

In vivo evaluation of human dental pulp stem cells differentiated towards multiple lineages

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Abstract

An increasing number of investigations supports that adult stem cells have the potential to differentiate into matured cell types beyond their origin, a property defined as plasticity. Previously, the plasticity of stem cells derived from dental pulp (DPSC) has been confirmed by culturing cells in lineage-specific media *in vitro*. In the current study, the *in vivo* differentiation or maturation potential of DPSC was further analysed, by transplanting human DPSC/collagen scaffold constructs into subcutaneous tissue of immunocompromised mice. Cells received odontogenic, adipogenic or myogenic pre-induction, whereas control samples received no stimulation. Also blank collagen scaffolds were implanted. The results indicated that seeded cells produced tissue within the implanted constructs after 3 weeks of implantation. According to morphological and phenotypical changes, the pre-induced DPSC showed the ability to further differentiate along odontogenic, myogenic and adipogenic pathways *in vivo*. Moreover, DPSC without pre-treatment were able to spontaneously differentiate along odontogenic and adipogenic directions *in vivo*. However, only limited mature morphological changes were detected in histology. In summary, stem cells derived from human dental pulp form a suitable source for tissue engineering and cell-mediated therapy, although additional analyses should be considered. Copyright © 2008 John Wiley & Sons, Ltd.

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1. Introduction

The occurrence of adult stem cells in dental pulp has been confirmed in different species, including humans (Gronthos *et al.*, 2000), dogs (Iohara *et al.*, 2004), rats (Zhang *et al.*, 2005) and mice (Mina and Braut, 2004). Such stem cells derived from dental pulps (DPSC) have the ability to differentiate into odontoblast-like cells, and produce hard tissue under both *in vitro* and *in vivo* conditions. Furthermore, the

cells show plasticity, i.e. the cells can differentiate into specialized lineages that are distinct from their original tissue (Pittenger *et al.*, 1999). Gronthos *et al.* (2002) reported that human DPSC could differentiate into adipocyte-like cells, which produced lipid clusters and expressed adipocyte-specific transcripts. Also, the neurogenic potential of DPSC was proven (Gronthos *et al.*, 2002). Pierdomenico *et al.* (2005) found that human DPSC derived from the dental pulp fragments without further enzyme digestion, showed *in vitro* osteogenic and adipocytic, but no chondrocytic differentiation. Another study proved that c-kit⁺/CD34⁺/STRO-1⁺ sorted cells from a primary culture of human dental pulp were able to undergo osteogenic, adipogenic and myogenic differentiation (Laino *et al.*, 2006; Pappacio *et al.*, 2006).

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In our own previous *in vitro* research, the multilineage differentiation ability of two types of DPSC was analysed (Zhang *et al.*, 2006a). It was shown that, even after prolonged cryopreservation, both human DPSCs were capable of differentiation along five directions under the stimulation of appropriate inductive media, i.e. neurogenic, osteogenic/odontogenic, adipogenic, myogenic and chondrogenic pathways. Although phenotypical changes were confirmed by immunofluorescent staining and PCR, typical morphological changes were not always detectable. At that time, we hypothesized that the *in vitro* environment was insufficient as appropriate surrounding. Moreover, limited *in vivo* data are available. Therefore, the current study was performed in order to evaluate the differentiation or maturation potential of human DPSC towards multiple lineages in an animal model. Differentiation in our study set-up is initiated in an *in vitro* culturing phase. After implantation *in vivo*, the cells have the opportunity to differentiate or mature further. We specifically aim to implant in the 'ectopic' environment of the subcutaneous tissue, which is not externally giving signals to induce differentiation of the cells. Only implantation of a construct into such an atypical environment is adequate to prove that the obtained differentiation is actually the result of the implanted cells themselves, and not from tissue ingrowth from the environment.

2. Materials and methods

2.1. Cells

In this study the same human DPSC were used as in our previous *in vitro* study (Zhang *et al.*, 2006a). All procedures were performed obeying national guidelines for working with human materials. Briefly, the coronary pulp of an impacted third molar from a 22 year-old male patient was dissected, digested by 3 mg/ml collagenase type I, and cultured in alpha-minimal essential medium (α -MEM; Gibco BRL, Life Technologies B.V. Breda, The Netherlands) with 20% fetal calf serum (FCS; Gibco BRL) and 50 μ g/ml gentamycin (Gibco BRL). Cells of passage 2 were collected and stored in liquid N₂.

2.2. Materials

As scaffold material, circular-shaped crosslinked collagen type-1 matrices with a diameter of 6 mm and a height

of 4 mm were used (Daamen *et al.*, 2003). In brief, insoluble type I collagen was purified from bovine achilles tendons, swelled in acetic acid and homogenized. Porous collagen matrices were made by freezing and subsequent lyophilization. Then, the matrices were crosslinked using 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). Scaffolds of the required shape were punched out. For initial analysis, one sample was fixed, dehydrated, dried in a critical point drier, and examined using a Jeol 6310 SEM microscope. The rest of the matrices were disinfected in 70% ethanol, washed in sterilized phosphate-buffered saline (PBS) buffer, and then incubated in α -MEM/10% FCS/50 μ g/ml gentamycin for 2 h.

