

AUS DEM LEHRSTUHL FÜR
MUND-, KIEFER-, GESICHTSCHIRURGIE
PROF. DR. DR. TORSTEN E. REICHERT
DER MEDIZINISCHEN FAKULTÄT
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A TWO-STEP STRATEGY FOR NEURONAL DIFFERENTIATION IN VITRO OF
HUMAN DENTAL FOLLICLE CELLS

Inaugural – Dissertation
zur Erlangung des Doktorgrades
der Medizin

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Dekan: Prof. Dr. Dr. Torsten E. Reichert

1. Berichterstatter: PD Dr. Christian Morszeck

2. Berichterstatter: Prof. Dr. med. Dr. h.c Joachim Grifka

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A two-step strategy for neuronal differentiation *in vitro* of human dental follicle cells

Florian Völlner^{a,b}, Wolfgang Ernst^a, Oliver Driemel^c, Christian Morsczeck^{a,b,*}

^a Institute of Human Genetics, Franz-Josef Strauss Allee 11, University of Regensburg, 93053 Regensburg, Germany

^b Department of Operative Dentistry and Periodontology, Franz-Josef Strauss Allee 11, University of Regensburg, 93053 Regensburg, Germany

^c Department of Oral and Maxillofacial Surgery, Franz-Josef Strauss Allee 11, University of Regensburg, 93053 Regensburg, Germany

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ABSTRACT

Human dental follicle cells (DFCs) derived from wisdom teeth are precursor cells for cementoblasts. In this study, we recognized that naïve DFCs express constitutively the early neural cell marker β -III-tubulin. Interestingly, DFCs formed β -III-tubulin-positive neurosphere-like cell clusters (NLCCs) on low-attachment cell culture dishes in serum-replacement medium (SRM). For a detailed examination of the neural differentiation potential, DFCs were cultivated in different compositions of SRM containing supplements such as N2, B27, G5 and the neural stem cell supplement. Moreover, these cell culture media were combined with different cell culture substrates such as gelatin, laminin, poly-L-ornithine or poly-L-lysine. After cultivation in SRM, DFCs differentiated into cells with small cell bodies and long cellular extrusions. The expression of nestin, β -III-tubulin, neuron-specific enolase (NSE) and neurofilament was up-regulated in SRM supplemented with G5, a cell culture supplement for glial cells, and the neural stem cell supplement. DFCs formed NLCCs and demonstrated an increased gene expression of neural cell markers β -III-tubulin, NSE, nestin and for small neuron markers such as neuropeptides galanin (GAL) and tachykinin (TAC1) after cultivation on poly-L-lysine. For a further neural differentiation NLCC-derived cells were sub-cultivated on laminin and poly-L-ornithine cell culture substrate. After 2 weeks of differentiation, DFCs exposed neural-like cell morphology with small neurite-like cell extrusions. These cells differentially express neurofilament and NSE, but only low levels of β -III-tubulin and nestin. In conclusion, we demonstrated the differentiation of human DFCs into neuron-like cells after a two-step strategy for neuronal differentiation.

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1. Introduction

Although the use of human embryonic stem cells poses multiple options for cell therapies of neural diseases, the development of therapeutic replacement strategies using adult stem cells remains a realistic alternative. Neural differentiation of bone marrow-derived mesenchymal stem cells was evaluated in various previous studies (Chen et al., 2006; Corti et al., 2004). Here a rather wide range of chemicals, growth factors and cell culture substrates have been used in efforts to initiate neural differentiation of mesenchymal stem cells with varying success (Chen et al., 2006; Corti et al., 2003; Hemptinne et al., 2004; Hermann et al., 2004, 2006; Ho et al., 2006; Krabbe et al., 2005). Dental stem cells are related to bone marrow-derived mesenchymal stem cells but they are less well characterized for neural

differentiation (Morsczeck et al., 2008). Dental cells are nonetheless easily accessible and therefore an interesting alternative for cell therapy approaches (Morsczeck et al., 2008). Shi and coworkers isolated SHED (stem cells from human exfoliated deciduous teeth) that can differentiate into a variety of cell types, including neural cells (Miura et al., 2003). Recently, neural stem cells could be isolated from dental tissues, which could be propagated as neurospheres under serum-free medium conditions supplemented with growth factors (Widera et al., 2007). Today, the formation of neurospheres is the most common way of isolating and expanding neural stem cells *in vitro* (Campos, 2004). Isolated periodontal stem cells were highly proliferative (Widera et al., 2007). Growth factor deprivation and retinoic acid treatment led to the acquisition of neural morphology and stable expression of markers of neural differentiation by more than 90% of the cells (Widera et al., 2007). In contrast to these periodontal-derived neural stem cells, dental pulp-derived stem cells or dental follicle cells (DFCs) are – like bone marrow-derived mesenchymal stem cells – plastic-adherent cells.

In our study we investigated neural differentiation potential of dental follicle cells, which were isolated from the ectomesenchymal

* Corresponding author at: Department of Operative Dentistry and Periodontology, University of Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany. Tel.: +49 941/9446161; fax: +49 941/9446025.

E-mail address: Christian.morsczeck@klinik.uni-regensburg.de (C. Morsczeck).

dental follicle (Morsczeck, 2006; Morsczeck et al., 2005a,b). Ectomesenchymal cells are derived from the neural crest, which also give rise to the peripheral nervous system (Pardal et al., 2007). In this context, Pardal et al. (2007) recently discovered neural crest stem cells of the peripheral nervous system, which generated dopaminergic neurons *in vivo*. This fact makes very promising the use of ectomesenchymal cells for neural tissue cell therapies. In previous studies, we demonstrated that DFCs can differentiate into osteogenic cells under *in vitro* and *in vivo* conditions (Morsczeck et al., 2005a,b). Recently, Kemoun et al. (2007) demonstrated that isolated dental follicle cells expressed the mesenchymal stem cell marker STRO-1. Moreover, they confirmed that follicle cells have multipotential mesenchymal precursor cell properties after differentiating toward multiple mesenchymal-derived cell types, such as cementoblasts, chondrocytes and adipocytes (Kemoun et al., 2007). However, they did not demonstrate neural cell differentiation potential for dental follicle cells.

The neural differentiation potential of DFCs under *in vitro* conditions was therefore evaluated for the first time. In this study, various cell culture media conditions were examined and compared in combination with different surface modifications for neurosphere-like cell cluster (NLCC) formation in DFCs. We investigated furthermore the expression of neural cell-specific cell markers in DFCs by immunocytochemistry and quantitative real-time RT-PCR. For a further neural differentiation, NLCCs were cultivated on poly-L-ornithine and laminin.

2. Materials and methods

2.1. Cell culture

Normal human impacted third molars were collected from adults (18 and 24 years). Coronal parts of the dental follicle were derived from unerupted or partially erupted wisdom teeth. The surfaces of these tissues were cleaned and minced by using a sterilized scalpel. Dental follicle precursor cells were isolated as described previously (Kemoun et al., 2007; Morsczeck et al., 2005a,b). Dental follicle precursor cells were seeded into cell culture flasks in regular cell culture medium DMEM [Dulbecco modified Eagle medium (with L-glutamine) supplemented with 10% fetal calf serum and penicillin/streptomycin (all purchased from PAA, Pasching, Austria)] and incubated at 37 °C in 5% CO₂ in a humidified atmosphere.

