&D Systems) [53]. To achieve differentiation induction, cells were cultured for up to 3 weeks. Chondrogenic induction of DPC populations was performed using adherent cells as well as cell pellets that were prepared by centrifuging cells (2.5×10^5) in 15-ml conical polystyrene tubes (Corning) at 190 g for 5 min. After cells had been incubated in differentiation induction media, they were washed with phosphate-buffered saline (PBS) (Nissui Pharmaceutical, Tokyo, Japan) and fixed with 4% paraformaldehyde (Wako Pure Chemical) in PBS. Cells were then stained with either 1% Alizarin Red S (Merck) for odontogenic differentiation, 0.18% Oil Red O (Merck) for adipogenic differentiation, or 1% Alcian blue (pH 1.0) (Merck) for chondrogenic differentiation. For cell pellet analysis, frozen sections (10 μ m thick) were stained with 1% Alcian blue.

mRNA expression

Total cellular RNA was isolated and reverse-transcribed using a method described previously [57]. qRT-PCR analyses were performed using TaqMan® gene expression assays in a StepOne Plus® RT-PCR system (Thermo Fisher Scientific). The following human genes were targeted: IBSP as a marker of odontogenic differentiation, LPL as a marker of adipogenic differentiation, and COL10A1 as a marker of chondrogenic differentiation (assay IDs: Hs00173720_m1, Hs01012569_m1, and Hs00166657_m1, respectively; Thermo Fisher Scientific). The eukaryotic 18S rRNA gene (Thermo Fisher Scientific, catalogue number: 4319413E) was used as an endogenous control for expression. All experiments were performed in triplicate.

Transplantation

DPC populations (approximately 2×10^6) in primary culture (at 4.0 PDL) were mixed with 40 mg hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Kobayashi Medical, Osaka, Japan) and incubated for 90 min at 37°C. After cells had been centrifuged at 440 g for 7 min, the resulting cell pellets

with HA/TCP were transplanted into subcutaneous space of anesthetised adult healthy female immunocompromised beige mice (CrI: NIH-LystbgFoxn1nuBtkxid) (n=4) (Charles River Laboratories Japan, Kanagawa, Japan), using a method described previously [29, 55]. These experiments were performed with the approval of the Animal Experiments Committee of The Nippon Dental University School of Life Dentistry at Tokyo. All mice were kept under specific pathogen free conditions and housed 2-5 per cage with ad lib access to food and water and 12 h dark cycle. The mice were euthanised three months after transplantation and no adverse event was observed. Transplants were harvested from the immunocompromised mice and fixed with 4% paraformaldehyde in PBS, then decalcified with buffered 10% ethylenediaminetetraacetic acid (pH 8.0) and embedded in paraffin. Paraffin-embedded sections (5 µm thick) were stained with haematoxylin and eosin and qualitatively analysed as described previously [4].

Immunocytochemical staining

Cells were fixed with 4% paraformaldehyde in PBS. After they had been washed in PBS, cells were incubated with mouse anti-human STRO-1 antibody (MAB1038; R&D systems, 10 µg/ml) or mouse anti-human CD146 antibody (NCL-CD146; Leica Biosystems, Baden-Württemberg, Germany, 1:25 dilution) and stained with Histostain®-SP kit (AEC, broad spectrum; Thermo Fisher Scientific), in accordance with the manufacturers' instructions. Mouse anti-human isotype control (Thermo Fisher Scientific) was used as a negative control. The detection criteria were as follows: at least one positive cell detected in three visual fields with x20 magnification was classified as a positive finding, while a lack of positive cells was classified as a negative finding.

Senescence-associated β-galactosidase assay

The SA-β-Gal assay was performed as described previously [58]. Briefly, cells were washed in PBS, fixed for 5 min at room temperature in 2% formaldehyde/0.2% glutaraldehyde, then washed and incubated at 37°C (without CO₂) in fresh SA-β-Gal stain solution. Staining was evident in 2–4 h and reached a peak within 12–16 h.

