ORIGINAL ARTICLE

Age of the donor affects the nature of *in vitro* cultured human dental pulp stem cells

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Abstract *Objectives:* The dental pulp stem cells (DPSCs) of six donors (three young donors aged < 19 years and three adult donors aged > 25 and < 30 years) were characterized for their stem cell marker expression and differentiation potential to study the effect of donor age on DPSCs *in vitro*.

Methods: DPSCs were cultured in αMEM supplemented with 20% fetal calf serum (conventional conditions) or on fibronectin-coated flasks with neurobasal medium supplemented with B27, bFGF and EGF (alternative conditions). DPSCs were characterized by immunofluorescence staining to detect the neural crest/mesenchymal stem cells markers P75 and CD146, respectively. The differentiation potential was tested by the induction of DPSCs into osteogenic, adipogenic and glial lineages and then by detecting the corresponding markers osteocalcin, lipidtox and S100ß, respectively.

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Results: The DPSCs of the young donors expressed CD146 only under the conventional conditions and expressed P75 regardless of the culture conditions. However, the DPSCs of adult donors expressed CD146 only under the alternative conditions and expressed P75 only under conventional conditions. Only the DPSCs of the young donors differentiated into the glial linage. The DPSCs of the adult donors differentiated more efficiently into the adipogenic linage. Osteogenic differentiation was comparable.

Conclusion: Donor age affects the expression of stem cell markers and differentiation potential of DPSCs. Moreover, the effect of culture conditions on DPSCs is age dependent.

1. Introduction

Dental pulp is loose connective tissue surrounded by specialized dentin-forming cells called odontoblasts. It has an abundant extracellular matrix comprising fibronectin, laminin, collagen, elastin, hyaluronic acid, dermatan sulfate and chondroitin sulfate where mainly fibroblasts and dental pulp stem cells (DPSCs) are found (Yildirim, 2013) The cranial neural crest forms most of the cell types in the dental pulp (Chai et al., 2000; Miletich and Sharpe, 2004). Accordingly, it is called ectomesenchyme and explains some of the DPSC characteristics that are similar to those of neural crest stem cells (NCSCs), such as sphere-forming ability and differentiation into glial, myogenic and melanocytic lineages (Abe et al., 2012; Al-Zer and Kalbouneh, 2015; Janebodin et al., 2011; Paino et al., 2010; Sasaki et al., 2008). However, DPSCs of mesodermal origin (intrapulpal blood vessels) also exist in the dental pulp (Cho et al., 2003). These mesodermal DPSCs possess definitive stem cell properties, such as multi-differentiation and self-renewal (Gronthos et al., 2000). They are heterogeneous and can differentiate into osteogenic, chondrogenic and adipogenic lineages *in vitro,* as well as into dentin pulp-like complex *in vivo* (Gronthos et al., 2002, 2000; Honda et al., 2007; Ishizaka et al., 2013; Jo et al., 2007; Liu et al., 2006; Spath et al., 2010); they also have phenotypic characterics similar to those of bone marrow mesenchymal stem cells (MSCs) (Joe et al., 2007; Shi et al., 2001). Thus, proper attention should be given to the origin of the different stem cell populations residing in the dental pulp to help in the isolation of the appropriate populations desired for cell-based therapies and to allow the precise manipulation of DPSCs *in vitro* and *in vivo*. For example, although mesenchyme-derived DPSCs are suggested for bone regeneration therapies (Graziano et al., 2008), neural crest-derived DPSCs are suggested for peripheral nerve regeneration therapies (Al-Zer and Kalbouneh, 2015). In the current study, we investigated the effect of donor age on the enrichment and/or extinction of some DPSC populations *in vitro*. Few studies have investigated the effect of donor age on DPSC characteristics (Bressan et al., 2012; Horibe et al., 2014; Kellner et al., 2014; Wu et al., 2015; Yi et al., 2017). The similarities and contradictions between those studies and our study are discussed in the discussion section.

2. Materials and methods

2.1. Sample collection

Human wisdom teeth from six donors (three young donors aged < 19 years and three adult donors aged between 25 and 30 years) were collected because of dental reasons. This study was approved by the Institutional Review Board (IRB) of Jordan University Hospital, which conforms to the World Medical Association Declaration of Helsinki. The teeth were transported to the laboratory and processed for culturing immediately after extraction.

