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ORIGINAL ARTICLE

Comparison of human dental follicle cells (DFCs) and stem cells from human exfoliated deciduous teeth (SHED) after neural differentiation in vitro

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Abstract Dental stem cells from human exfoliated deciduous teeth (SHED) and dental follicle cells (DFCs) are neural crest-derived stem cells from human dental tissues. Interestingly, SHED and DFCs can successfully differentiate into neuron-like cells. We hypothesized that SHED and DFCs have the same neural cell differentiation potentials. To evaluate neural cell differentiation, we cultivated SHED and DFCs in four different serum-replacement media (SRMs) and analyzed cell morphology, cell proliferation, and gene expression patterns before and after differentiation. In a standard cell culture medium, SHED and DFCs have not only similar cell morphologies, but they also have similar gene expression patterns for known stem cell markers. However, only SHED expressed the neural stem cell marker Pax6. After cultivation in SRMs, cell proliferations of DFCs and SHED were reduced and the cell morphology was spindle-like with long processes. However, differentiated DFCs and SHED had different neural cell marker expression patterns. For example, gene expression of the late neural cell marker microtubule-associated protein 2 was upregulated in DFCs and downregulated in SHED in SRM with the B27 supplement. In contrast, SHED formed neurosphere-like cell clusters in SRM with the B27 supplement, epidermal growth factor, and fibroblast growth factor-2. Moreover, SHED differentially expressed the glial cell marker glial fibrillary acidic protein, which in contrast was weakly or not expressed in DFCs. In conclusion, SHED and DFCs have different neural differentiation potentials under the same cell culture conditions.

Keywords Dental follicle · Stem cells · Neural differentiation · Real-time RT-PCR · Serum-replacement medium

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Introduction

The human dental follicle (dental sac) is a tissue of the tooth germ, which can be easily isolated after wisdom tooth extraction. Recently, we isolated undifferentiated cells from the dental sac [1, 2]. These human dental follicle cells (DFCs) can differentiate into cells of the periodontium such as alveolar osteoblasts, periodontal ligament (PDL) fibroblasts, and cementoblasts [1, 3]. DFCs can be distinguished from bone marrow-derived mesenchymal stem cells, osteoblasts, or PDL fibroblasts by real-time reverse transcription polymerase chain reaction (RT-PCR) analyses.

Interestingly, DFCs express neural progenitor cell markers such as Notch-1 and nestin [1, 2]. We were able to differentiate DFCs into neural-like cells after cultivation in serum-replacement medium (SRM) [4]. After differentiation, DFCs exposed neural-like cell morphology with small neurite-like cell extrusions. These cells differentially express late neural cell markers such as neurofilament, but only low levels of early neural cell markers such as beta-III-tubulin and nestin. Recently, Kaltschmidt and colleagues

demonstrated the isolation of neural stem cells from the human PDL that were able to differentiate into neural-like cells under in vitro conditions. These neural stem cells are probably closely related to the recently discovered PDL stem cells [5, 6]. Shi and colleagues reported the isolation of stem cells from human exfoliated deciduous teeth (SHED) that are also capable of differentiation into neuron-like cells. After in vivo transplantation, SHED survived in mouse brain along with the expression of neural markers [7]. All isolated human dental stem cells are neural crest-derived dental progenitor cells and are capable of neural differentiation. The hypothesis of this study argues that human dental stem cells such as SHED and DFCs have the same neural differentiation potential after cultivation in SRMs.

To verify this hypothesis, we compared DFCs and SHED after neural differentiation. In our study, SRMs were used that are known for the cultivation and differentiation of neural progenitor cells. We investigated cell morphology, cell proliferation, and gene expression profiles of neural cell markers before and after differentiation.