For the induction of neural differentiation, various cell culture media were used. DFCs were used at passage 5 for experiments. Cell culture media were based on serum-replacement media (SRM) comprising Neurobasal medium (PAA) containing different supplements and growth factors. We tested different neural stem cell differentiation media (NSCM) protocols in our study. These protocols were combined with different cell substrates (see below). For neural differentiation protocol NSCM I DFCs were cultured in Neurobasal medium containing L-glutamine, the G5-supplement and neural stem cell supplement (all PAA) for one week. The G5 supplement contains 100 µg/ml biotin, 1 µg/ml EGF, 500 ng/ml FGF-2, 500 µg/ml human transferrin, 360 ng/ml hydrocortisone, 500 µg/ml insulin, 520 ng/ml sodium selenite. For protocol NSCM II DFCs were cultivated in the Neurobasal medium containing L-glutamine, the B27 supplement (all PAA) in combination with growth factors (20 ng/ml EGF (Sigma-Aldrich, St. Louis, USA) and 20 ng/ml FGF-2 (Sigma-Aldrich)) for 4 days and without growth factors but with retinoic acid for additional 7 days. The B27 supplement were purchased from PAA and contains undisclosed concentrations of biotin, L-carnitine, cholesterol, corticosterone, ethanolamine, D(+)-galactose, glutathione (reduced), lecithin, linoleic acid, linolenic acid, phosphatidylcholin,

progesterone, putrescine, retinol, retinyl acetate, sodium selenite, T3 (triiodo-L-thyronine), DL- α -tocopherol (vitamin E), DL- α -tocopherol acetate, albumin, catalase, insulin, superoxide dismutase, and transferrin. For NSCM III, cells were cultivated in Neurobasal medium supplemented with B27 and retinoic acid without growth factors for 7 days. DFCs were plated at a cell density of 25,000 cells/cm² for neural differentiation. Cell culture media were changed every second day.

For further neural differentiation, cells were incubated as follows. DFCs were cultivated in NSCM I on poly-L-lysine to form neurosphere-like structures. These pre-differentiated DFCs were further differentiated by protocol NDSS: DMEM/F12 Media (PAA), Insulin–transferrin–sodium selenite supplement (ITTS, Roche, Mannheim, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin, and 40 ng/ml FGF-2 for 14 days. The medium was supplemented with 0.5 µM retinoic acid (Sigma-Aldrich) between days 7 and 14 of differentiation. The surface was modified with poly-L-ornithine and laminin.

2.2. Preparation of culture substrates

The expansion and induction of neural differentiation under different cell culture conditions were performed in 6-well tissue grade polystyrene plates. The surfaces of wells were precoated with substrates of interest. The following concentrations were applied for surface modifications: gelatin (0.2 mg/cm²), poly-L-lysine (2 µg/cm²), laminin (5 µg/cm²), poly-L-ornithine (10 µg/cm²) or fibronectin (2 µg/cm²). Substrates were purchased from Sigma-Aldrich and BD Biosciences (Heidelberg, Germany).

2.3. Immunocytochemistry

Fixed cells were washed in Tris-buffered saline (BS) (0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5), then blocked with a solution composed of TBS, 0.1% Triton-X100 (only for intracellular antigens), 1% bovine serum albumin (BSA) and 0.2% Teleostean gelatin (Sigma-Aldrich) (fish gelatin buffer [FGB]). The same solution was used during the incubations with antibodies. Primary antibodies were applied overnight at 4 °C. Fluorochrome-conjugated species-specific secondary antibodies were used for immunodetection.

The following antibodies and final dilutions were used. Primary antibodies: mouse anti-neuron-specific enolase (anti-NSE) (1:200; AbD serotec); rabbit anti-gial fibrillary acidic protein (GFAP) (1:1,000; Dako Denmark A/S, Glostrup); mouse anti-rat nestin (1:500; BD PharMingen, San Diego, USA); mouse anti-Map 2a+2b (1:250; Sigma-Aldrich); mouse anti- β -III-tubulin (1:500; clone 5G8; Promega, Madison, USA); mouse anti-(PAN) neurofilament medium and heavy (1:1000 Invitrogen, Karlsruhe, Germany); secondary antibodies: donkey anti-goat, -mouse, -rabbit or -rat conjugated with Alexa Fluor 488 (Invitrogen), goat anti-mouse, -rabbit or -rat conjugated with Alexa Fluor 488, RHOX (1:500). Nuclear counterstaining was performed with 4',6'-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) at 0.25 µg/µl (Sigma-Aldrich). Specimens were mounted on microscope slides using a Prolong Antifade kit (Invitrogen). Epifluorescence observation and photodocumentation were accomplished using a Leica microscope. For negative controls, the primary antibodies were omitted.

2.4. Real-time reverse transcription (RT)-PCR

Total RNA was isolated from cells with the RNeasy Kit (Qiagen, Hilden, Germany). Genomic DNA contamination was eliminated with Rnase-free DNase for on-column digestion (Macherey-Nagel,

Düren, Germany). Total RNA from human total brain was derived from Clontech (Mountain View, USA). First-strand cDNA synthesis was performed by using 0.4 µg total RNA and the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). We used the TaqMan[®] Fast Universal PCR Master Mix (Applied Biosystems, Foster City, USA). Sequences for primers and probes were selected using the Universal ProbeLibrary Assay Design Center (www.roche-applied-science.com) and can be obtained from the authors. For quantification, we used the delta/delta calculation method described previously (Winer et al., 1999). The house-keeping genes for GAPDH and GUS were used as reference genes in all applications. PCR primers were established with total RNA from the brain (Clontech, Mountain View, USA). For negative control the reverse transcriptase omitted in the first-strand synthesis kit (-RT, data not shown). PCR primers were established by using cDNAs made from commercial available total RNA from various human tissues (BD Bioscience, Franklin Lakes, NJ, USA).

3. Results

3.1. Gene expression of neural cell markers in naïve DFCs

DFCs were isolated and initially cultivated in regular cell culture medium. The growth curve demonstrated cell proliferation of DFCs at passage 6 (Fig. 1). Before evaluation of neural cell differentiation, the expression of neural cell markers was investigated in naïve DFCs by immunocytochemistry. These cells express not only the neural progenitor cell marker nestin as previously described (Morsczeck et al., 2005a,b) but also neural cell makers, which represent different developmental stages. In immunocytochemical stainings, DFCs expressed the early neural cell marker β -III-tubulin ($94\% \pm 4.7$ SEM), but late neural differentiation markers such as neurofilament and NSE were weakly expressed if at all (Fig. 2A). However, the glial cell marker GFAP was not detected in undifferentiated cells (data not shown). Interestingly, DFCs formed nestin and β -III-tubulin-positive neurosphere-like cell clusters after cultivation on low-attachment cell culture dishes. Here, cells were cultivated in serum-free cell culture medium containing the B27 supplement and EGF and FGF-2 (Fig. 2B). The gene expression of neurofilament and β -III-tubulin was up-regulated in NLCCs (Fig. 2C).