2.2. Tissue culture

The teeth were cleaned from the gingival and periodontal tissues, immersed briefly in 80% alcohol before the pulp tissue was extracted, cut into small pieces and digested with 3 mg/ml of collagenase (Sigma, MO, USA) and 4 mg/ml of dispase (Sigma, Japan) for 1 h at 37 °C. The resulting cell suspension was cultured on non-coated flasks in αMEM (Sigma, UK) supplemented with 20% fetal calf serum (FCS) and 2 mM l-glutamine (conventional conditions). Alternatively, the cell suspension was cultured on flasks previously coated with 5 μg/cm^2 of human fibronectin (BD Biosciences, Bedford, USA) in neurobasal medium (Invitrogen, Paisley, UK), supplemented with B27 supplement without vitamin A (Invitrogen, NY, USA), 20 ng/ml of basic fibroblast growth factor bFGF (Peprotech, Rocky Hill, NJ), 20 ng/ml of epidermal growth factor EGF (R&D Systems, Minneapolis, MN), 2.5 μM insulin (Sigma, MO, USA) and 2 mM l-glutamine (Invitrogen, Paisley, UK) (alternative conditions). The medium was changed 2–3 times a week. The doubling time was calculated by counting cells before and after each passage. All the subsequent experiments were performed at least in triplicate utilizing DPSCs from the third passage.

2.3. Fluorescent immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 10 min and then in methanol for 5 min. Thereafter, the cells were blocked for 30 min in 10% goat serum (DAKO, Glostrup, Denmark), 1% bovine serum albumin (Sigma, USA) and 0.2% glycine (Sigma, USA). To enhance the staining of osteocalcin and lipidtox, 0.3% Triton x-100 (Sigma, USA) was added to the blocking step. The cells were incubated with the primary antibodies, mouse monoclonal anti-CD146 (1:500; Millipore, CA, USA), mouse monoclonal anti-CD271 (1:200; BD Biosciences), rabbit polyclonal anti-S100ß (1:500; DAKO, Glostrup, Denmark) and mouse polyclonal osteocalcin (1:500; Millipore, CA, USA) overnight at 4 °C. Bound antibody was then visualized using Alexa Fluor-conjugated goat-anti mouse/rabbit secondary antibodies (1:500; Invitrogen, Rockford, USA). Lipidtox (1:200; Gibco, OR, USA) was

incubated for two hours at room temperature because pre-conjugated HCS LipidTOX green neutral lipid stain was used.

2.4. Differentiation induction

Conventional condition-cultured DPSCs were induced into osteogenic, glial and adipogenic lineages. The cells (5,000 cells/well) were cultured on plain 8-well chambers [except for glial differentiation induction where the wells were coated with 4 μg/ml of laminin (Gibco, CA, USA)] for 1 week before differentiation induction. Osteogenic differentiation was induced by incubating the cells with osteogenic differentiation medium (Gibco, NY, USA) for 3 weeks. Thereafter, immunofluorescence of osteocalcin was performed. For glial differentiation, the cells were supplemented with αMEM containing 1 mM mercaptoethanol (Sigma, Germany) without FCS for 24 h and then for 3 days with 20% FCS αMEM containing 35 ng/ml of retinoic acid (Sigma, China). Thereafter, the cells were incubated in 20% FCS-αMEM supplemented with 5 μM forskolin (Sigma, USA), 10 ng/ml of bFGF, 5 ng/ml of platelet-derived growth factor (PDGF) (Peprotech, London, UK), and 200 ng/ml of recombinant human neuregulin-ß1 (Sigma, USA) for the following 3 weeks. Immunofluorescence

detection of the Schwann cell marker S100ß was then performed. Finally, the induction of adipogenic lineage differentiation was achieved by culturing cells with adipogenic differentiation medium (Gibco, NY, USA) for three weeks before performing lipidtox staining.

3. Results

3.1. The morphology of DPSCs does not vary with the donor age

The doubling time and morphology of DPSCs were not affected by the donor age under both culture conditions (conventional and alternative). Typical fibroblast-like morphology was observed with a doubling time of approximately 30–36 h (Fig. 1).