Materials and methods

Isolation of DFCs and cell culture

DFCs were isolated as described previously [1, 2]. Briefly, the attached dental follicle was separated from the mineralized tooth. DFCs were isolated from a 20-year-old donor. The follicle tissue was cleaned in phosphate-buffered saline (PBS) and then digested in a solution of collagenase type I, hyaloronidase (Sigma-Aldrich, Munich, Germany), and DNAse I (Roche, Mannheim, Germany) for 1 h at 37°C. Single cells were seeded into T25 flasks in MesenchymStem Medium (MSCM; PAA, Pasching, Austria) at 37°C in 5% CO_2. Nonadherent cells were removed by change of media, whereas DFCs have attached on the plastic surface. SHED were kindly provided by Dr. Songtao Shi from the University of Los Angeles [7]. Cells were grown and used at passage 6 for experiments.

Neural differentiation

For neural differentiation, SRMs were used. DFCs after passage 5 were used for experiments. For neural differentiation, cells were cultivated at an initial cell density of 25,000 cells per square centimeter on six-well plates with Nunclon Delta surface modification (Nunc, Wiesbaden, Germany). SRM formulations for neural differentiation (NDM) are listed in Table 1. DFCs and SHED were cultivated for 7 days and cell culture media were changed every third day.

Immunofluorescence studies

For immunofluorescence studies, cells were cultivated overnight on cover slips (10,000 cells per cover slip). Cells were washed with PBS and were fixed after treatment in 4% paraformaldehyde (10 min). Cells were incubated for 30 min with blocking solution containing 10% normal goat serum lyophilized solid (Calbiochem) and 0.3% Triton X-100 (Sigma-Aldrich) in PBS to reduce nonspecific background staining. This was followed by overnight incubation of the primary antibody at 4°C. Antibodies used included STRO-1 IgM (dilution 1:20) and bTAN 20 (Notch-1) IgG (dilution 1:250). All antibodies of this study were obtained from the Developmental Hybridoma Study Bank (Iowa City, IA, USA). After washing with PBS, cells were labeled for 2 h at room temperature with the secondary antibody antimouse IgM fluorescein isothiocyanate conjugate (488 nm; Sigma-Aldrich) or Cy3 IgG antirat (Sigma-Aldrich). Cells were rinsed, mounted with DakoCytomation Flourescent Mounting Medium (Dako Cytomation), and viewed with a fluorescence microscope (Axioskop 2, Zeiss, Oberkochen, Germany). The expression of Stro-1 and Notch-1 was quantified as percentage of positive cells per field of view. Bars in Fig. 1 represent the mean of four experiments ± standard deviation.

Reverse transcription-PCR and quantitative RT-PCR

Total RNA was isolated from cells with NucleoSpin® RNA II (Macherey-Nagel, Düren, Germany). In order to digest genomic DNA contamination, the isolated RNA was treated with DNAse I (Macherey-Nagel, Düren, Germany). The cDNA synthesis was performed using 400 ng total RNA and the RevertAid™ M-MuLV Reverse Transcriptase Kit (Fermentas, St. Leon-Rot, Germany). For conventional PCRs, we used the GoTaq Green Master Mix (Promega) and a T3000 Thermocycler (Whatmann-Biometra, Göttingen, Germany). A Tris/borate/ethylenediaminetetraacetic acid–agarose gel electrophoresis was used for PCR product analysis. PCR primer sequences and PCR product sizes are listed in Table 2. Quantitative PCR was performed with TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Foster City, USA). Sequences for primers and probes were obtained from the Universal ProbeLibrary from Roche (http://www.roche-applied-science.com). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed with the iCycler (Bio-Rad, Munich, Germany). The iCycler Version 3.1.7050 software was used for estimation of threshold cycles. All PCRs were run in duplicates. For quantification, the delta/delta calculation method was used as described previously [8]. The gene expression of the house-keeper genes glyceraldehyde-3-phosphate dehydro-

Table 1 Serum-free cell culture medium for neural differentiation (NDM)

Medium	Components
NDM I	Neurobasal Medium (NBM, PAA, Pasching, Austria), G5 supplement (PAA), and Neural Stem Cell Supplement (NSCS, PAA)
NDM II	NBM, N2 supplement, 20 µg/mL EGF (Sigma-Aldrich), and 20 µg/mL FGF-2 (Sigma-Aldrich)
NDM III	NBM, B27 Neuro Mix (PAA), EGF, and FGF-2
NDM IV	NBM and B27 Neuro Mix

genase (GAPDH) and beta-glucuronidase (Gus) was used for normalization.