3.2. Pre-differentiation of DFCs (first step)

For a detailed analysis of the neural differentiation of DFCs we examined cell morphology, NLCC formation and gene expression

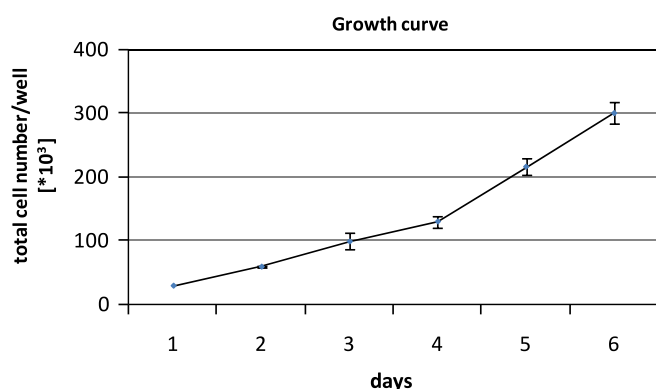


Fig. 1. Growth curve of DFCs. Cells were plated at a density of 5000 cells/cm² and cultured in serum-containing regular cell culture medium for 6 days. Cells were harvested each day and counted with a hemocytometer. Values expressed are the mean \pm SEM.

of neural cell markers after applying different cell culture conditions (Figs. 3 and 4). DFCs cultivated in regular cell culture medium (DMEM) expressed transcripts of nestin, β -III-tubulin, neurofilament, neuron-specific enolase and MAP-2 at all points in time of the study. DMEM-treated DFCs demonstrated a fibroblastic cell morphology on all tested cell culture surfaces (Fig. 3) and neural cell markers were almost constitutively expressed on all different surface modifications (Fig. 4). However, gene expression of nestin was up-regulated on poly-L-lysine (Fig. 4). In contrast, the expression of neural cell markers was up-regulated in SRM; especially after cultivation in NSCM I (Fig. 4F). Cell morphologies of DFCs in SRM varied with different substrate surface modifications. DFCs formed NLCCs on poly-L-lysine with each tested combination of SRM. Most of dental follicle cell-derived NLCCs had a diameter of about 50–150 µm (Fig. 5). In contrast to poly-L-lysine we detected only surface adherent cells on gelatin and laminin. Here, cells displayed relatively long axon-like cell extensions (Fig. 3). On laminin/poly-L-ornithine, DFCs differentiated into surface adherent cells in NSCM I and NSCM III (Fig. 3) and formed adherent NLCCs after 4 days and free-floating NLCCs after additional 7 days of treatment with NSCM II protocol (Fig. 3). However, neural cell markers nestin and β -III-tubulin were constitutively expressed in NSCM II and NSCM III but differentially expressed in NSCM I (Fig. 4). Interestingly, gene expression of NSE was differentially expressed in cells after the formation of NLCCs on poly-L-lysine and in NSCM II on laminin/poly-L-ornithine. For an evaluation of real-time RT-PCR data, a statistical test (multiple ANOVA, analysis of variance) was done. The dependence of neural cell marker expression on cell culture media and cell culture substrate demonstrated a peak neural differentiation on poly-L-lysine surface with use of cell culture medium NSCM I and high expression levels of NSE in NSCM II and NSCM III (Fig. 4).

We made a real-time RT-PCR analysis (Table 1) for marker genes of neural subpopulations for a further categorization of differentiated cells (Lemke et al., 1997; Martinez-Gutierrez and Castellanos, 2007). The expression of choline acetyltransferase characteristically for cholinergic neurons was detected neither before nor after differentiation of DFCs (data not shown). Calretinin (CALB2), a calcium-binding protein involved in calcium signaling, is often expressed in GABAergic neural cells. DFCs expressed CALB2 at all timepoints, but were not differentially affected by culture conditions (Table 1). Parvalbumin (PVALB) is present in GABAergic interneurons of the nervous system and was expressed after cultivation in DMEM on poly-L-lysine and after differentiation in NSCM II on laminin, but not expressed in naïve DFCs on polystyrene. The neuropeptides galanin (GAL) and tachykinin (TAC1), the precursor of substance P, are small or small/intermediate neuron markers. DFCs in DMEM and polystyrene expressed GAL and TAC1 at low levels. Gene expression of both markers was increased after differentiation in SRM, especially in NLCCs on poly-L-lysine and on laminin/poly-L-ornithine (Table 1). Therefore NLCCs probably contain small- and intermediate-sized neurons or neural precursor cells. Interestingly, the large neuron marker vasoactive intestinal peptide (VIP) was also expressed weakly after differentiation with NSCM I and NSCM II (Table 1). We found that tyrosine hydroxylase (TH), a marker of dopaminergic neurons, was detectable only in DFCs after cultivation in NSCM II and NSCM III on polystyrene. However, this marker was expressed after cultivation on poly-L-lysine and gelatin. Glutamate decarboxylase 1 (GAD1), a marker of GABAergic neurons, was down-regulated or not differentially expressed after differentiation. In contrast, the gene of the neurotransmitter transporter serotonin (SLC6A4), a marker of serotonergic neurons, was differentially expressed after cultivation in SRM.

