Neural differentiation of dental pulp stem cells

PhD thesis

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Budapest

2010

INTRODUCTION

One of the first signs of mammalian tooth development is seen as the oral epithelium starts to invaginate into the underlying neural crest-derived mesenchyme. The mesenchymal cells are derived from the dorsal-most aspect of the neural tube, and contribute to many tissues, including the peripheral nervous system and cranium. From this aspect, it would be of great interest to identify a neural progenitor pool in the adult human dental tissues, and investigate its regenerative potential for nervous system defects such as neurodegenerative diseases or disorders arising from stroke or injuries.

AIMS OF THE STUDY

1. Developing a differentiation method to convert DPSCs robustly into functionally active neurons. Compare our results to the features observing when applying other methods for neural differentiation, on DPSCs.
2. Describing the morphological changes and the molecular (mRNA, protein and ionic channel expression) background of the differentiation process.
3. Transplantation of uninduced, and *in vitro* pre-induced, fluorescently labeled DPSCs into healthy, and cold lesioned brains of rats. Describing the localization and the distribution of the engrafted cells between brain regions. Defining their specific marker-expression pattern and voltage-dependent ionic current characteristics *in vivo*. Comparing the data obtained from differentiated and non-induced DPSCs.

EXPERIMENTAL PROTOCOLS

Isolation and culture of human DPSCs: Normal impacted human third molars were collected from adults (19-55 yrs of age) at the Departments of Maxillofacial Surgery, Prostodontics and Periodontology, Semmelweis University, Hungary, under approved guidelines set by the National Institutes of Health Office of Human Subjects Research. Dental pulp tissues were minced and then digested in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase type II for 1 hr at 37°C. After centrifugation, cells were resuspended in culture media, and passed through a 70 μm strainer to obtain single-cell suspension. Cells were seeded in culture dishes and maintained under standard conditions (37°C, 100% humidity, 5% CO2) in αMEM medium, supplemented with 100 μM ascorbic acid 2-phosphate, 2 mM L-glutamine, 100

U/ml penicillin, 100μg/ml streptomycin and 10% fetal calf serum (FCS). Subconfluent cultures were regularly passaged by treatment with 0.05% trypsin in phosphate-buffered saline (PBS, pH 7.4) and seeded at a density of 10^4 cells/cm^2.

Osteogenic differentiation: DPSCs were cultured with 1% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10-8 M dexamethazone, 50 μg/ml L-ascorbic acid 2-phosphate, 10 mmol/L β-glycerophosphate in αMEM for 20 days without passaging, but replacing the medium twice a week, after which calcium accumulation was detected by 2% Alizarin red S (pH 4.2, buffered with ammonium hydroxide) staining.

Neural differentiation: For neuronal induction, cultured morphologically homogeneous dental pulp cells (DPSC cultures, passage 1 to 4) were seeded at 20000 cells/cm^2 in poly-L-lysine coated 10 cm Petri dishes, 6-well plates and coverslips in DMEM/F12 (1:1), 2.5% FCS, 100 U/ml penicillin, and 100μg/ml streptomycin, and cultured for 24 h. Epigenetic reprogramming (step 1) was performed using 10 μM 5-azacytidine in DMEM/F12 containing 2.5% FCS and 10 ng/ml bFGF for 48 h. Neural differentiation (step 2) was induced by exposing the cells to 250 μM IBMX, 50 μM forskolin, 200 nM TPA, 1 mM dbcAMP, 10 ng/ml bFGF, 10 ng/ml NGF, 30 ng/ml NT-3, 1% of insulin-transferrin-sodium selenite premix (ITS) in DMEM/F12 for 3 days. At the end of the neural induction treatment, cells were washed with PBS, and then neuronal maturation (step 3) was performed by maintaining the cells in Neurobasal A media supplemented with 1 mM dbcAMP, 1% N2, 1% B27, and 30 ng/ml NT-3 for 3-7 days. All solutions were freshly prepared immediately prior to use. Following each step, cells in one well of the 6-well plates were lysed in lysis buffer containing 1% β-mercaptoethanol, and stored for RT-PCR analysis. After the final step, coverslips were fixed for immunocytochemistry analyses, or used for electrophysiological recordings.

To compare our results to studies obtained by other, previously published neural differentiation methods we adapted two protocols developed for BMSCs to apply them on DPSCs. Protocol 1: Cells were differentiated with 10 ng/ml bFGF, 200 nM TPA, 250 μM IBMX and 50 μM forskolin, in DMEM/F12 supplemented with ITS. The cells were fixed for immunocytochemistry right before and 24 h post-induction. Protocol 2: Cells were preinduced for 1 day with DMEM/F12, with 20% FCS, and 10 ng/ml bFGF. The preinduction medium was removed, cells were washed with PBS and then changed to serum-free induction medium that consisted of DMEM containing 2% DMSO, 200 μM BHA, 25 mM KCl, 2 mM valporic acid, 10 μM forskolin, 1 μM hydrocortisone and 5 μg/ml insulin.