Cell proliferation assay

For the estimation of cell proliferation, a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. In the MTT test, tetrazolium salts are transformed by active enzymes of the cells into intracellular formazan deposits; amount of color produced is directly proportional to the number of viable cells. For this assay, SHED and DFCs (5×10^3 cells per square centimeter) were attached on the surface in MSCM for 24 h and cells were cultivated in indicated SRMs or MSCM for additional 48 h. The MTT assay was performed after 48 h of cultivation. Here, cells were treated with 5 mg/mL MTT for 4 h at 37°C. The cell culture medium was removed, and cells were lysed by the addition of 0.1 M HCl/isopropyl alcohol. The metabolized MTT was evaluated by optical density (OD) in a spectrophotometer at 540 nm. The ratio (relative cell proliferation) was calculated by following formula: [$OD_{540\ nm}$ cells grown in SRM]/[$OD_{540\ nm}$ cells grown in MSCM]×100%. Bars in Fig. 3b represent the mean from three independent biological replicates (±standard deviation).

Results

Characterization of DFCs and SHED by immunohistochemistry and RT-PCR

DFCs and SHED had fibroblast-like cell morphology. In immunofluorescence studies, DFCs and SHED expressed Notch-1 and Stro-1, which are markers of progenitor cells (Fig. 1). Twenty-seven percent of SHED and 35% of DFCs were positively stained for the mesenchymal stem cell marker Stro-1, but more than 90% of investigated DFCs and SHED expressed Notch-1. Dental stem cells expressed the embryonic stem cell marker Oct-4 (POU class 5