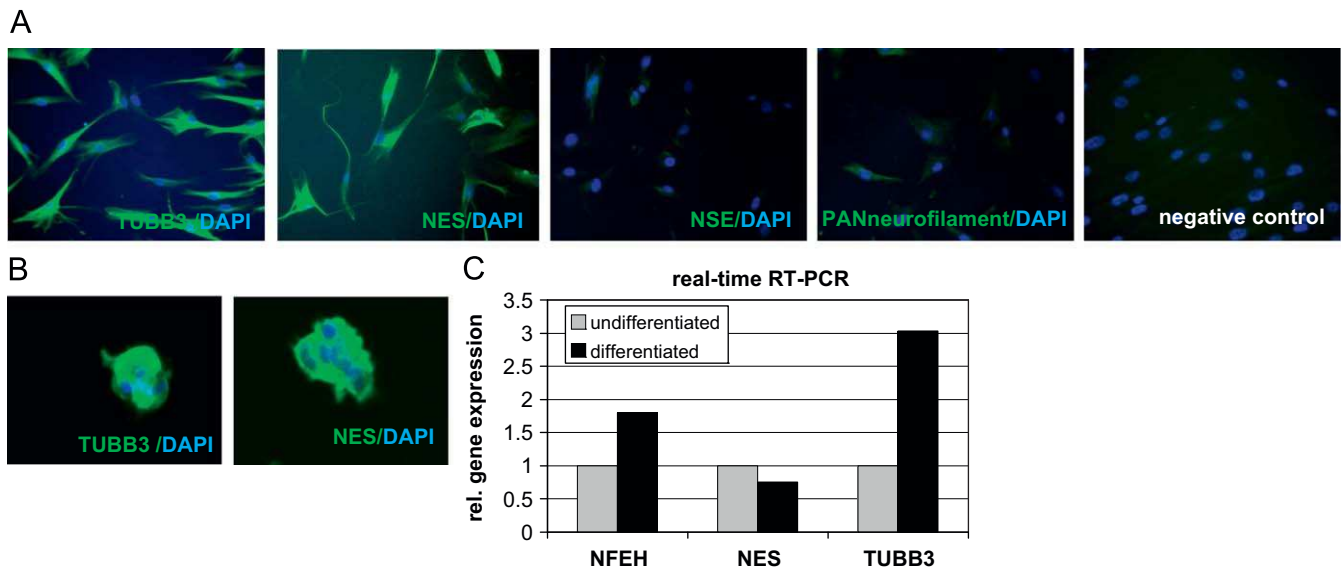


Fig. 2. Analysis of neural differentiation of DFCs on low attachment cell culture dishes. (A) Immunofluorescence study for the expression of neural cell marker β -III tubulin (TUBB3), neuron-specific enolase (NSE), PANneurofilament and neuronal progenitor cell marker nestin (NES) in naïve DFCs. Antibody staining was applied as described under Section 2. For negative control the primary antibody was omitted (negative control). (B) Cells were cultivated in Neurobasal medium containing the B27 supplement in combination with EGF and FGF-2. Here, DFCs formed nestin and β -III-tubulin-positive NLCCs. For negative controls the primary antibody was omitted. (C) Gene expression of neurofilament (NEFH), β -III-tubulin (TUBB3) and nestin (NES) in DFCs before (undifferentiated) and after the formation of NLCCs (differentiated); determined by real-time RT-PCR. Total RNA from naïve DFCs before differentiation was used for calibration (relative gene expression = 1).

3.3. Neural differentiation of pre-differentiated DFCs (second step)

To achieve more advanced neural differentiation we produced NLCCs in NSCM I and differentiated these cells with a protocol that was recently described for neural differentiation of dental pulp stem cells (Arthur et al., 2008). After cultivation on poly-L-ornithine and laminin NLCCs attached, spread and formed single cells with neuron-like morphologies (Fig. 6E–H). These cells differentially expressed neuron cell markers such as neuron-specific enolase and neurofilament, which were weakly expressed in undifferentiated DFCs (Fig. 6A–D). In contrast, early neural cell markers, nestin and β -III-tubulin, which were strongly expressed in naïve DFCs, were down-regulated after the second differentiation step. We detected by real-time RT-PCRs that gene expression of nestin, increased after the formation of NLCCs, and was down-regulated after a further differentiation step. Moreover, the early neuron-specific marker β -III-tubulin was constitutively expressed after the second step of differentiation. In contrast, gene expression of NSE and neurofilament was increased after differentiation (Fig. 6I). Here, gene expression of NSE was remarkably increased after the second differentiation step on poly-L-ornithine and laminin. Gene markers for neural subpopulations demonstrated a differential expression of the neuropeptide TAC1 and the calcium-binding protein CALB2 (Fig. 6J). By contrast, expression levels of SLC6A4, GAD1 and GAL were lower than in undifferentiated or pre-differentiated DFCs. All other tested marker genes of neural subpopulations were not expressed in differentiated DFCs. The astrocyte marker GFAP was not detected before and after differentiation (data not shown).

4. Discussions

DFCs can differentiate into various cell types like cementoblasts, adipocytes or chondrocytes. However, less was known

about neural differentiation potential of DFCs (Kemoun et al., 2007; Morsczech et al., 2005a, b). In our study naïve DFCs strongly expressed early neural cell markers and on a low level markers for mature neural cells (Campos, 2004; Corti et al., 2003; Wachs et al., 2003; Widera et al., 2007). The expression of cell markers can be identified in naïve DFCs by both immunocytochemistry and quantitative (real-time) RT-PCR. The real-time RT-PCR is the most sensitive technique for a quantitative analysis of gene expression (Peirson et al., 2003). In our study, we were interested in also recording minor effects on neural differentiation after applying different cell culture conditions. Gene expression profiles of neural cell markers were therefore estimated by real-time RT-PCR after neural differentiation.

This study characterized human dental follicle cells after cultivation in three different SRMs, which comprise the B27 supplement or the G5 supplement for maintenance and differentiation of neural stem cells (Wachs et al., 2003). The B27 supplement was frequently used for neural stem cell cultures and for neural differentiation of somatic stem cells (Engelhardt et al., 2004, 2005; Miura et al., 2003; Wachs et al., 2003; Widera et al., 2007). NSCM II was a two-step neural differentiation protocol adapted from a successful protocol for neural differentiation of mesenchymal stem cells (Hermann et al., 2004, 2006). Here, Storch and colleagues were able to trans-differentiate human mesenchymal stem cells into NLCCs and later into neural-like cells. In our study the formation of NLCCs was evident with NSCM II (day 11) in combination with the cell culture substrate laminin/poly-L-ornithine, but only the expression of NSE does support this tendency of neural differentiation. However, we also made use of a less-often utilized serum-free medium (NSCM I) composed of the G5-supplement and a specific neural stem cell supplement. The G5-supplement was originally designed for cell-growth and differentiation of glial cells and contains growth factors such as EGF and FGF (Michler-Stuke and Bottenstein, 1982). Recently, it was also used for neural differentiation of mesenchymal stem cells