Analysis of gene expression profiles

DNA microarray analyses of representative clones were performed to compare the gene expression profiles of genes related to multipotency. Samples of total RNA of CL 4, CL 21, CL 29, CL 35, and CL 38 were collected from 24.1 to 29.1 PDL. After total cellular RNA from each clone had been isolated and reverse-transcribed, DNA microarray analyses were performed by Cell Innovator Inc. (Fukuoka, Japan), using Affymetrix GeneChip® Human Gene 1.0 ST Arrays (Affymetrix, Thermo Fisher Scientific). Raw data were processed for gene-level analysis with median polish summarisation and quantile normalisation by Affymetrix® Expression Console™ 1.1 software (Affymetrix, Thermo Fisher Scientific) to obtain normalised intensity values. The expression ratio between clones was calculated from the signal intensity values of each probeset. Unsupervised hierarchical clustering analyses, which are represented in heat maps of the signal intensity values, were performed with Multiple Experiment Viewer software [59]. Expression profile data were analysed using IPA software (Ingenuity, QIAGEN, North Rhine-Westphalia, Germany) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) [60] for gene

ontology analysis. The PathVisio database [61] was used for pathway analysis. The databases of IPA and Gene Ontology [62] and the scientific literature were used to compile lists of 'stemness'- or 'differentiation'-related genes (Additional files 8,9: Tables S5, S6).

Abbreviations

MSC

mesenchymal stem cell; MCAM:melanoma cell adhesion molecule; ENG:endoglin; THY1:Thy-1 cell surface antigen; KIT:KIT proto-oncogene, receptor tyrosine kinase; NT5E:5'-nucleotidase ecto; DPC:dental pulp cell; qRT-PCR:quantitative reverse transcription polymerase chain reaction; IBSP:integrin binding sialoprotein; LPL:lipoprotein lipase; COL10A1:collagen type X alpha 1 chain; CL:clone; PDL:population doubling level; SA- β -Gal:senescence-associated β -galactosidase; CCNA2:cyclin A2; CCNB1:cyclin B1; CCNB2:cyclin B2; CCNE2:cyclin E2; CDC2:cell division cycle 2; CDK2:cyclin dependent kinase 2; IPA:Ingenuity Pathway Analysis; ND:not determined; MYC:MYC proto-oncogene, bHLH transcription factor; TFRC:transferrin receptor; BMP2:bone morphogenetic protein 2; DLL3:delta-like canonical Notch ligand 3; RPA3:replication protein A3; ICAM1:intercellular adhesion molecule 1; ATP8B1:ATPase phospholipid transporting 8B; DSP:desmoplakin; INHBA:inhibin beta A subunit; NNAT:neuronatin; OXTR:oxytocin receptor; SERPINE1:Serpine family E member 1; SORT1:sortilin 1; SRGN:serglycin; ADGRA2:adhesion G protein-coupled receptor A; ANTXR1:anthrax toxin receptor 1; COL1A2:collagen type I alpha 2 chain; COL3A1:collagen type III alpha 1 chain; ITGA8:integrin subunit alpha 8; MEM α : minimum essential medium alpha; FBS:fetal bovine serum; ascorbic acid:L-ascorbic acid phosphate magnesium salt n-hydrate; PE:phycoerythrin; PBS:phosphate-buffered saline; HA/TCP:hydroxyapatite/tricalcium phosphate; DAVID:Database for Annotation, Visualization and Integrated Discovery

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Declarations

Ethics approval and consent to participate

The clinical portion of this study was performed with the approval of the Committee of Ethics at the Nippon Dental University School of Life Dentistry at Tokyo; animal experiments were performed with the approval of the Animal Experiments Committee of The Nippon Dental University School of Life Dentistry at Tokyo.

Consent for publication

Informed consent was obtained from the patient for the publication of this report.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TK, TI, YI, and TWT designed the study. TK, DT, MN, and TWT performed the experiments and analysed the data. All authors read and approved the final manuscript.

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Tables

Table 1 Ratios of STRO-1 and/or CD146 expression in clones at 17.6 PDL and 40.1–56.1 PDL.

PDL	STRO-1	+	+	-	-	Not determined
	CD146	+	-	+	-	
17.6		45/50 (90%)	5/50 (10%)	0/50 (0%)	0/50 (0%)	0
40.1–56.1		23/36 (64%)	5/36 (14%)	8/36 (22%)	0/36 (0%)	14

PDL, population doubling level

Table 2 Ratios of clones with odontogenic and/or adipogenic differentiation potentials at 24.1 PDL and 40.1–60.7 PDL.