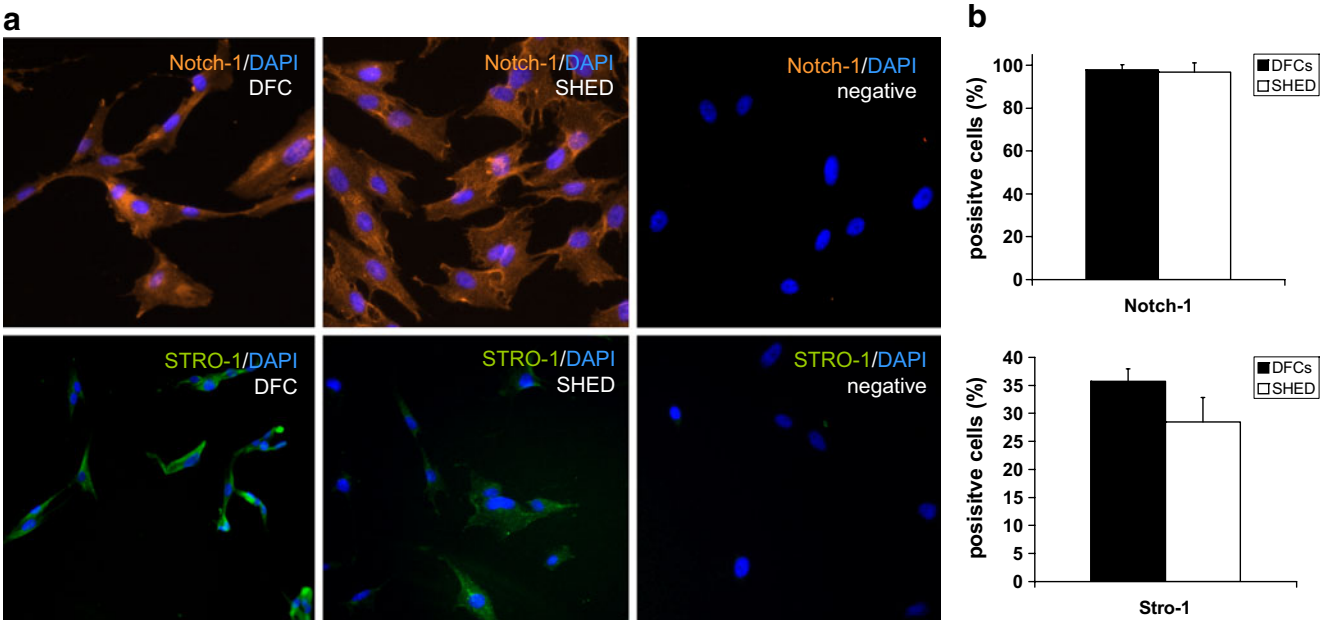

Fig. 1 Immunofluorescence studies for STRO-1 and Notch-1 in DFCs and SHED. Antibody stainings were applied as described under the "Materials and methods" section. For the negative control, the first antibody was omitted. Stem cell markers Notch-1 and STRO-1 were expressed in DFCs and SHED. Bars represent the mean of four experiments ± standard deviation

Table 2 Primers for PCRs

Gene	Primer sequence (forward; reverse)	Annealing temperature (°C)	Product length (bp)
ABCg2	5′-GGG TTC TCT TCT TCC TGA CGA CC-3′ 5′-TGG TTG TGA GAT TGA CCA ACA GAC C-3′	60	399
Oct-4	5′-GAA GGA TGT GGT CCG AGT GT-3′ 5′-GTG AAG TGA GGG CTC CCA TA-3′	60	183
CD166	5′-CGT CTG CTC TTC TGC CTC TT-3′ 5′-TAA ATA CTG GGG AGC CAT CG-3′	60	175
CD105	5′-CAC TAG CCA GGT CTC GAA GG-3′ 5′-CTG AGG ACC AGA AGC ACC TC-3′	60	165
CD73	5′-CGC AAC AAT GGC ACA ATT AC-3′ 5′-CTC GAC ACT TGG TGC AAA GA-3′	60	241
SOX2	5′-ACA CCA ATC CCA TCC ACA CT-3′ 5′-GCA AAC TTC CTG CAA AGC TC-3′	55	244
CD146	5′-GTC TGC GCC TTC TTG CTC-3′ 5′-TTC CAC CTC CAC CAG CTC-3′	55	99
CD13	5′-GGG CAC AAT CCA CAC GTA G-3′ 5′-TCA CGG TGG ATA CCA GCA C-3′	55	107
Notch-1	5′-GCA CTG CGA GGT CAA CAC-3′ 5′-AGG CAC TTG GCA CCA TTC-3′	55	200
GAPDH	5′-CGT CTT CAC CAC CAT GGA GA-3′ 5′-CGG CCA TCA CGC CAC AGT TT-3′	57	320
Nestin	5′-GTG GCA CAC ATG GAG ACG-3′ 5′-GAG CGA TCT GGC TCT GTA GG-3′	55	250
Pax6	5′-TCG GTG GTG TCT TTG TCA AC-3′ 5′-CAC ACA TCC GTT GGA CAC C-3′	55	128

homeobox 1) and cell surface markers CD166 (activated leukocyte cell adhesion molecule), CD105 (endoglin), CD146 (melanoma cell adhesion molecule), CD13 (alanyl (membrane) aminopeptidase), and CD73 (5′-nucleotidase, ecto) (Fig. 2). They furthermore expressed neural stem cells associated markers such as SRY (sex-determining region Y)-box 2 (SOX2), nestin, and ATP-binding cassette, subfamily G, member 2 (ABCg2). Both DFCs and SHED expressed vimentin, which is a typical marker of mesenchymal cells. Interestingly, the progenitor cell marker paired box gene 6 (Pax6) was only expressed in SHED (Fig. 2).