MTT assay: To determine viable cell number in the cultures, an indirect method measuring metabolic activity of mitochondrial enzymes was used. Control and treated cells were incubated with MTT (0.2 mg/ml) diluted in the appropriate media, in each phase of the differentiation for 60 min at 37°C in 96-well plates. Culture medium was removed and formazan was solubilized in DMSO (100μl). The extent of reduction of MTT to formazan within cells was quantified by using a spectrophotometer at a wavelength of 480 nm with the reference wavelength of 650 nm. Absorbance is directly proportional to the number of living cells in culture. Changes in cell viability were estimated by calculating the relative absorbance values normalized to absorbance values of the confluent culture from the same sample on each plate, which were used as internal controls (the cell number of the confluently grown wells on each plate remained constant 9500±380 cells/well during the 9 days long experiment series).

RT-PCR: Total RNA from DPSCs was isolated by lysis buffer supplemented with 1% β-mercaptoethanol and RNA was cleaned up using an RNeasy Plus Micro Kit with on-column DNase digestion. The concentration of the RNA was determined by the Ribogreen method. The integrity of the RNA was verified by electrophoresis on a 1% agarose gel and 200 ng total RNA was used per sample for cDNA synthesis, using random primers in a total volume of 50 μl. cDNA was subsequently amplified by polymerase chain reaction (PCR) using specific oligonucleotide primers and conditions described and successfully used previously, optimized with the gradient PCR method. PCR reactions were performed with an Eppendorf Mastercycler gradient PCR machine.

Real-time PCR: For quantitative PCR amplification, 5% of the cDNA synthesis reaction was used with the real time PCR primers and target-specific fluorescence probe. The probes and primers were selected from the Applied Biosystem Assay on Demands database for the specific markers (VIM, NES, N-tub, NSE, NF-M, GFAP) and for the human acidic ribosomal phosphoprotein P0 (RPLP0), which was used as an internal control. Universal Mastermix containing AMP-erase was used for amplification in a total volume of 20 μl. For detection of fluorescence signal during the PCR cycles, a LightCycler 480 was used with the default setting (50°C for 2 min, 95°C for 10 min, 45 cycles: 95°C for 15 s, 60°C for 1 min). Each treatment was repeated five times and each sample was measured in duplicate. Changes in gene expression levels were estimated by calculating the relative expression values normalized to the RPLP0 level from the same sample.

Immunocytochemistry: DPSCs grown on poly-L-lysine-coated glass coverslips were fixed with 4% PFA in PBS for 20 minutes at room temperature (RT), then permeabilized with 0.1% Triton X-100 (in PBS) for 8 min. To block nonspecific binding, fixed cultures were incubated in PBS containing 4% bovine serum albumin (BSA; 90 min at RT), then reacted with primary antibodies at 4 °C overnight. Antibodies were diluted in 4% BSA as follows: anti-N-tub 1/200, NeuN 1/50, NF-M 1/200, and GFAP 1/1500. IgG anti-mouse and anti-rabbit Alexa fluor 488 conjugated secondary antibodies were diluted 1/750 and applied for 1 hr at RT. After washing, the preparations were mounted with Mowiol containing 10 mg/ml bisbenzimide. Labeled sections were examined by fluorescent microscopy, and images were captured with a cooled CCD camera connected to a PC running image acquisition software (SPOT Advanced). The digitized images of DAPI and specific stainings were merged using Adobe Photoshop.

Cell transplantation and cortical injury: Three days old Wistar rats (male, n=60) were anesthetized (halothane and nitrous oxide mix) and the head was fixed in a stereotaxic frame. 4-5x10^5 Vybrant DiD-labeled cells in a volume of 25 µl of Neurobasal Medium were injected into the liquor of the left lateral ventricle. To investigate the effect of cortical lesion on the implantation of the cells, a cortical injury (cold lesion) was applied on the same day before (n=25), or 7 day after the transplantation (n=25). To achieve cortical deficit rats were anesthetized (halothane and nitrous oxide mix) and the head was fixed in a stereotaxic frame. The scalp was incised over the left motor cortex. The cold lesion stamp was precooled to -60˚C with a mixture of dry ice and acetone and was lowered by a micromanipulator to touch the exposed cranial bone for 1.5 min. Following the lesion the wound was closed and the animal regained consciousness.