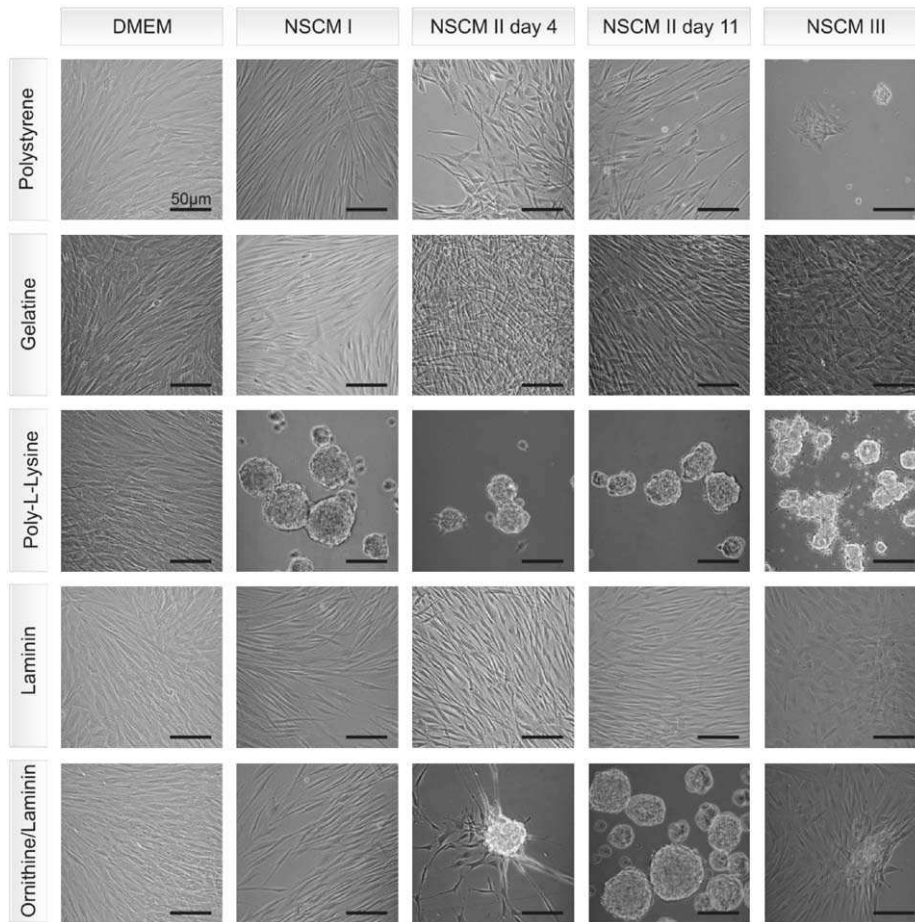


Fig. 3. Light microscope images displaying DFCs after cultivation in SRM. Cells were cultivated in regular cell culture media with serum (DMEM), NSCM I and NSCM III for 7 days on different surface modifications. For cultivation in NSCM II DFCs were pre-differentiated in Neurobasal-medium with B27 supplement, 20 ng/ml EGF and 20 ng/ml FGF-2 without retinoic acid for 4 days (NSCM II day 4). Here, cells formed surface adherent NLCCs. These pre-treated DFCs were then cultivated without growth factors but with retinoic acid for additional 7 days (NSCM II day 11). DFCs formed NLCCs on poly-L-lysine in NSCM I, NSCM II and NSCM III and on poly-L-ornithine/laminin in NSCM II. DFCs were surface adherent and had fibroblastic cell morphology in DMEM. In contrast to DMEM single DFCs became neural-like cells after cultivation in NSCM I, NSCM II and NSCM III on gelatin, laminin and polystyrene. Similar morphologies were observed on laminin/poly-L-ornithine in NSCM I and NSCM III.

(Hemptinne et al., 2004). Interestingly, in our study this medium was the most favorable for a pre-differentiation of DFCs into neural precursor cells.

For our study surface modifications were used that previously facilitated neural differentiation of stem cells. Gelatin-coated wells, for example, were successfully used for neural and glial cell differentiation of embryonic stem cell-derived neural progenitor cells (Goetz et al., 2006; Zhang et al., 2006). In this study, gelatin does not improve neural differentiation of DFCs. For the differentiation of neural stem cells, cell culture dishes were often modified with laminin or a combination of laminin and poly-ornithine or poly-D-lysine (Engelhardt et al., 2004, 2005; Ho et al., 2006; Widera et al., 2007). We identified (as mentioned above) the formation of NLCCs from DFCs after cultivation on laminin/poly-L-ornithine in combination with NSCM II. For mesenchymal stem cells, Ho et al. (2006) demonstrated that the induction of the neural stem cell marker, nestin, depends on a laminin substrate. In contrast, Storch and colleagues described the expression of nestin in naïve mesenchymal stem cells (Hermann et al., 2004; Hermann et al., 2006). These experiments suggest that the expression of neural cell markers is also strongly dependent on cell isolation procedures and the quality of donor tissues. For neural differentiation Storch and colleagues successfully applied poly-L-lysine

for the neural differentiation of mesenchymal stem cells (Hermann et al., 2004, 2006). In correspondence to this examination we found that poly-L-lysine is the most favorable surface modification for the formation of NLCCs and an increased expression of neural cell markers, especially early markers such as β -III-tubulin. In this context, it is remarkable that the expression of the neural progenitor cell marker, nestin, was significantly up-regulated ($p < 0.05$; $n = 3$; Student's *t*-test, statistics not shown in Fig. 4) after cultivation on poly-L-lysine in regular cell culture medium in comparison to each other tested cell culture surface. Interestingly the formation of NLCCs and the up-regulation of the neural cell marker NSE correlate very well in this study.

To obtain differentiated DFCs with typical neural cell morphology a second step of neural differentiation was required. In contrast to naïve DFCs, these cells strongly express neurofilament. However, neural differentiation of DFCs was not possible without a pre-differentiation step. Neural differentiated DFCs differentially express the neuropeptide, TAC1, which is thought to function as a neurotransmitter which interacts with nerve receptors and smooth muscle cells. Interestingly, TAC1 was also identified in neural differentiated mesenchymal stem cells (Cho et al., 2005).