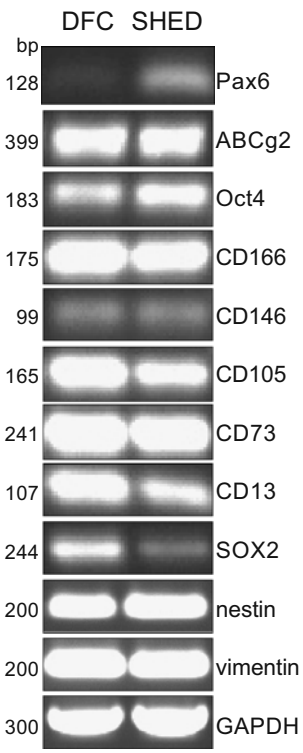

Fig. 2 Comparison of cell marker gene expression of DFCs and SHED (both passage 5) by RT-PCR. Cells were cultured in MSCM before analysis. The gene expression of GAPDH was used as a reference gene

Cell proliferation and neural differentiation in vitro of DFCs and SHED in SRMs

SHED and DFCs were fibroblast-like and grew until confluence after 7 days of cultivation in MSCM (Fig. 3a). The cell proliferation was reduced after cultivation in NDM I, NDM III, and NDM IV in comparison to cell proliferation in MSCM (Fig. 3b). SHED and DFCs were spindle-like in shape and adhered to the plastic tissue culture dishes after cultivation in SRMs (Fig. 3a). In NDM I (Fig. 3a), DFCs had small cell bodies (asterisks) and long neurite-like extensions