Immunohistochemistry: Four weeks after birth, animals were decapitated, the brains were removed and fixed in 3% formalin and cut on a vibratome into 50 µm coronal sections. Mouse/rabbit monoclonal/polyclonal antibodies were used to detect N-tubulin (1:200), NF-M (1:200), NeuN (1:100), and GFAP (1:800). The number of incorporated cells was determined in a minimum of three 50 µm thick sections per animal and brain region. Labeled sections were examined by fluorescent microscopy, and images were captured as described at the immunocytochemistry method.

Patch-Clamp Recordings: Voltage-clamp recordings were performed using the whole-cell technique at room temperature with an Axopatch 200B amplifier. Micropipettes were fabricated by a P-97 Flaming/Brown type micropipette puller from glass capillary tubes and

had a resistance of 5 to 10 MΩ when filled with pipette solution. Capacitative currents were compensated with analog compensation. Linear leak currents were not compensated. Currents were filtered at 2 kHz and sampled at 5 kHz. Pulse generation, data acquisition, and analysis were performed using the pClamp 6.03 software. For whole-cell recordings, the electrodes were filled with a solution containing (in mM): KCl 130.0, $CaCl_2$ 0.5, $MgCl_2$ 2.0, EGTA 5.0, HEPES 10.0 (pH 7.2). The extracellular solution contained (in mM): NaCl 145.0, KCl 3.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, D-glucose 10.0, HEPES 10.0, supplemented when appropriate with 1 mM tetrodotoxin or 5 mM tetraethyl ammonium chloride (TEA) (final concentrations).

For electrophysiological recordings of the transplanted DPSCs, recipient animals were decapitated, and 300-400 μm coronal sections were prepared using a vibratome. Slices were transferred to the stage of an upright microscope and perfused with Krebs solution containing (in mM): NaCl 127.0; KCl 1.8; KH_2PO_4 1.2; $MgSO_4$ 1.3; $NaHCO_3$ 26.0; $CaCl_2$ 2.4; glucose 15.0, adjusted to pH 7.4 and supplemented when appropriate with 1 μM tetrodotoxin (TTX, Alomone Diagnostics) or 5 mM tetraethyl ammonium chloride (TEA) (final concentrations).

Vybrant DiD-positive cells were readily identified using a digital camera, and patch-clamp recordings were obtained under visual control. Cells were recorded from the neocortex (n=11, from 5 different human donors), and the progenitor zones of the brain: subventricular zone (SVZ) and subgranular zone (SGZ) (n=13 from 5 different human donors). Glass microelectrodes were pulled from borosilicate glass (1.5 mm diameter; wall thickness, 340 μm, Warner Insruments) and had a resistance of 10.0-15.0 MΩ. Pipette solution contained (in mM): K-gluconate (120.0), KCl (20.0), MgCl2 (2.0), HEPES (10.0), EGTA (0.5), Mg-ATP (2.0), adjusted to 7.18-7.23 pH with KOH. Liquid junction potentials were not compensated. After establishing the whole-cell configuration, the resting membrane potential and cell internal resistance were measured.

Electrophysiological Measurements and Protocols: Input resistance (IR) was determined from the currents elicited by a 10-mV test pulse depolarizing the cell membrane from –70 mV to –60 mV, 40 ms after the onset of depolarizing pulse. Membrane conductance was determined from currents elicited by depolarization from a holding potential of –70 mV to +10 mV. Currents were measured 40 ms after the onset of the pulses. Membrane capacitance (Cm) was estimated by using the time constant of the membrane, which was calculated by fitting the double-exponential function to the measured currents elicited by a 10-mV test pulse depolarizing the cell membrane from -70 mV to -60 mV. Current patterns were obtained by clamping the cell membrane from a

holding potential of –70 mV to values ranging to +10 mV at intervals of 10 mV. Pulse duration was 50 msec. To isolate the voltage-gated KDR and TTX-sensitive Na+ currents, the voltage step from –70 to –60 mV was used to subtract the time- and voltage-independent currents. The amplitudes of KDR were measured at the end of the pulse. Transient Na+ current amplitudes were measured at the peak value. The changes in current amplitudes were expressed as changes in current densities (pA/pF).

Statistical Analysis: All values are expressed as mean ± SEM. Statistical comparisons were performed in Sigma-Plot 10.0 software. The differences between groups for real-time PCR and MTT assays were evaluated via One Way Repeated Measures of ANOVA followed by post-hoc Dunnett-tests. Statistical significance for patch clamp recordings was evaluated using the two-sample Student's t-test; $P \leq 0.05$ was considered significant.