3.2. The expression of stem cell markers varies according to the donor age

The expression of the NCSC marker P75 and MSC marker CD146 varied according to the donor age (Figs. 2 and 3). The DPSCs of the young donors expressed P75 regardless of
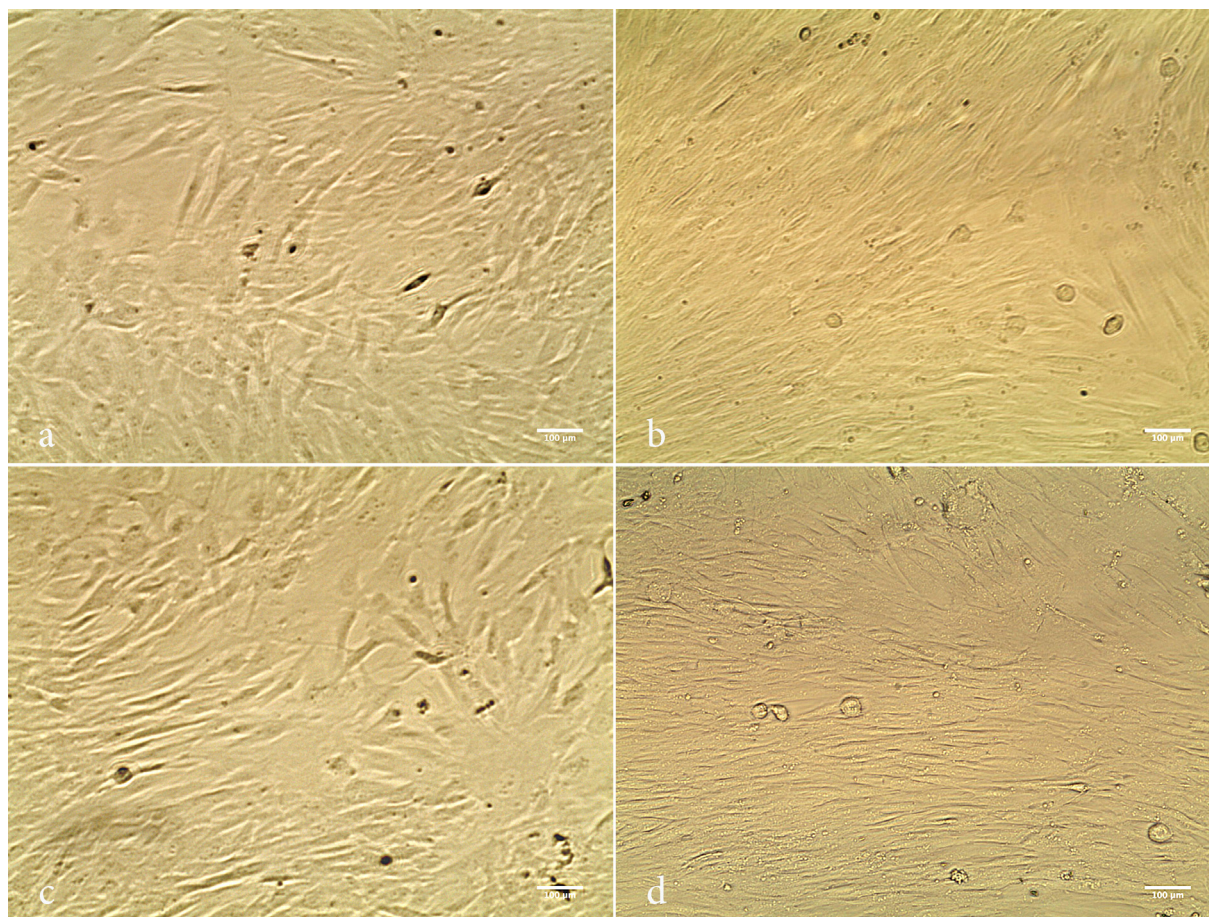