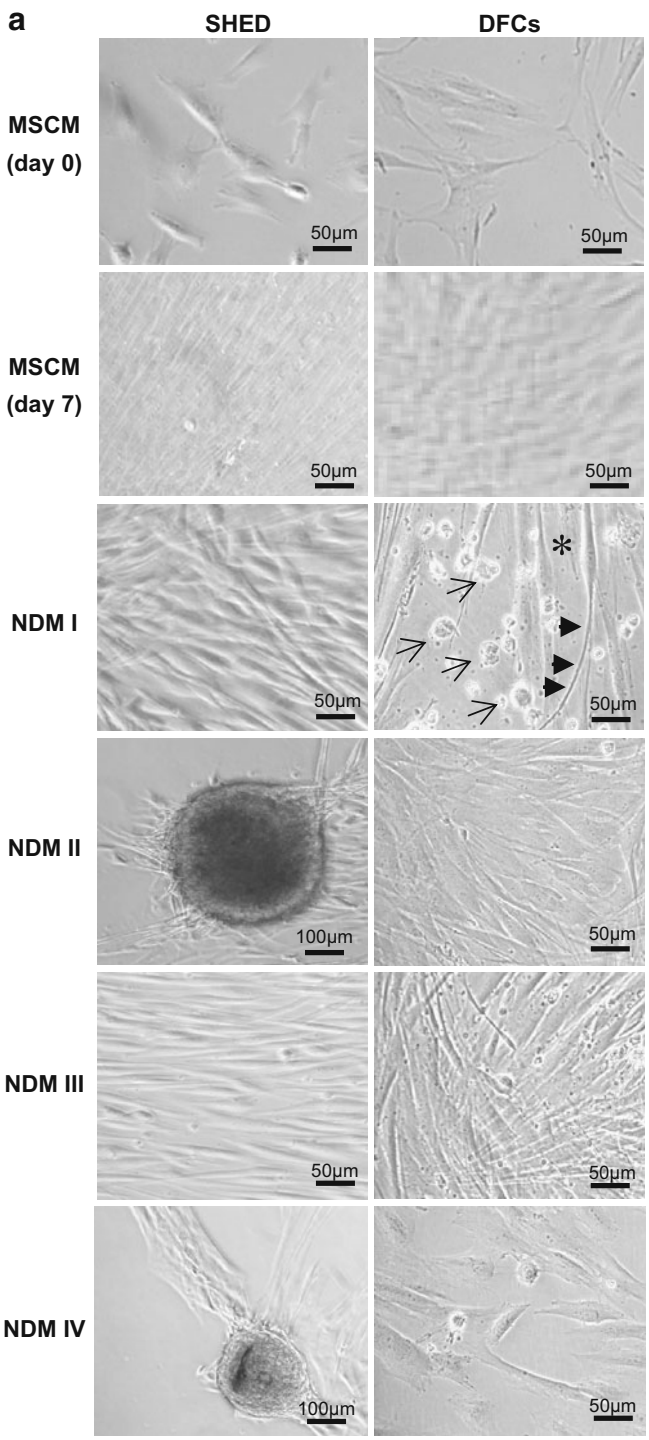

Fig. 3 Differentiation of DFCs and SHED. **a** Phase contrast microscopy of DFCs and SHED before [MSCM (day 0)] and after 7 days of cultivation in MSCM and after cultivation in NDM I, NDM II, NDM III, and NDM IV. **b** Cell proliferation (MTT assay) of dental stem cells in SRMs. DFCs and SHED showed a reduced cell proliferation after cultivation in NDM I, NDM III, and NDM IV. For the control, dental cells were cultivated in MSCM. Bars represent the mean from three independent biological replicates (±standard deviation)

(triangles). However, small cell debris was also observable in NDM I (arrows). SHED but not DFCs formed adherent neurosphere-like cell clusters in NDM IV and NDM II (not shown for NDM III).

The qRT-PCR assay demonstrated that neural cell markers were upregulated and downregulated after cultivation in SRMs (Fig. 4). In standard cell culture medium, SHED and DFCs expressed the neural progenitor