RESULTS

Isolation, culture, passage and osteogenic differentiation of DPSCs: Dental pulp stem cell cultures consist of morphologically homogenous, spindle-shaped cells, harboring elements that express stem cell and progenitor markers. Following their release by enzymatic digestion from the host tissue, and cultivation under standard conditions, they adhere to the tissue culture grade plastic surface and start dividing. Two to three weeks after plating, DPSCs isolated from a normal human impacted third molar reach confluence in a standard T75 culture dish.

Under our experimental conditions, the cultures plated in parallel with those prepared for neuronal differentiation showed overt signs of mineralization by accumulating insoluble calcium deposits. Thus, this observation confirms the previous findings that DPSCs are capable of differentiation into at least two different cell types, which are normally derived from different germ layers.

Differentiation of dental pulp stem cells into a neuronal lineage: Dental pulp stem cells give rise to neuronal cells consistently and reproducibly when induced by activators of the PKC and cAMP signaling pathways. Noninduced cells display flat morphology typical of fibroblasts, but become more rounded after pretreatment with 5-azacytidine and bFGF for 48 hours. After 2 hours of treatment with the inducing mixture, DPSCs grow processes and start moving towards the high cell-density areas. During the three days of induction, cells anchor their position in the network structure, the previously developed processes disappear, and

their morphology reverts to flat and round cell shapes observed during earlier stages of differentiation. In the final maturation step, cells diverging radially from the centers begin to grow neurite-like processes. After 10 days of differentiation, the vast majority of cells display complex neuronal morphology, expressing both bipolar and stellate forms. However, a small portion of cells retain their flat shape, and are attached beneath the processes of the neuronal cells. These elements are presumably committed towards glial fates, or serve as a stanchion for the developing neuronal cells. Therefore, they might be indispensable for neuronal survival. Taken together, our three step differentiation procedure over 10 days results in a robust differentiation of cells towards neural lineages in essentially all surviving cells that initially showed all characteristics of dental fibroblasts. However, exclusion of the intracellular cAMP increasing components (dbcAMP, forskolin, IBMX) or the PKC activator TPA from our differentiation protocol resulted in the development of incomplete neural-like morphology by the 10th day of the differentiation procedure. When we applied recently described differentiation protocols for periodontal ligament-derived stem cells or for DPSCs, we found the formation of some neuronal-like structures, but the proportion of these cells compared to the entire population appeared to be very small, probably due to the high proliferation capacity of the overgrowing undifferentiated early passage DPSCs that we used in our studies.

When DPSC cells were treated using Protocol 1 with a mixture containing substances such as TPA, IBMX and forskolin, the morphology of cells observed by phase contrast microscopy changed also rapidly. After 4 hours of treatment initiation, the cytoplasm of the cells retracted toward the nucleus in many cells, taking a more spherical shape and extending processes. The percentage of cells with modified morphology decreased within 24 hours. When differentiation factors were removed from the medium, cell morphology reverted to the original. Following the application of Protocol 2 (bFGF, KCl, forskolin, DMSO and BHA) neuronal morphology changes were temporary, followed by an irreversible distraction of the culture: after 48 hours, the majority of cells were seen to have a rounded morphology followed by the death of almost the entire culture, except for a few spindle-shaped cells expressing none of the neuronal markers investigated by us.

Cell viability profile during the differentiation process: Viability of human dental pulp stem cells during differentiation was evaluated by MTT assay. Four DPSC cultures collected from different patients were used for cell viability assays. The cell density of the confluent wells was constantly between 30000 ± 1200 cells/cm^2 during the ten-day experimental protocol,

which was confirmed each time by cell counting. There was a minor decrease (approximately 10%) in viability in response to pretreatment, then the switch to inductive conditions resulted in a significant loss of viable cells ($p<0.001$), but all of the remaining (approximately 40 percent of control) remained vital, showing almost exclusively neural morphological characteristics. In the maturation phase, there was a minor recovery in cell viability, but the number of viable cells was still significantly lower than it had been initially.

Changes in the expression of neuronal markers during induction: Transcripts for mesenchymal vimentin, the neural progenitor marker nestin, the neuronal N-tubulin and NSE were already detectable in the noninduced and pretreated DPSCs, confirming the presence of neural progenitors in the mesenchymal fibroblastic cultures. During induction, NF-M was strongly expressed, while vimentin and nestin became downregulated. A transient decrease in N-tubulin expression was also observed. During the maturation phase, the glial marker GFAP became detectable, the neuronal NGN2 was strongly upregulated, NF-M was downregulated, while nestin and N-tubulin expression increased. NSE was present at every examined time point during differentiation, and its expression progressively increased under maturing conditions. As expected, expression of β-actin, which served as an endogenous control, was constant in all phases of differentiation.