Fig. 1 Effect of donor age on the morphology of DPSCs. The DPSCs of both donor groups (young and adult) were cultured under two different conditions: conventional and alternative. The DPSCs showed fibroblast-like morphology, and no difference was observed between the two donor groups. (a) DPSCs of the young donors cultured under the conventional conditions, 10 × . (b) DPSCs of the young donors cultured under the alternative conditions, 10 × . (c) DPSCs of the adult donors cultured under the conventional conditions, 10 × . (d) DPSCs of the adult donors cultured under the alternative conditions, 10 × . Scale bar = 100 μm.
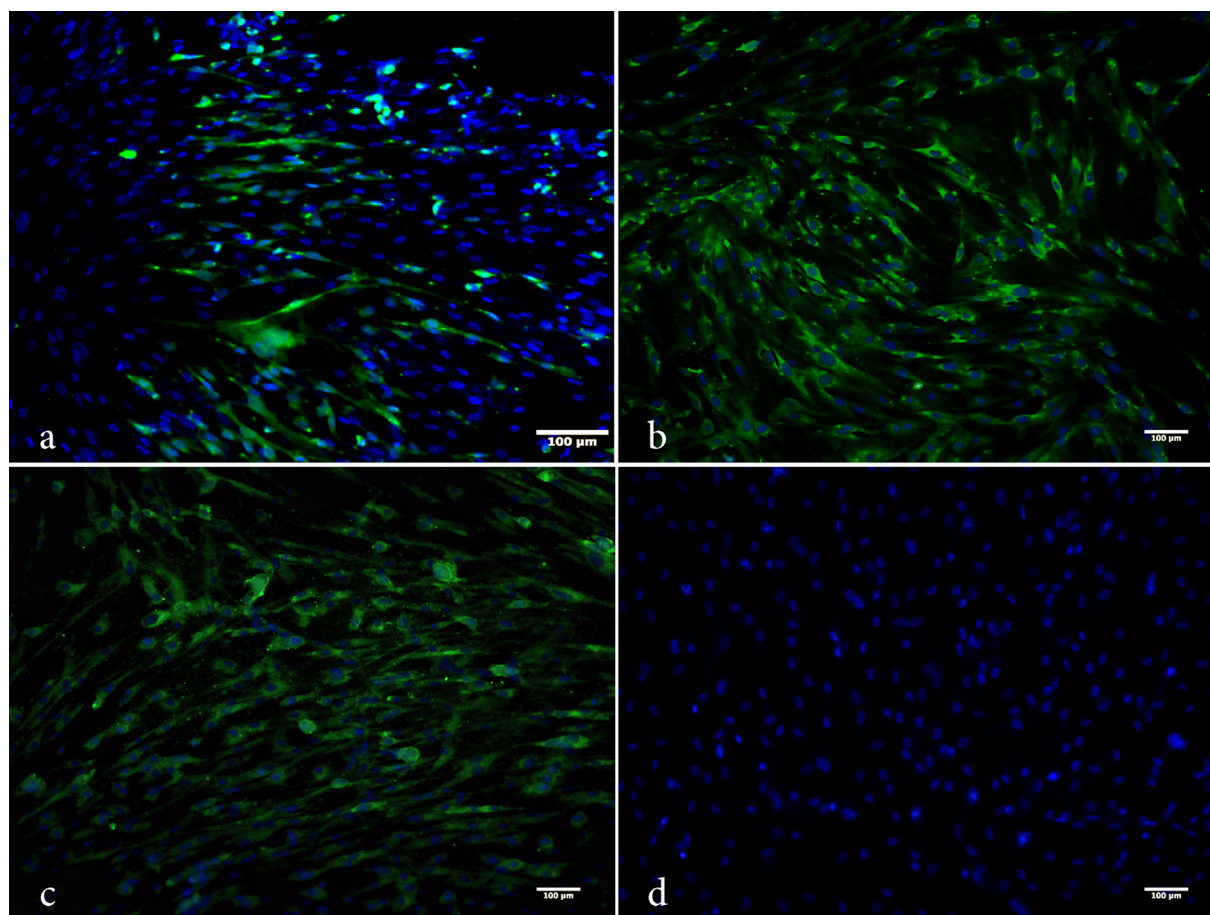
Fig. 2 Effect of donor age on the neural crest stem cell marker (NCSC) P75. The DPSCs of both donor groups (young and adult) were subjected to immunofluorescence to detect P75 expression. The DPSCs were cultured under two different conditions: conventional and alternative. The DPSCs from the young donors expressed P75 under conventional (a) and alternative culture conditions (c), 10 × . The DPSCs from the adult donors expressed P75 under the conventional conditions (b) and were negative under alternative conditions (d), 10 × . Scale bar = 100 µm.

the culture conditions (Fig. 2a and c). However, the DPSCs of the adult donors expressed P75 only under the conventional culture conditions (Fig. 2b and d). The DPSCs of the young donors expressed the MSC marker CD146 under the conventional culture conditions only (Fig. 3a and c), while those of the adult donors expressed CD146 only under the alternative conditions. Additionally, CD146-positive cells formed large net-like structures (Fig. 3d).

3.3. The differentiation potentials of DPCSs are affected by the donor age

3.3.1. Osteogenic potential: Almost all the cells of both donor age groups were positive for osteocalcin expression (Fig. 4a-b).
3.3.2 Glial potential: The DPSCs obtained from the young donors expressed the Schwann cell marker S100ß, whereas those of the adult donors failed to differentiate and were negative for S100ß (Fig. 4c-d).
3.3.3. Adipogenic potential: The DPSCs of the adult donors showed strong lipidtox staining, while few positive cells were observed in the DPSCs obtained from the young donors (Fig. 4e-f).

4. Discussion

Dental and mesenchymal stem cells have drawn the attention of regenerative medicine researchers
because they show multi-differentiation potentials and because of their fundamental roles as promoters, enhancers, and playmakers of translational regenerative medicine (Ballini et al., 2018). Multiple oral stem cell populations have been isolated and reported, such as dental pulp stem cells, periodontal stem cells and recently reported periapical cyst-mesenchymal stem cells that, interestingly, have immunomodulatory and pro-osteogenic activities in the local environment (Tatullo et al., 2017).