PDL	Odontogenic	+	+	-	-	Not determined
	Adipogenic	+	-	+	-	
24.1		8/28	4/28	10/28	6/28	22
		(29%)	(14%)	(36%)	(21%)	
40.1-60.7		8/12	4/12	0/12	0/12	38
		(67%)	(33%)	(0%)	(0%)	

PDL, population doubling level

Table 3 Clones with a variety of differentiation potentials.

Clone No.	Differentiation potential				
	Tripotent			Bipotent	Unipotent
	CL 4	CL 21	CL 35	CL 29	CL 38
Odontogenic	□	□	□	□	□
Chondrogenic	□	□	□	□	□
Adipogenic	□	□	□	□	□

CL, clone

Table 4 Fourteen representative genes related to multipotency and ‘stemness or differentiation’.

Genes positively correlated with multipotency

Symbol	Gene name
<i>ATP8B1</i>	ATPase phospholipid transporting 8B1
<i>DSP</i>	Desmoplakin
<i>ICAM1</i>	Intercellular adhesion molecule 1
<i>INHBA</i>	Inhibin beta A subunit
<i>NNAT</i>	Neuronatin
<i>OXTR</i>	Oxytocin receptor
<i>SERPINE1</i>	Serpin family E member 1
<i>SORT1</i>	Sortilin 1
<i>SRGN</i>	Serglycin

Genes negatively correlated with multipotency

Symbol	Gene name
<i>ADGRA2</i>	Adhesion G protein-coupled receptor A2
<i>ANTXR1</i>	Anthrax toxin receptor 1
<i>COL1A2</i>	Collagen type I alpha 2 chain
<i>COL3A1</i>	Collagen type III alpha 1 chain
<i>ITGA8</i>	Integrin subunit alpha 8

Figures

Figure 1

Differentiation potentials and tissue regeneration characteristics of human dental pulp cell populations. (a) Expression characteristics of cell surface molecules of dental pulp cell populations at 17.8 PDL analysed by flow cytometry. (b) Cell morphologies of dental pulp cell populations at 4.0 PDL. (c) Alizarin Red S staining of dental pulp cell populations cultured in odontogenic differentiation medium for 21 days. (d) Oil Red O staining of dental pulp cell populations cultured in adipogenic differentiation medium for 8 days. (e, f) Alcian blue staining of dental pulp cell populations cultured in chondrogenic

differentiation medium. (e) Adherent cells after 8 days of induction. (f) Cell pellet after 21 days of induction. The border of the pellet is indicated with a dashed line. (g–i) Gene expression levels of differentiation marker genes in each differentiated dental pulp cell population, analysed by qRT-PCR. Grey bar: differentiation induced cells; white bar: control cells. n = 3. Data are shown as mean (standard deviation). (g) Expression of IBSP for cells cultured in odontogenic differentiation medium. (h) Expression of LPL for cells cultured in adipogenic differentiation medium. (i) Expression of COL10A1 for cells cultured in chondrogenic differentiation medium. (j) Haematoxylin and eosin-stained section of regenerated dentin/pulp-like complex tissues after transplantation of dental pulp cell populations with HA/TCP into immunocompromised mice. d: dentin-like structure; ct: connective tissue; arrows: odontoblast-like cells; ha: HA/TCP carriers. Scale bars in (b–f, j) = 50 μ m. Abbreviations: PDL, population doubling level; qRT-PCR, quantitative reverse transcription polymerase chain reaction; HA/TCP, hydroxyapatite/tricalcium phosphate.

Figure 2

Expression characteristics of surface markers and differentiation potentials of clones. (a–c) Representative immunocytochemical stainings of clones. (a) STRO-1-positive, (b) CD146-positive, and (c) negative control. (d–f) Representative histochemical stainings of clones. (d) Alizarin Red S staining for

odontogenic differentiation. (e) Oil Red O staining for adipogenic differentiation. (f) Alcian blue staining for chondrogenic differentiation (adherent cells). Scale bars = 50 μ m.

Figure 3

Gene expression profiles of representative clones. (a) Unsupervised hierarchical clustering heat map of genes with altered expression in five representative clones. (b) Unsupervised hierarchical clustering heat map of genes with altered expression correlating positively or negatively with multipotency. (c) Venn diagram representing the strategy for analysis of gene expression profiles related to multipotency and 'stemness or differentiation'. (d) Unsupervised hierarchical clustering heat map of 95 probesets related to multipotency and 'stemness or differentiation'.

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