To quantify the changes in the mRNA expression of neuronal marker genes at different time points, we performed real-time PCR assays. The expression of each target gene was normalized to that of the RPLP0 housekeeping gene, and expressed as fold change relative to the noninduced sample. The decrease in the expression was sharp for vimentin and nestin, and moderate for N-tubulin in response to neurogenic stimulation ($p<0.05$). The expression of NSE, NF-M and the glial intermediate filament GFAP increased during induction and maturation ($p<0.05$).

Prior to the transplantation, brain region-specific gene expression pattern of control and 10-days differentiated DPSCs were compared. Transcripts for Dlx1 and En1 were strongly detectable in the differentiated cultures. The expression of En1 was only remarkable in the differentiated populations, although Dlx1 was found to also be expressed in the control cells, but for much lower extent. Mash1 was present in a very small extent both in the uninduced and differentiated cultures. Pax6 remained silent after any treatment. β-actin was constant in both phases of the differentiation.

Immunocytochemical analysis was performed in the final maturation stage of the differentiation, to correlate with mRNA expression data. The protein expression pattern of the

DPSCs fixed on the 10th day of neural differentiation showed a mixed population harboring neural cells expressing the early appearing neuronal microtubule marker N-tubulin, intermediate filament subunit NF-M, the glial intermediate filament marker GFAP, or postmitotic nuclei protein NeuN. Expression of N-tubulin, NF-M and NeuN was observed in more than half of the neuronally committed DPSCs. On the contrary, immunostaining for glial specific markers was seen only on less than 10% of the cells.

Electrical properties of differentiated DPSCs: After normalizing the currents with membrane capacities, three subsets of cells became separable. The first subpopulation (n=5, type 1) displayed fast inactivating Na+ currents which were completely blocked by 1 mM TTX, with a threshold between -60 and -50 mV, and a maximum around -40.1 ± 4.0 pA/pF at -10 mV (from a holding potential of -70 mV). 1 mM TTX completely blocked the sodium currents of type 1 cells having neuronal morphology. Delayed, outwardly rectifying currents (KDR) were also demonstrated in these cells. 5 mM TEA partially reduced the KDR currents. The threshold of the current was at -30 mV, and the maximum current densities were around 88.3 ± 6.1 pA/pF at +10 mV. The passive membrane conductance in these cells was about 1.7 ± 0.4 nS. Another subset of the cells (type 2, n=21) expressed TTX sensitive Na+ currents with the same threshold as observed in type 1 cells, but their maximal amplitudes were only about -19.0 ±5.1 pA/pF around 0 mV. KDR currents were observed after depolarization over a threshold of -30 mV as seen in type 1 cells, but the maximal amplitudes were only 60.8 ± 9.8 pA/pF. The inhibitory effect of TEA was less pronounced in type 2 cells than in type 1 ones. The passive membrane conductance was 2.5 ± 0.8 nS. The third group of cells (type 3, n=12), as well as the noninduced DPSCs, displayed a passive membrane conductance of 25 ± 10.1 nS, and did not exhibit any voltage dependent ion currents. However, in contrast to noninduced cells, they showed neuronal-like morphology.

Fate of Vybrant DiD labeled DPSCs after transplantation: In order to compare and study the potential for in vivo neuro- and gliogenesis of neurally induced and uninduced DPSCs, we used uninjured and cold lesioned (CL) rat brain models to show the differences between their developments. These rats were sacrificed four weeks after transplantation of DPSCs into the lateral ventricle of the brain.

In uninjured animals, both the uninduced and differentiated cells were found to be present on the pial surface and in the normal progenitor zones of the brain. Most of these cells harbored by the progenitor zones of the brain were found to be incorporated in the subcallosal zone (SCZ), in the subventricular zones (SVZ) and in the subgranular zones (SGZ) of the

hippocampus. However, their distribution among the different brain regions was dependent on their in vitro treatment. More than 80% of the in vitro pre-differentiated, labeled DPSCs was incorporated in the progenitor zones of the uninjured brain, and less, than 20% of these cells adhered to the pia and expressed mesenchymal marker vimentin. In contrast, in the case of in vitro uninduced DPSCs, this distribution was approximately reversed.

Nondifferentiated cells basically only expressed the mesenchymal marker vimentin. Immunostaining for glial or neuronal markers was seen on less, than 5% of the nondifferentiated cells. In contrast, much higher extent of the engrafted pre-differentiated DPSCs was positive for N-tubulin, NF-M, NeuN or GFAP.