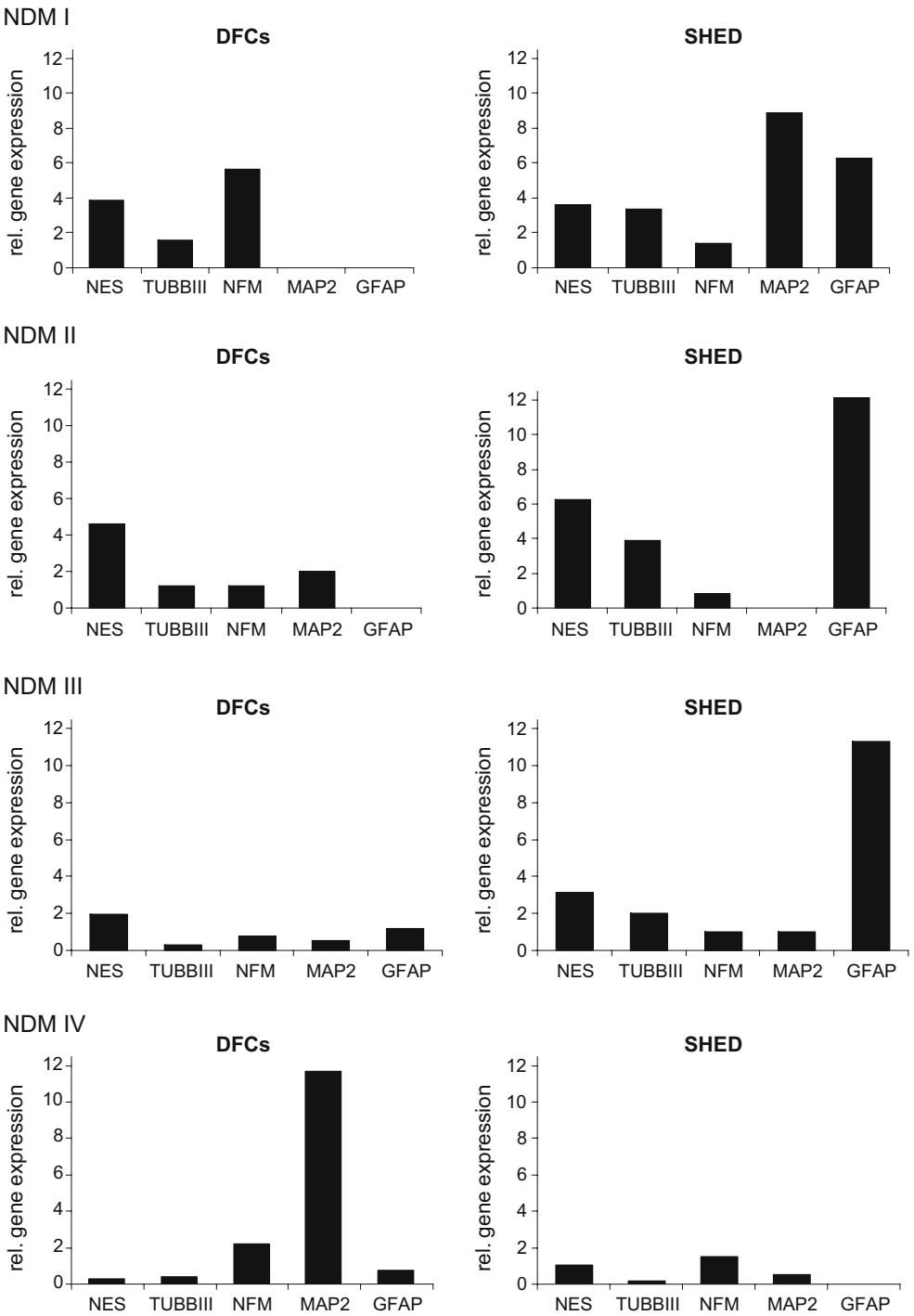

Fig. 4 Comparison gene expression changes of neural cell markers in SHED and DFCs after cultivation in NDM I, NDM II, NDM III, and NDM IV media. The delta/delta method was used for the quantitation of gene expression. The gene expression of undifferentiated DFCs and undifferentiated SHED was defined as one (calibrator). Samples were measured in duplicates and bars represent the mean of measurements

cell marker nestin, the early neural cell marker β-III tubulin (TUBB3), the glial cell marker glial fibrillary acidic protein (GFAP), and cell markers for mature neurons such as neurofilament (NFM) and microtubule-associated protein 2 (MAP2). However, GFAP was very weakly expressed in undifferentiated DFCs. After cultivation in NDM I or NDM II, DFCs expressed differentially nestin and neurofilament, but they did not express GFAP. We found that MAP2 and neurofilament were upregulated after cultivation in NDM IV and that GFAP was very weakly expressed in NDM IV and NDM III (Fig. 4). In contrast, SHED differentially expressed GFAP after cultivation in NDM I, II, and III, but they did not express GFAP in NDM IV. Moreover, MAP2 was highly expressed in NDM I, but it was not expressed after cultivation in NDM IV (Fig. 4).

Discussion

Gene expression of stem cell markers in undifferentiated DFCs and SHED

This study investigated the hypothesis that DFCs and SHED have the same neural differentiation potential under in vitro conditions. Before neural differentiation analyses, we investigated the gene expression pattern of stem cell markers in DFCs and SHED. In this study, SHED and DFCs expressed stem cell markers such as Oct-4 and ABCg2 that were also markers of PDL stem cells [9, 10]. The expression of ABCg2 is considered to be an indicator for side populations in the human PDL, which represent a particular population of stem cells [9]. We, therefore, believe that both dental cell types contain side populations similar to PDL stem cells. However, this will be examined in further studies. Additionally, cell markers were expressed that were associated with multipotent stem cells (Oct-4), neural stem cells (SOX2, Notch-1, and nestin), endothelial cells (CD146), and monocytic cells (CD13) [10–15]. In this context, two points are of interest: (1) CD146 was detected on almost all kinds of undifferentiated dental cells and (2) CD13 was expressed on embryonic-like dental pulp stem cells [16]. Interestingly, Pax6, a marker of retinal stem cells, was expressed in SHED only and may indicate a good neural cell differentiation potential. This characterization of SHED and DFCs demonstrated that both cell types express markers for stem cells and especially for neural stem/precursor cells. Interestingly, we identified a higher number of Stro-1-positive SHED than previously described (27% vs. 9%) [7]. However, Miura et al. used flow cytometry, which may explain the difference to the result of our study [7].