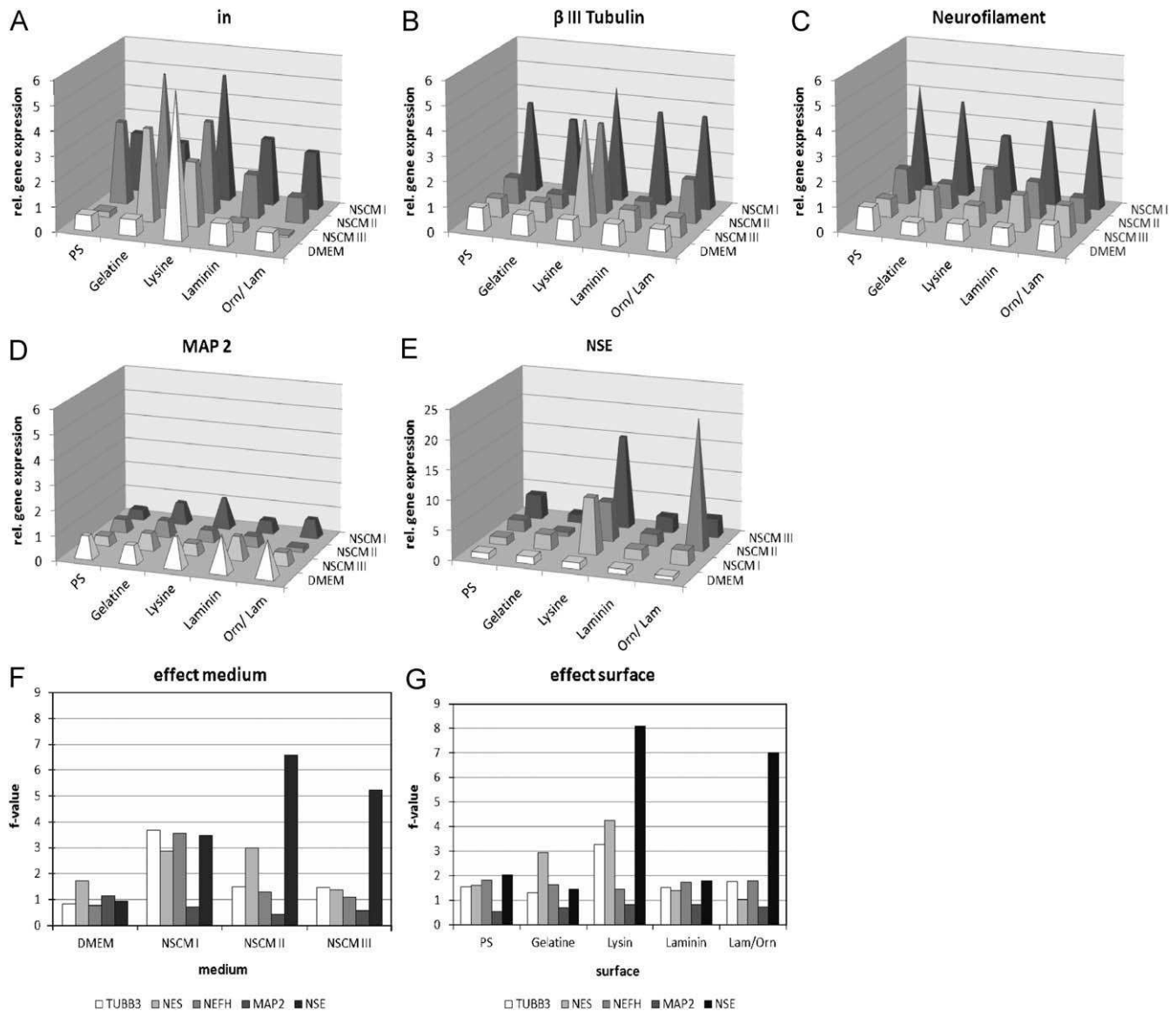


Fig. 4. Real-time RT-PCR analysis for relative gene expression of neural cell markers in DFCs after cultivation in SRM on different cell culture substrates: Real-time RT-PCR results (A–E). Columns of each diagram represent the average of three biological replicates ($n = 3$). Total RNA from naïve DFCs before differentiation were used for calibration (relative gene expression = 1). PCRs were done in duplicates for each sample (F and G). For the evaluation of neural cell marker expression dependent on cell culture medium (medium) and cell culture substrate (surface) f -values were calculated by multivariate analysis of variance (MANOVA). An up-regulation of neural cell markers were detectable for DFCs cultivated in NSCM I (nestin, β -III tubulin, neurofilament) and on poly-L-lysine (β -III-tubulin, nestin). *Abbreviations:* PS: polystyrene; lysine: poly-L-lysine; orn/lam: poly-L-ornithine/laminin.

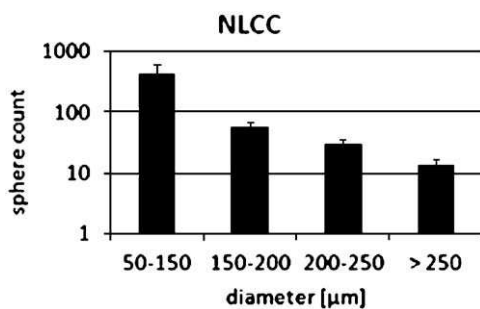


Fig. 5. Assessment of the neurosphere-like cell cluster (NLCC) size of DFCs after differentiation with NSCM I on poly-L-lysine. For this test 250,000 cells were seeded and NLCCs were counted and sizes were measured after differentiation (average \pm SEM; $n = 3$).

Our study investigated neural differentiation of human DFCs for the first time. We demonstrated that naïve DFCs express early neural cell markers. In our study, naïve and differentiated DFCs did not express the glial cell marker GFAP. We conclude that DFCs are neural precursor without potential for glial cell differentiation. Further investigation will determine whether the expression of glial cell markers depends on appropriate cell culture conditions. DFCs differentiate into cells at an early stage of neural differentiation that was demonstrated by an up-regulation of early neural cell markers and by the formation of NLCCs. After a second step of differentiation, late neural cell markers, NSE and neurofilament were up-regulated. We conclude that DFCs displayed characteristics of neural progenitor cells and they are a promising alternative for new cell therapy approaches.

Table 1
Real-time RT-PCR assay with specific primers for markers of neural subpopulations after cultivation in SRM on different cell culture substrates.

| | DMEM | | | | | | | | NSCM I | | | | | | | | | |
|-------------------|--------------|--------|--------------|-----------|--------------|-------|-------|-------------------|----------|--------------|-------|--------------|-----------|--------------|-------|-------|-------------------|--|
| | Dopaminergic | | Serotonergic | GABAergic | Neuropeptide | | | Ca-binding system | | Dopaminergic | | Serotonergic | GABAergic | Neuropeptide | | | Ca-binding system | |
| | TH | SLC6A4 | GAD1 | GAL | TAC1 | VIP | PVALB | CALB2 | TH | SLC6A4 | GAD1 | GAL | TAC1 | VIP | PVALB | CALB2 | | |
| Polystyrene | n. d. | 0.640 | 0.275 | 0.673 | 0.417 | n. d. | n. d. | 0.227 | n. d. | 0.877 | 0.174 | 0.114 | 0.759 | n. d. | n. d. | 0.071 | | |
| Gelatine | + | 1.405 | 0.459 | 0.664 | 1.043 | n. d. | n. d. | 0.741 | n. d. | 2.353 | 0.359 | 0.488 | 4.105 | + | n. d. | 0.167 | | |
| Poly-L-lysine | + | 1.212 | 0.206 | 1.532 | 1.774 | n. d. | + | 0.750 | + | 3.121 | 0.113 | 9.779 | 5.359 | + | n. d. | 0.205 | | |
| Laminin | n. d. | 0.383 | 0.207 | 0.296 | 0.830 | n. d. | n. d. | 0.324 | n. d. | 1.137 | 0.181 | 0.261 | 1.582 | + | n. d. | 0.134 | | |
| Ornithine/laminin | n. d. | 0.209 | 0.069 | 0.333 | 0.481 | n. d. | + | 0.140 | n. d. | 0.554 | 0.077 | 0.267 | 2.124 | n. d. | n. d. | 0.116 | | |
| | NSCM II | | | | | | | | NSCM III | | | | | | | | | |
| | Dopaminergic | | Serotonergic | GABAergic | Neuropeptide | | | Ca binding system | | Dopaminergic | | Serotonergic | GABAergic | Neuropeptide | | | Ca binding system | |
| | TH | SLC6A4 | GAD1 | GAL | TAC1 | VIP | PVALB | CALB2 | TH | SLC6A4 | GAD1 | GAL | TAC1 | VIP | PVALB | CALB2 | | |
| Polystyrene | + | 3.056 | 0.114 | 4.536 | 3.428 | + | n. d. | 0.181 | + | 5.909 | 0.484 | 4.932 | 5.793 | + | n. d. | 0.361 | | |
| Gelatine | + | 5.247 | 0.234 | 0.762 | 3.478 | + | n. d. | 0.112 | + | 6.960 | 0.371 | 0.925 | 2.172 | + | n. d. | 0.156 | | |
| Poly-L-lysine | + | 1.123 | 0.186 | 12.023 | 5.805 | + | n. d. | 0.421 | + | 4.153 | 1.028 | 68.872 | 2.909 | n. d. | n. d. | 1.523 | | |
| Laminin | + | 3.942 | 0.149 | 2.729 | 6.397 | + | + | 0.129 | + | 2.038 | 0.094 | 1.629 | 3.091 | + | n. d. | 0.098 | | |
| Ornithine/laminin | + | 0.763 | 0.065 | 17.068 | 2.617 | n. d. | + | 0.229 | n. d. | 1.241 | 0.059 | 1.583 | 2.214 | + | n. d. | 0.085 | | |

Relative gene expression is displayed for marker genes of neuropeptide, GABAergic, dopaminergic or serotonergic neurons (for details text). Total RNA isolated from naïve DFCs were used for calibration (relative gene expression = 1). Numbers represent the average of three biological replicates (n = 3). Marker gene expression identified differentiated cells only were marked with "+". Abbreviations: n.d.: not detected, PVALB: parvalbumin, CALB2: calbindin 2, GAL: galanin, TAC1: tachykinin, TH: tyrosine hydroxylase, SLC6A4: neurotransmitter transporter serotonin, GAD1: glutamate decarboxylase 1, VIP: vasoactive intestinal peptide.