Migration of the DPSC-derived SCZ, SVZ, SGZ cells, due to cortical lesion: In cases when we developed a fronto-parietal cortical injury by cold lesioning the brain at the time of the cell transplantation or one week after, four weeks after the transplantation, Vybrant DiD-labeled cells became observable in the cortical region ipsilateral to the lesion. Migrating cells expressed mature neuronal markers NF-M, NeuN or glial GFAP in a similar ratio like in the uninjured animals. In contrast, we could not observe the presence of Vybrant DiD-labeled cells in the cortex of noninjured animals. The average extent of N-tubulin, NF-M, NeuN and GFAP positive cells compared to the total number of Vybrant DiD labeled cells in the specific brain regions in the injured cortex did not depend on the timing of injury.

Physiological properties of the transplanted DPSC-derived cells: In uninjured rats, 10 of 13 engrafted DPSCs settled in the progrenitor zones displayed KDR currents, which were sensitive to 5mM TEA. The maximum KDR current values measured on these cells were around 92 ± 33 pA at +10 mV. 6 of 13 cells expressed voltage dependent (VD) sodium currents (INa) sensitive for 1 μM TTX, displaying -13 ± 5 pA maximum amplitudes at +0 mV. The average resting membrane potential was -40.3 ± 3.6 mV, the average internal cell resistance was 3.0 ± 1.5 GΩ.

In CL rats 10 of 11 recorded DPSCs located in the cortical region displayed KDR currents being sensitive to 5mM TEA. The maximum KDR current values measured on these cells were around 226 ± 117 pA at +10 mV. 10 of 11 cells expressed INa sensitive for 1 μM TTX displaying -37 ± 35 pA maximum amplitudes at +10 mV. The average resting membrane potential was -57.8 ± 11.7 mV, the average internal cell resistance was 1.3 ± 0.9 GΩ.

When we compared these current values between uninjured and CL animals statistically significant increase of the KDR current values was found at -40 to +10 mV ($p<0.05$), and

significant increase of INa was demonstrated at +0 and +10 mV ($p<0.05$) following cold lesion. The average membrane potential increased and the input resistance decreased in DPSCs recorded in the CL rats, although in a non-significant manner.

DISCUSSION

When we applied Protocol 1, DPSC cells exhibited a transient neural differentiation. Very similar observations were made following treatment of human BMSCs with the PKC activator TPA, the phosphodiesterase inhibitor IBMX, the adenylate cyclase activator forskolin and bFGF: stimulation of the cells resulted in drastic morphological changes accompanied by the expression of neural markers such as neurofilaments, N-tubulin, NSE and GFAP. However, their expression was transient, and the cells reverted to the original fibroblastic BMSCs within 48 hours. Altogether, these findings indicate that the differentiation potential of Protocol 1 is limited. When we used Protocol 2, again we observed only transient morphological changes followed by the death of most of the cells. This was most probably due to the presence of DMOS/BHA and extremely high, 25 mM K+ concentration. Therefore due to these positive and negative results, when we further developed our differentiation protocol, normal K+ concentration, PKC/cAMP activation and bFGF was used.

In our three-step differentiation study, we described for the first time that neural crest derived adult human dental pulp stem cells can be differentiated into cells that not only express several neural markers, but also simultaneously display voltage dependent sodium and potassium currents. We have developed a relatively rapid and highly reproducible differentiation protocol that selectively supports the development and survival of neural progenitor cells residing in dental pulp derived cultures. The complex stimulatory effect of our protocol involves the influence of bFGF and 5-azacytidine on cell plasticity and neurogenesis, the inductive effect of the cross-talk between the protein kinase C and cyclic AMP pathways and the maturation inducing effect of the combination of known differentiation promoting agents. When applied to DPSCs, our protocol results in a mixture of immature and more mature neural cells, and presumably it does not support the survival of mesenchymal fibroblastic elements that have no potential for neural commitment. Beside the stimulatory agents, another important factor that might affect the efficiency of the differentiation is the plating cell density. In preliminary experiments when we used either higher or lower initial cell concentrations, we found that both the number of cell clusters formed during the induction and the proportion of cells growing neuronal processes under

maturing conditions were considerably lower. Our observations are in line with previous data showing that physical cell-to-cell contacts may be crucially important for neuronal commitment of DPSCs as well as of neural progenitor cells obtained from other tissue sources.