In this study, DPSCs taken from the extracted wisdom teeth of six donors (three young donors and three adult donors aged between 25 and 30 years) were cultured and then characterized by stem cell marker expression and differentiation potential to study the effect of donor age on the existence and differentiation potential of DPSCs. DPSCs were cultured under two culture conditions inspired by previous studies—conventional and alternative conditions. The conventional conditions were utilized to support mesenchyme-derived
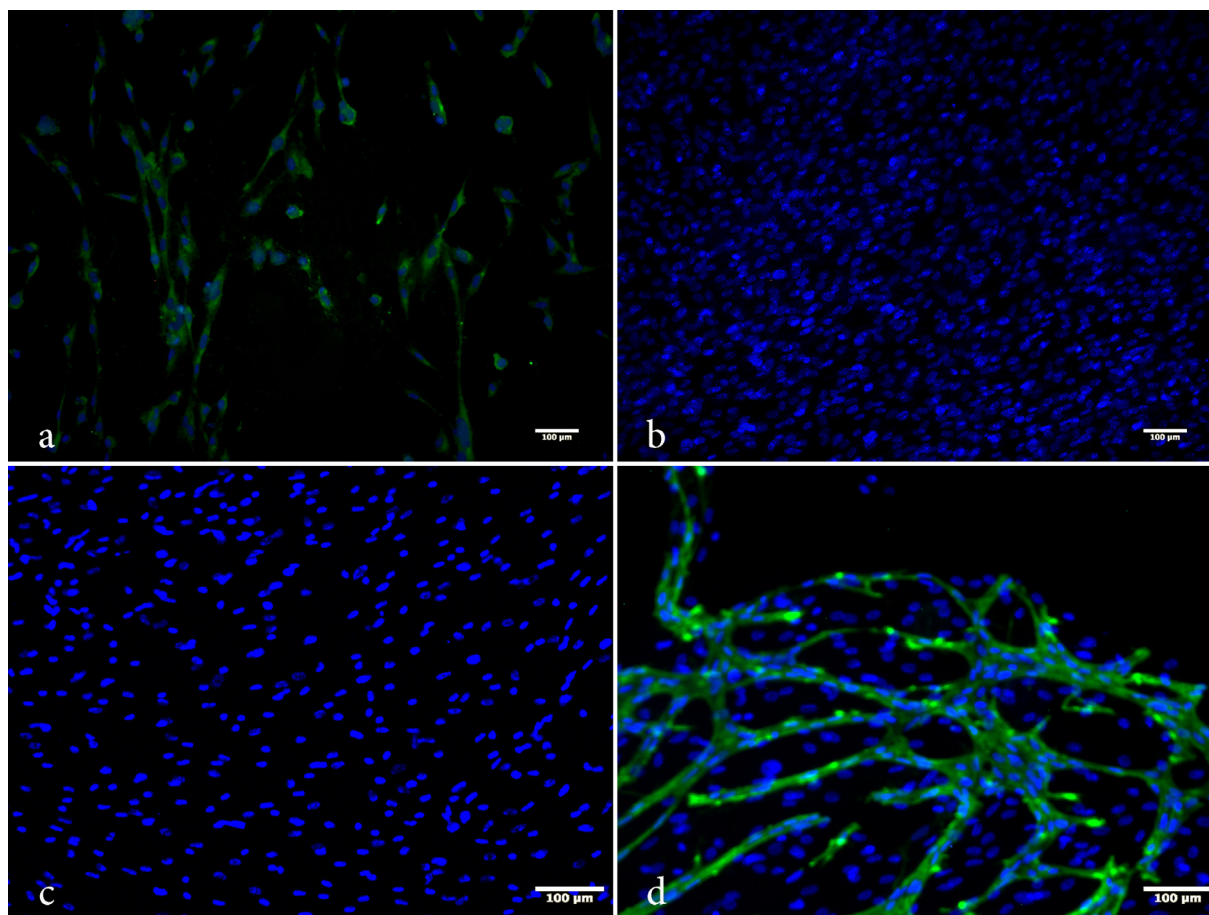
Fig. 3 Effect of donor age on the mesenchymal stem cell marker CD146. The DPSCs of both donor groups (young and adult) were subjected to immunofluorescence to detect CD146 expression. The DPSCs were cultured under two different conditions: conventional and alternative. The DPSCs from the young donors expressed CD146 under conventional conditions (a) and were negative under alternative culture conditions (c), 10×. The DPSCs from the adult donors did not express CD146 under the conventional conditions (b) and were positive for CD146 under the alternative conditions (d), 10 × . The CD146-positive cells from the adult donors formed large net-like structures. Scale bar = 100 μm.

DPSC survival, whereas the alternative conditions were utilized to support neural crest-derived DPSC survival (Al-Zer et al., 2015; Ramírez-García et al., 2017; Stemple and Anderson, 1992). All the experiments utilized cells with a low passage number (passage number three) because telomerase attrition and the loss of DPSC stemness were observed in DPSCs under extended passage (Bressan et al., 2012; Mokry et al., 2010). Additionally, the cultivation of DPSCs in media containing a high concentration of bovine serum for extended periods has critical side effects on cell chromosomal stability (Suchanek et al., 2013).

Few studies have investigated the effect of donor age on DPSCs, and variable characteristics were studied. DPCSs isolated from different age ranges and cultured under different culture conditions vary in stem cell marker expression as well as in their differentiation potential (Bressan et al., 2012; Horibe et al., 2014; Kellner et al., 2014; Wu et al., 2015; Yi et al., 2017). Interestingly, Horibe et al. reported that the isolation of mesenchymal DPSC subsets using granulocyte-colony stimulating factor (G-CSF) mobilization renders the DPSCs to overcome the decline in stemness occurring with age and called these DPSCs mobilized DPSCs (MDPSCs).