Neural differentiation potentials of SHED and DFCs after cultivation in SRMs

Neural differentiation of adult stem cells is of great interest for an autologous cell replacement therapy. Bone marrow-derived mesenchymal stem cells can differentiate into neuron-like cells under in vitro conditions [12, 17–19]. Interestingly, neural cell markers are also expressed in undifferentiated mesenchymal stem cells [12, 19–21]. We found that naive DFCs and SHED express neural cell markers, which illustrate the neural crest origin of human dental stem cells. In recent publications, mesenchymal stem cells were differentiated into neural cells after treatment with simple chemicals such as retinoic acid and dimethyl sulfoxide [12, 22]. However, simple chemical treatment protocols for neural differentiation should be regarded with caution, since the rapid changes in cell morphology after treatment with chemically inductive media are possibly caused by rapid disruptions of the cytoskeleton [22, 23]. We, therefore, used different SRMs for neural differentiation that contain essential supplements for the maintenance and differentiation of neural stem/precursor cells. Mesenchymal stem cells, for example, formed nestin-positive neurosphere-like cell cluster aggregates in SRMs supplemented with growth factors like fibroblast growth factor (FGF)-2 or epidermal growth factor (EGF) [13, 14, 24]. Similar results were also achieved with SHED and stem cells derived from the PDL [6, 7]. Widera et al., for example, obtained neural stem cells from the PDL that are capable of differentiation into neurons or glial cells [6]. Miura et al. [7] demonstrated that SHED can form neurosphere-like cell clusters that express late neural cell markers such as neurofilament. In our study, SHED formed neurosphere-like cell clusters as well, but the expression of late neural cell markers was not upregulated. These neurospheres usually specifies an early step of neural differentiation, but it is important to note that they do not contain solely undifferentiated neural cells [25]. In our study, a reduced cell proliferation and a typical neuron-like appearance also indicate the neural differentiation of DFCs and SHED. However, DFCs did not form neurosphere-like cell clusters in this study, but these cells made these cell clusters after cultivation on poly-L-lysine in a previous study [4]. In this study, early neural cell markers such as nestin were upregulated.

We evaluated gene expressions of neural cell markers with reliable quantitative real-time RT-PCR assays [8, 26]. DFCs differentially expressed MAP2, a marker for mature neurons [21], in NDM IV, but it was not expressed in NDM I. In contrast, SHED differentially expressed MAP2 in NDM I, but MAP2 was downregulated in NDM IV. This is an example for different reactions of SHED and DFCs after an identical stimulus. So, SHED and DFCs can be distinguished after cultivation in SRM. Moreover, the gene expression of GFAP was upregulated only in SHED after differentiation. This result shows that SHED can differentiate into both neurons and glial cells. In contrast, a glial cell differentiation can be excluded for DFCs in this study. Similar results were also obtained with a two-step strategy for neural differentiation of DFCs [4]. Interestingly, DFCs do not express the transcription factor Pax6, which promotes the maturation of astrocytes in progenitors [27].

Conclusion

This study shows that both SHED and DFCs differentiate toward neuron-like cells. However, we could demonstrate that SHED and DFCs do not have the same neural differentiation potential, so our initial hypothesis has to be

rejected. Our study demonstrates furthermore that SRMs are helpful for the characterization of dental stem cells.

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Conflicts of interest The authors declare that they have no conflicts of interest.

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