Our RT-PCR and real-time PCR results show that noninduced DPSCs already express neuronal markers such as nestin, N-tubulin and NSE, as well as low levels of NGN2 and NF-M. However, we could not detect substantial expression of GFAP in these undifferentiated DPSC cells. These data indicate that already at the nontreated stage, at least a subset of DPSCs have high potential for neural differentiation. Following pretreatment and induction, the progenitor marker nestin became downregulated. This phenomenon, in conjunction with cell viability data, suggests that stimulation of neural differentiation results in the loss of a large fraction of the cells, and at the same time triggers neurogenic mechanisms in the surviving population. This is supported by the time-dependent changes of expression of the intermediate/mature neural markers NSE and GFAP as their levels gradually elevated during the first two steps of the protocol, while NF-M expression dramatically increased during induction by combined PKC-cAMP activation. Only a small percentage of DPSCs started growing processes and developed neuronal morphology during the PKC-cAMP activation period suggesting that the visible changes in morphology occur much slower than the alterations in gene expression. Our data suggest that approximately 40 percent of the cells survive the combined, sustained activation of the PKC and cAMP systems. Based on the results of our MTT assay and studies reporting about the proportions of DPSC cultures expressing stem cell markers, the proportion of cells that survive the induction period is clearly much higher than that of the cell population that has progenitor characteristics in the initial cell population. During maturation, the cell number only moderately increased, presumably due to limited cell proliferation. These data suggest that not only the subset of DPSCs originally expressing stem/progenitor markers has the capability of differentiating towards neural cells. Other cells of the DPSC cultures may also differentiate in response to the inductive treatment.

In the final maturation phase of the protocol described in our study, the expression levels of N-tubulin, NSE, NGN-2 and GFAP increased, and NeuN immunoreactivity was also detected. Vimentin and nestin expression increased as well, but they did not increase over the initial levels measured in noninduced DPSCs. The mRNA level of the late neuronal marker NF-M, which is known to be expressed prior to final maturation, decreased after peaking on the first

day of induction. These data suggest that a fraction of induced cells entered the final developmental stage of neurogenesis. For the expression profile of these markers, similar observations were made previously using a fundamentally different protocol for neural differentiation.

In addition to mRNA and protein expression results, the characteristics of ion currents obtained by patch clamping indicate that the differentiation of adult human dental pulp cells results in the development of a mixed, heterogeneous cell population. Within this population are subsets that contain neurally committed cells in different proportions. Embryonic stem cell derived neural stem cells were shown to express voltage-sensitive sodium currents only after a few days' exposure to inductive media. The progressive increase of current amplitudes during neuronal maturation was documented by several studies. Our investigations indicate that a subset of differentiated DPSCs express sodium currents displaying mature characteristics, while another subpopulation also exists among the differentiated DPSCs that displays premature currents. Therefore, subsets of differentiated DPSCs may represent distinct developmental stages. We observed similar heterogeneities among the amplitudes of the evaluated outward delayed rectifier potassium currents. KDR currents are non-inactivating potassium currents that play a major role in membrane repolarization during an action potential, and were reported to appear during neurogenesis prior to the expression of voltage dependent sodium currents. Their density, as well as their TEA sensitivity, increases during maturation. Therefore, it may not be surprising that the subset of differentiated DPSCs displaying more mature, characteristic type of sodium currents, expressed KDR currents with increased amplitudes and higher TEA sensitivity (type 1). On the other hand, the type 2 subpopulation exhibited a premature sodium current pattern, and produced KDR currents with lower amplitudes and moderate TEA sensitivity. These data provide important additional functional evidence for the neuronal differentiation of human DPSCs. A recent pioneer work reported that voltage dependent potassium currents could not be detected on DPSCs that underwent neuronal differentiation and displaying voltage gated sodium currents. The reason for the discrepancy between the two studies is not clear, but it might be attributed to the different inductive protocols resulting in different levels of neural differentiation.

Another neuronal feature investigated on DPSCs by patch clamping was the passive conductance, which is known to be very small in maturing neurons. Passive membrane conductance values measured on type 1 and type 2 DPSCs were lower by one order of magnitude than those measured on type 3 cells. Although these cells had some morphological

features typical of neurons, they did not produce any voltage dependent ion currents. These data suggest that type 3 DPSCs may have remained in an early progenitor stage, or are as yet undescribed, non-differentiated neuron-like fibroblastic byproducts of the differentiation process.

Regarding our *in vivo* study, we found for the first time about the potential of human DPSCs to get integrated into injured or healthy adult brain tissue. Prior to the transplantation, we characterized the region-specific gene expression pattern of our control and differentiated DPSC cultures. During the early development, expression of Pax6 is primarily detectable in diencephalon, metencephalon, myelencephalon and some regions of the telencephalon. En1 is restricted to the developing midbrain-hindbrain junction, substantia nigra, and dopaminergic neurons of the ventral neural tube. Dlx1 transcripts are mainly present in cells derived from telencephalon, ganglionic eminences, diencephalon, thalamus, and certain cells of the hypothalamus. Both non-induced and differentiated DPSCs strongly expressed the Dlx1 gene; moreover, the level of En1 transcription was detectable in the in vitro differentiated cultures. These data suggest that both the non-treated and differentiated cultures harbor a subset of cells capable of neural development, although the proportion of these cells is much higher in the cultures that were formerly exposed to the inductive treatment. Four weeks after injecting the cells into the cerebrospinal fluid of noninjured, or CL animals, we found them engrafted in the SVZ, SCZ, SGZ, and/or the cortex of the brain. This observation confirms that Dlx1 expressing DPSCs are capable of integrating into the adult forebrain. Although En1 was expressed in the differentiated cultures, we found only a very few cells engrafted into the striatum or substantia nigra of the animals. These data might indicate that the intact, mature dopaminergic tissue supports the development and integration of dopaminergic precursor cells only in a very limited manner.