The donors' age ranged between young adults (19–30 years) and older adult donors (44–70 years), and MDPSCs from the older adult and young adult donors were similar in their capacities for migration, differentiation and stem cell marker expression.

In this study, the morphology of the cells and doubling time of both donor groups, the young and the adult, were not affected by the donor age. DPSCs showed a fibroblastic morphology and a doubling time of approximately 30–36 h. Similar results were previously reported (Bressan et al., 2012; Horibe et al., 2014; Kellner et al., 2014; Wu et al., 2015; Yi et al., 2017). In the Horibe et al. study, DPSCs and MDPSCs from young adult and older adult donors were cultured with DMEM containing 10% human serum. The morphology and colony-forming efficiency were similar between DPSCs from young and older adult donors; similar results were found for MDPSCs from young and older adult donors. However, the proliferation activity in DPSCs/MDPSCs from young donors was higher than that in DPSCs/MDPSCs from older adult donors (Horibe et al., 2014). Another study compared DPSCs cultured under conventional conditions from 27 patients (12–21 years) and 7 patients (22–30 years old) for four passages.
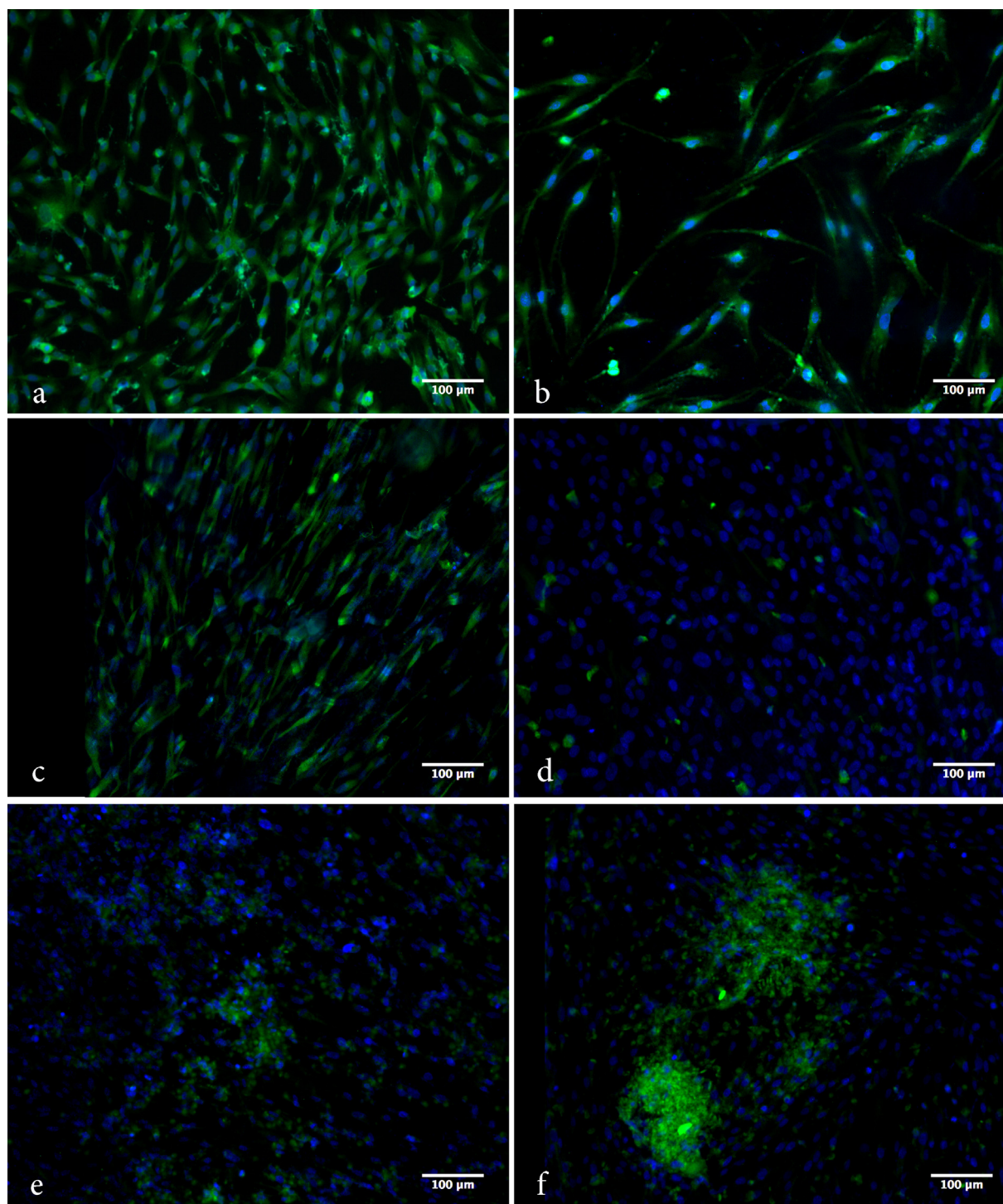
Fig. 4 Effect of donor age on the DPSC differentiation potential. The DPSCs of both donor groups (young and adult) cultured under the conventional condition were induced to differentiate into the osteogenic, glial and adipogenic lineages. The induced DPSCs were subjected to immunofluorescence for the expression of osteogenic, glial and adipogenic differentiation markers osteocalcin, S100ß and lipidtox, respectively. High expression of osteocalcin was observed in induced DPSCs from the young (a) and adult (b) donors, 10×. DPSCs obtained from the young donors expressed the Schwann cell marker S100ß (c), whereas DPSCs from the adult donors failed to differentiate and were negative for S100ß (d), 10×. The DPSCs of the adult donors showed strong lipidtox staining (f), whereas a weaker signal was observed in the DPSCs obtained from the young donors (e), 10 × . Scale bar = 100µm.