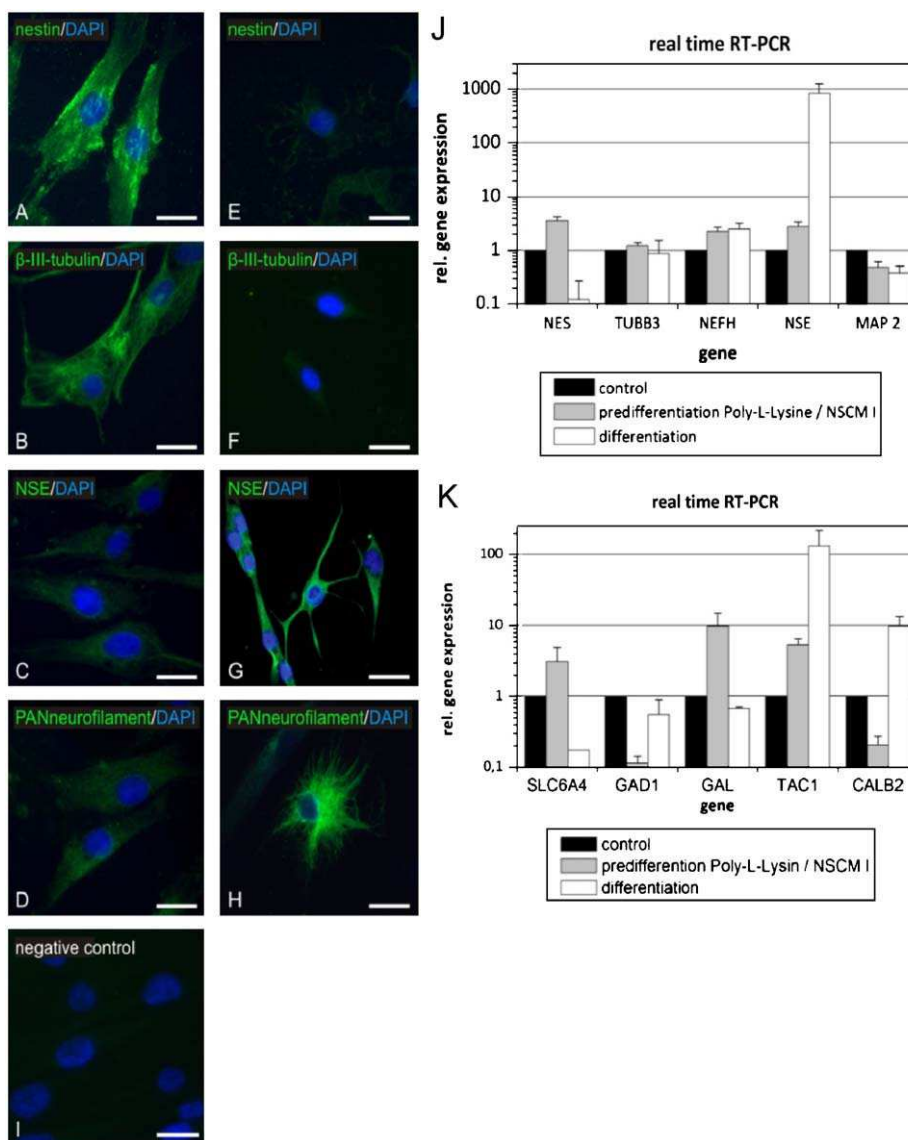


Fig. 6. Immunocytochemical analyses of undifferentiated DFCs (A–D) and after the second step of neural differentiation (E–H) demonstrated a down-regulation of nestin and β -III-tubulin, but an up-regulation of NSE and PANneurofilament. DFCs after the second step of differentiation acquired typical neural cell morphology. Scale bars = 25 μ m. For negative control the primary antibody was omitted (I). (J) In a real-time RT-PCR analysis the gene expressions of NES, TUBB3, NEFH, NSE and MAP-2 were documented for neural differentiation of DFCs. (K) The expression of marker genes for neural subpopulation was furthermore analyzed by real-time RT-PCR. *Abbreviations:* CALB2: calbindin 2, GAL: galanin, TAC1: tachykinin, SLC6A4: neurotransmitter transporter serotonin, GAD1: glutamate decarboxylase 1, NEFH: neurofilament (heavy chain), NSE: neuron-specific enolase, MAP-2: microtubule associated protein 2, TUBB3: β -III-tubulin.

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Zusammenfassung

In der modernen Medizin ist der Einsatz von Stammzellen zunehmend unverzichtbar. So ermöglicht das *Tissue Engineering* durch die Kombination autolog gewonnener Zellen und biokompatibler Trägermaterialien heute bereits die Herstellung künstlicher Gewebe. Diese können als zur Unterstützung oder Substitution von degenerierten Geweben oder Organen in den Patienten replantiert werden (1,2). Dentale Follikelzellen (dental follicle cells, DFCs) stellen hierzu eine leicht zugängliche Stammzellquelle dar. Es handelt sich um adulte Stammzellen, die aus dem Zahnsäckchen von Weisheitszähnen ohne abgeschlossenes Wurzelwachstum gewonnen werden können und ektomesenchymalen Ursprungs sind (3). Unter *in-vitro*-Bedingungen sind DFCs plastikadhärent und besitzen klonogene Eigenschaften. Sie konnten bereits zu Osteoblasten, Chondrozyten und Adipozyten, sowie in Zementoblasten und parodontale Ligamentzellen differenziert werden. DFCs exprimieren mesenchymale Stammzellenmarker wie zum Beispiel STRO-1, ein Marker für multipotente Stammzellen, aber auch neuronale Progenitorzellmarker wie Notch-1 und Nestin (4-6). Dies lässt auf ihre Herkunft von Zellen aus der Neuralleiste schließen, aus der auch das periphere Nervensystem entsteht (7). Allerdings konnte bislang nicht gezeigt werden, ob humane DFCs in neurale Zellen differenziert werden können.

Ziel dieser Arbeit war es, das neuronale Differenzierungspotential der humanen dentalen Follikelzellen zu untersuchen. Hierzu wurden die DFCs in einem ersten Schritt in drei verschiedenen serumfreien, neuronalen Differenzierungsmedien (*neural stem cell media*, NSCM I-III) auf unterschiedlich beschichteten Zellkulturoberflächen (Polystyrol, Gelatine, Ornithin oder Laminin) kultiviert. Nach einer Kultivierungszeit von 7-14 Tagen wurden die Zellen lichtmikroskopisch und mittels quantitativer realtime RT-PCR charakterisiert. Interessanterweise lösten sich die DFCs auf den mit Poly-L-Lysin und L-Ornithin beschichteten Oberflächen in Kombination mit den unterschiedlichen Differenzierungsmedien von der Oberfläche ab und bildeten im Medium schwimmende Zellkonglomerate (*neurosphere like cell cluster*, NLCC). Es ist bekannt, dass *neurospheres*, die aus Zellen des zentralen Nervensystem gewonnen werden, ein besonders hohes neuronales Differenzierungspotential besitzen (8). In der quantitativen realtime RT-PCR zeigten die NLCCs der DFCs besonders auf Poly-L-Lysin und dem NSCM I (Neurobasalmedium mit G5 supplement) einen Anstieg der Genexpression sowohl des neuronalen Progenitorzellenmarkers Nestin (*NES*), als auch des neuronalen Zellmar-

kers β -III-Tubulin (*TUBB3*). β -III Tubulin zählt zu den neuronenspezifischen Markern und ist in frühen Stadien der neuronalen Differenzierung zu finden. Marker von späten Stadien, wie zum Beispiel das Neurofilament, die neuronenspezifische Enolase (*NSE*) oder das „Mikrotubuli assoziiertes Protein 2“ (*MAP 2*) zeigten einen nur schwachen Anstieg der Expression gegenüber undifferenzierten Follikelzellen.