Mash1 and Ngn2 were described to be expressed in complementary populations of neural progenitors. Mash1 expression more likely contributes to the development of GABAergic neurons, while NGN2 is associated to the appearance of glutamatergic phenotypes. Our results clearly show that in vitro differentiated DPSCs have potential to develop cortical neurons. However, when compared to the Mash1 transcription level, the much higher NGN2 expression indicates the commitment of induced DPSCs to progress excitatory neuronal features.

The waste majority of the cells that were injected into the cerebrospinal fluid of the animals adhered to the pial surface and differentiated into fibroblasts, or migrated into the

forebrain. In noninjured animals, DPSCs incorporated into the normal progenitor zones of the brain, like into the subventricular zone (SVZ), subcallosal zone (SCZ) and subgranular zone (SGZ). In vitro differentiation did not affect the localization, however, had a great influence on the cell distribution between these regions. DPSCs engrafted into the SVZ, SCZ and SGZ displayed very similar immunohistochemical, and passive or active electrophysiological properties, as the normal neural progenitors of the adult brain. Four weeks after transplanting them into CL animals, both control and in vitro induced DPSCs were found to be engrafted in the injured brain region however, their protein expression was radically distinct. Non-differentiated DPSCs expressed mainly the mesenchymal vimentin, suggesting that the growth factors secreted by some cells due to the injury stimulated them towards scar formation. In contrast, many of the pre-differentiated DPSCs were able to penetrate deeper into the lesioned cortex, and express neuronal- and glial- specific immunoreactive and functional markers, indicating that they have the capability and commitment to regenerate the host tissue. In comparison to the DPSCs harbored by the SVZ, SCZ or SGZ, the increased INa, KDR, NFM and NeuN expression in the pre-differentiated cells that were found to be localized in the lesioned cortex may suggest that these cells were induced to progress maturation. However, we could not detect any synaptic currents being displayed by these cells. According to previously published data obtained by using an ES cell line, in the case of DPSCs, spontaneous maturation was found to be suppressed *in vivo* for some reason as well, despite the promising in vitro results. Moreover, the over activated immune system of the CL animals completely eliminated the DPSCs from the lesioned brain region within 6 weeks, therefore the development and maturation of these cells remained incomplete.

In some experiments when we performed the cold lesion after the transplantation, we could observe, that DPSCs originally engrafted in the progenitor zones started to migrate towards the injured brain region. Migration of the normal neural progenitors of the brain along the rostral migration stream, towards the olfactory bulb is well known in rodents. However, only a few publications mention that SVZ/SCZ/SGZ cells may be capable of migration towards the cortex. Four weeks after the lesion, we were able to detect DiD-labeled cells inside of the lesioned tissue, and obtain very similar immunohistochemical and electrophysiological data to those we have observed on DPSCs that were injected directly into previously injured animals. These data show that neural properties and fate of the differentiated DPSCs depend much more on their final localization, than the relative time of the injury to the transplantation of the differentiated DPSCs.

CONCLUSIONS

Overall, our observations confirm the presence of pluripotent cells in the adult dental pulp that can form both nonmesenchymal and mesenchymal derivatives. These results obtained in our in vitro multistep activation neurogenic model indicate that DPSCs may differentiate into cells with distinct neuronal phenotypes after exposure to specific signals and thus may help understand the molecular background of neuronal differentiation of adult neural progenitor cells.

According to our *in vivo* study, we propose that at the single-cell level, grafted DPSC-derived neurons undergo morphological and functional integration into the host brain circuitry. Immunohistochemical and electrophysiological data both reveal that Vybrant DiD-labeled DPSCs can provide an appropriate model for studying neuro- and gliogenesis in vivo, since they show progression in their neural specific marker expression and electrophysiological properties after the brain injury, displaying a behavior very similar to that of the normal SVZ, SGZ and SCZ cells.

Eventually, these neurally committed DPSCs might represent a new, easily available pool for cell therapy of degenerative disorders of the central nervous system.

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New therapeutical targets in ulcerative colitis: The importance of ion transporters in the human colon

IBD, Accepted: 2010 June 22