The DPSCS of young donors showed a faster doubling time in the second and third passages than those of patients older than 21 years. Nevertheless, evidence of older patient cells dividing unusually quickly and of younger patient cells dividing slowly was found (Kellner et al., 2014). Additionally, Wu et al. investigated four age groups (children, adolescence, adults, and

older adult donors) and found that the proliferation activity of DPSCs cultured under conventional conditions was significantly reduced and apoptosis significantly increased in the older adult patients (older than 54 years), whereas they were comparable in younger age groups (Wu et al., 2015). Bressan et al. studied a range of ages between 16 and 66 years and reported that a reduced proliferation and a prolonged doubling time of DPSCs were mostly observed in older adult patients (older than 55 years) (Bressan et al., 2012). Finally, Yi et al. studied the DPSCs of young (16–25 years) and older adult (60–70 years) donors cultured under conventional conditions and reported a longer doubling time and a reduced proliferation activity in the older adult donors (Yi et al., 2017). Age-related proliferation reduction, a prolonged doubling time, reduced stem cells marker expression and reduced differentiation abilities of DPSCs in all the above-mentioned studies occurred in older adult or late adulthood patients but not in adolescents or young adults (Bressan et al., 2012; Horibe et al., 2014; Kellner et al., 2014; Wu et al., 2015; Yi et al., 2017), findings that agree with this study.

Two stem cell markers were investigated, P75 and CD146. Many studies have utilized P75 to isolate NCSCs from the dental pulp and other neural crest derivatives (Hauser et al., 2012; Lee et al., 2007; Stemple and Anderson, 1992; Stevens et al., 2008; Widera et al., 2009), whereas CD146 was reported to be expressed by mesenchyme-derived DPSCs (Lei et al., 2014; Shi and Gronthos, 2003). Here, the DPSCs of the young donors expressed the NCSC marker P75 under both culture conditions (Fig. 2a and c), whereas the DPSCs of the adult donors expressed P75 only under the conventional culture conditions (Fig. 2b and d). P75 is expressed by NCSCs and Schwann cell precursors (Jessen and Mirsky, 2005), and each of these cell types has different survival and differentiation cues. Because the alternative culture conditions support NCSC survival but not Schwann cell precursor survival (Jessen and Mirsky, 2005; Woodhoo et al., 2004), we suggest that the young donor cells were of NCSC ontogeny, whereas the adult donor cells were more of Schwann cell precursor ontogeny because they were not detected under the alternative culture conditions. Regarding the MSC marker CD146, the DPSCs of the young donors expressed CD146 only under the conventional culture conditions (Fig. 3a and c), whereas the DPSCs of adult donors expressed CD146 only under the alternative condition; the signal was strong, and the positive cells formed large net-like structures (Fig. 3b and d). These results not only suggest that the number of NCSCs and MSCs in DPSC cultures vary with age but also that the effect of the culture conditions on DPSCs, either by enriching or causing extinction, is age dependent.

In the Horibe et al. study, fluorescence-activated cell sorting analysis of stem cell marker expression showed that the number of CD146-positive cells in DPSCs and MDPSCs from young donors was similar, while that in DPSCs from older adult donors was higher than that in MDPSCs from older adult donors, supporting our observation that the number of CD146-positive cells and culture condition effects are both age dependent. In our study, the highest CD146 expression was observed in the DPSCs of adult donors cultured under the alternative conditions. Whether this result is related to neural crest-derived DPSC differentiation as the donor ages into the mesenchymal phenotype needs more investigation (Hall, 2009a, 2009b). Finally, in contrast to our study results, Yi et al. reported no difference in CD146 expression between young (16–25 years) and older adult (60–70) donors (Yi et al., 2017).