Zur weiteren neuronalen Differenzierung wurden die NLCCs der Paarung Poly-L-Lysin und dem NSCM I (Neurobasalmedium mit G5 supplement) isoliert, separiert und auf einer Laminin/Poly-L-Ornithin-modifizierten Oberfläche und einem weiteren Differenzierungsmedium (DMEM/F12 mit Insulin–transferrin–sodium selenite supplement und FGF-2) für weitere 14 Tage kultiviert. Die erhaltenen Zellen waren plastikadhärent und zeigten unter dem Lichtmikroskop eine Nervenzell-ähnliche Morphologie mit einem kleinen Zellkörper und langen Zellausläufern. In der quantitativen realtime RT-PCR Untersuchung und in der immunzytochemischen Untersuchung exprimierten diese Zellen neben Neurofilament vor allem NSE, also Marker der späten neuronalen Differenzierung. Die Marker der frühen Stadien einer neuronalen Differenzierung wie Nestin und β -III-Tubulin hingegen wurden stark herunterreguliert.

In einer Subpopulationsanalyse zur Spezifizierung der differenzierten Zellen (9,10) zeigte sich ein differentieller Anstieg der Expression für die Neuropeptide Tachykinin (TAC 1) und Galanin. Tachykinin ist die Vorstufe zu der Substanz P und dient als Neurotransmitter zwischen peripheren Nervenzellen und der glatten Muskulatur. Interessanterweise wurde es auch bei neuronal differenzierten mesenchymalen Zellen gefunden (11). Galanin ist ebenfalls ein Peptid, welches in vielen unterschiedlichen Neuronen exprimiert wird und unter anderem die Neurogenese initiiert (12). Marker dopaminerger, serotonerger oder GABAminerger Neuronen konnten nicht nachgewiesen werden.

Zusammenfassend konnte in dieser Arbeit ein neuronales Differenzierungspotential humaner dentaler Follikelzellen mittels einer Zwei-Schritt-Strategie nachgewiesen werden. Es konnte gezeigt werden, dass sich in einem ersten Schritt aus naiven DFCs durch eine Modifikation der Oberfläche und des Mediums neurosphere like cluster bilden und Marker früher Stadien der neuronalen Differenzierung exprimieren. Weiter konnte gezeigt werden, dass sich aus diesen NLCCs nach einem weiteren Differenzierungsschritt Neuronen-ähnliche Zellen bilden, die späte neuronale Marker wie NSE und Neurofilament exprimieren. DFCs haben ein neuronales Potential und stellen somit eine vielversprechende Quelle für neue Therapieformen dar.

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Curriculum Vitae

Angaben zur Person

Name Dipl.-Ing. Univ. Florian Völlner
Anschrift Anton-Scherübl-Straße 8
D-93155 Hemau
Handy +49(0)179/4651753
Email florian.voellner@gmx.de
Geburtsort Regensburg
Geburtsdatum 28. 02. 1978
Nationalität Deutsch
Zivilstand ledig



Berufserfahrung

Seit 01/2011: Assistenzarzt an der Klinik und Poliklinik für Orthopädie der Universität Regensburg am Asklepios Klinikum Bad Abbach GmbH

Ausbildung

2004 – 2010: Studium der Humanmedizin an der Universität Regensburg
Abschluss: Ärztliche Prüfung
Gesamtnote: „gut“

1998 – 2004: Studium an der Fakultät für Maschinenwesen der Technischen Universität München

- Vertiefungsrichtungen: Medizintechnik
Systematische Produktentwicklung
- Abschluss: Diplom Ingenieur Universität
Gesamtnote: 1,8; Prädikat “cum laude”

1989 – 1998 Musikgymnasium der Regensburger Domspatzen
Abschluss: Abitur
Gesamtnote: 2,2

Praktika

08/2009 – 08/2010 Praktisches Jahr:

- Orthopädie (Asklepios Klinikum Bad Abbach und Uniklinik Balgrist, Zürich, Schweiz)

Dipl.-Ing. Univ. Florian Völlner * Anton-Scherübl-Str. 8 * D-93155 Hemau * +49 179 4651753
email: florian.voellner@gmx.de

- Chirurgie (Uniklinikum Regensburg)
- Innere Medizin (Barmherzige Brüder Regensburg)

| | |
|-------------------|---|
| 2007 – 2008 | Famulaturen: <ul style="list-style-type: none">• Pulmologie (30 Tage, Klinik Donaustauf)• Intensivstation 93 der Inneren Medizin II (30 Tage, Uniklinikum Regensburg)• Orthopädie (30 Tage, Asklepios Klinikum, Bad Abbach)• Unfallchirurgie (30 Tage, Uniklinikum Regensburg) |
| 01/2009 – 08/2009 | Studentische Hilfskraft in der Poliklinik für Zahnerhaltung und Parodontologie, Universität Regensburg |
| 08/2008 – 01/2009 | Studentische Hilfskraft am Institut für Humangenetik, Universität Regensburg |
| 04/2006 – 07/2008 | Studentische Hilfskraft im Schlaflabor des Bezirksklinikum Regensburg |
| 03/2003 – 05/2003 | Ingenieurspraktikum bei der BrainLAB AG, München |
| 11/2003 – 04/2004 | Studentische Hilfskraft am Zentralinstitut für Medizintechnik an der Technischen Universität München |

Sprach- und PC-Kenntnisse

| | | |
|--------------------|--|------------------------------|
| Computerkenntnisse | CATIA, Pro/ENGINEER, AutoCAD, Microsoft Windows, Microsoft Office, Lotus SmartSuite, C, Corel Draw, Origin, Photo Shop | |
| Sprachkenntnisse | Deutsch | Muttersprache |
| | Englisch | fließend in Wort und Schrift |
| | Französisch | Grundkenntnisse |

Außerberufliche Aktivitäten

| | |
|-------------|--|
| Hobbies | Badminton, Wandern, Squash, Fußball, Kochen |
| Musik | Chorsänger bei dem "Vokalensemble Cantaloupes e.V". Sänger bei dem Quartet "Völlner brothers" |
| Rotes Kreuz | Aktives Mitglied in der Bereitschaft Regensburg 1 |

Publikationen

- Morsczeck C, Völlner F, Saugspier M, Brandl C, Reichert TE, Driemel O, Schmalz G. "Comparison of human dental follicle cells (DFCs) and stem cells from human exfoliated deciduous teeth (SHED) after neural differentiation in vitro" Clin Oral Investig. (2009).
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Danksagung

Für meine Eltern und meine Familie...ohne Euch
wäre ich nicht der, der ich heute bin!

Eidesstattliche Erklärung

„ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Insbesondere habe ich nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeit erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt“.

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Dipl.-Ing. Univ. Florian Gerhard Völlner