To test the differentiation potential of DPSCs, three lineages were chosen: Schwann cells (ectodermal), osteoblasts (mesodermal) and adipocytes (mesodermal). After three weeks of osteogenic induction, DPSCs from both donor groups differentiated successfully into osteoblasts and expressed osteocalcin (Fig. 4a-b). This finding suggests that the donor age is not of high value in experiments intended to retrieve differentiated osteoblasts from DPSCs, an observation reported in previous studies (Bressan et al., 2012; Horibe et al., 2014; Wu et al., 2015). Horibe et al. reported no difference in the gene expression of osteocalcin in DPSCs/MDPSCs between older adult and young donors (Horibe et al., 2014). In the Wu et al. study, all DPSCs from the four different age groups under study differentiated successfully into osteoblasts (Wu et al., 2015). Finally, Bressan et al. reported, depending on the number of osteonectin-positive cells, that the osteogenic differentiation potential was maintained in DPSCs despite the age, with a small decline in the differentiation potential after the age of 54 years. Moreover, the regenerative abilities of DPSCs from young and older adult donors were comparable when they were seeded on hydroxyapatite nanostructured granules to repair bone critical size defects (Bressan et al., 2012). Only Yi et al. reported a decrease in the osteogenic potential of DPSCs from older adult donors as alkaline phosphatase activity, alizarin red staining and calcium quantitation were higher in the DPSCs from young donors. However, they reported no significant difference between these two groups in osteocalcin expression (Yi et al., 2017). We also observed that the number of osteocalcin-positive cells was not related to the number of CD146-positive or P75-positive cells, suggesting that many stem cell populations or progenitors are responsible for osteogenic differentiation in the dental pulp. This finding is similar to that reported previously by Alvarez et al. who used fluorescence-activated cell sorting with different surface marker combinations and found that different DPSC populations could differentiate into odontogenic lineages (Alvarez et al., 2015).

The DPSCs of the adult and young donor groups differentiated into adipocytes and secreted lipid droplets, and the DPSCs of adult donors showed a greater number of differentiated cells and a stronger signal (Fig. 4e-f). Accordingly, the DPSCs from adult donors would be more beneficial for experiments requiring adipocyte expression. Horibe et al. also reported that the gene expression of some adipogenic genes was slightly higher in DPSCs/MDPSCs from older adults than in those from young donors; however, the expression was highest in MDPSCs from older adult donors (Horibe et al., 2014). However, Yi et al. reported a reduced adipogenic differentiation ability of aged donors as oil red staining and quantitation analysis revealed that DPSCs from young donors had more lipid deposits than DPSCs from old donors (Yi et al., 2017).

Regarding glial differentiation, the DPSCs of the young donors differentiated into Schwann cells and expressed S100ß, whereas the DPSCs of the adult donors failed to differentiate and were negative for S100ß (Fig. 4. c, d). P75 is expressed by NCSCs and Schwann cell precursors (Jessen and Mirsky, 2005), but each of these cell types has different survival and differentiation cues. Because our differentiation medium supports NCSC survival but not Schwann cell

precursor survival (Jessen and Mirsky, 2005; Woodhoo et al., 2004), we suggest that the DPSCs of the young donor cells successfully differentiated into Schwann cells while the DPSCs of the adult donors failed to differentiate. Accordingly, DPSCs from young donors would be more beneficial for experiments requiring glial cells. To our knowledge, this is the first study to investigate the effect of donor age on the differentiation of DPSCs into Schwann cells.

Finally, it is worth mentioning that identifying a suitable source of stem cells for regenerative therapies is not sufficient. Developing safe *in vitro* culturing methods without using animal additives is a mandatory proceeding for any possible safe application in human models (Marrazzo et al., 2016). Furthermore, identifying suitable carriers/scaffolds plays a major role in therapy success or failure. Recently, the introduction of nanotechnologies into scaffold manufacturing has significantly improved the applicability of scaffolds (Barry et al., 2016). Hopefully, these improvements in the understanding of the nature of stem cell sources, advancement in culturing protocols and scaffold manufacturing can be combined and applied in safe, efficient therapies in the near future.

5. Conclusions

Although DPSCs are a feasible source for stem cell therapies and show multiple differentiation potentials, their heterogeneity complicates the isolation procedure of the appropriate stem cell population required for a specific therapy. Moreover, many factors can interfere with the DPSC number and survival *in vitro*. In our study, the age of the donor affected some characteristics of DPSCs. Additionally, the culture condition effects on DPSCs were age dependent. The DPSCs of adult donors differentiated better than those of the young donors into the adipogenic lineage, suggesting that adult DPSCs would perform better in therapies that require adipocytes. Only the DPSCs from young donors could differentiate into Schwann cells, suggesting that these DPSCs would perform better in peripheral nerve regeneration therapies, for example. These differences should be considered to optimize future studies regarding DPSC culture and differentiation. Accordingly, DPSCs should be investigated further before they can be considered for future therapies; the difference between the original *in vivo* DPSC characteristics and *in vitro* induced ones should be elucidated further to avoid incorrect conclusions about DPSC nature and potentials.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sdentj.2020.09.003.

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