2.3. Pre-induction and PCR analysis

DPSC cells were thawed, expanded in α -MEM/10% FCS/50 μ g/ml gentamycin. Upon confluency, the cells were incubated in different inductive media (Table 1) for 3 weeks. As controls, the same cells were cultured in medium without extra additives. For each differentiation pathway, cells were collected for RT-PCR analysis. The total RNA of the cells were isolated by a RNeasy Mini Kit (No. 74106; Qiagen, Hilden, Germany). PCR reactions were performed using the human-specific primers for dentine sialophosphoprotein (DSPP; sense, 5'-GGCAGTGACTCAAAGGAGC-3'; anti-sense, 5'-TGCTGTCAGTGTCACTGCTG-3'), peroxisome-proliferating activated receptor γ 2 (PPAR γ 2; sense, 5'-CCAGAGCATGGTGCCTTCGCT-3'; antisense, 5'-CA-GCAACCATTGGGTCAGCTC-3'), and muscle-specific transcription factor (MyoD1; sense, 5'-AAGCGACCTCTC-TTGAGGTA-3'; antisense, 5'-GCGCCTTTATTTTGATC-ACC-3'). As reference, GAPDH was also examined for all samples.

2.4. Cell labelling and seeding onto the scaffold

The cells were then labelled by the Vybrant™ carboxyfluorescein diacetate, succinimidyl ester (CFDA SE) fluorescent cell tracer kit (V-12883, Molecular Probes Invitrogen, Breda, The Netherlands). Briefly, the cells were incubated in a CFDA SE working solution (1 : 300) at 37 °C for 30 min. The labelled cells were then pelleted and resuspended at a cell density of 5 \times 10⁶ cells/ml.

Table 1. Culture media

Medium	Media	Serum	Supplements
Odontogenic	α -MEM	10% FCS	10 mM Na- β -glycerophosphate (β -GP), 10 ⁻⁸ M dexamethasone, 50 μ g/ml L-ascorbic acid and 50 μ g/ml gentamycin
Adipogenic	α -MEM	10% FCS	0.5 mM isobutyl-methylxanthine (IBMX), 0.5 μ M hydrocortisone, 60 μ M indomethacin
Myogenic	α -MEM	10% FCS, 5% HS*	0.1 μ M dexamethasone, 50 μ M hydrocortisone, 50 μ g/ml gentamycin
Non-inductive	α -MEM	10% FCS	50 μ g/ml gentamycin

*FCS, fetal calf serum; HS, horse serum.

The collagen matrices were seeded with cells at six scaffolds/ml suspension for 2 h, as described previously (van der Dolder *et al.*, 2003). Samples were checked by immunofluorescent microscopy immediately afterwards. The cell-loaded scaffolds were kept in the respective media overnight, washed three times with PBS buffer, and used for implantation the following day.

2.5. Implantation procedure

The cell/collagen constructs were implanted in 12 male 10 week-old BALB/C nude mice, after approval from the Radboud University Nijmegen Animal Ethics Committee (KUNDEC 2003-72). National guidelines for the care and use of laboratory animals were always obeyed. The mice were anaesthetized with 1.5–3% isoflurane, and their backs were disinfected with povidone–iodine. Four subcutaneous pockets were prepared on the dorsal side of each mouse, two at each flank. In each pocket, one implant was inserted. For each pre-induction, as well as for each analysis method, samples were implanted three-fold. Collagen materials without pre-loaded cells were used as blank controls.

2.6. Histological processing

All samples were retrieved after 3 weeks of *in vivo* implantation, as this was the maximum achievable time for detection of the fluorescent stain. Two preparation methods were followed for histology. Samples for routine histology, immunostaining and fluorescence microscopy were fixed in 4% paraformaldehyde, dehydrated in a series of ethanols, embedded in paraffin, serially sectioned, and mounted on Superfrost plus™ slides. A separate group of specimens for lipid staining and immunostaining was transported in liquid N₂ and embedded in OCT. Frozen sections were cut on a cryostat (HM 560, Microm GmbH, Germany), mounted on Superfrost plus™ slides and stored at –80 °C until use. The following analyses were performed:

1. *Vybrant™ labelling*. Non-stained paraffin sections were deparaffinized in xylol, rehydrated to water and observed by fluorescent microscopy.
2. *Haematoxylin and eosin (H&E) staining*. Paraffin sections were deparaffinized in xylol, rehydrated to water and stained with a routine H&E staining. Besides routine histology, the number of blood vessels on the samples with different pre-treatment was counted. At a magnification of ×100, a total of six fields was scored for each group, and data were compared by statistical analysis of variance (ANOVA) with *post hoc* Tukey testing.
3. *Von Kossa staining*. Paraffin slides were deparaffinized, rehydrated and subsequently incubated with fresh 5% silver nitrate (AgNO₃), exposed to ultraviolet light,

incubated in 2% sodium thiosulphate (Na₂S₂O₃) and counterstained with nuclear fast red. Sections were evaluated for calcified deposits, which stained black.

4. *Oil red O staining*. Frozen sections were fixed with 36% formaldehyde for 15 min in a 50 °C water bath, washed with MilliQ water, stained in haematoxylin and washed again. Then the slides were incubated in 2% (w/v) oil red O reagent, washed and mounted in glycerin jelly. Sections were evaluated by light microscopy for checking the lipid accumulation. For quantification, sections were only stained by oil red O and then destained in 50 µl 100% isopropanol. The optical density of the destained solution was then measured at 500 nm with a spectrometer.
5. *Immunofluorescence*. Paraffin sections were deparaffinized, rehydrated and treated with 3% H₂O₂ to quench endogenous peroxidase. Then, antigens were retrieved using a microwave. Frozen sections were warmed to room temperature and fixed in ice-cold acetone for 5 min. All slides were preincubated with 5% bovine serum albumin (BSA) and incubated in 1% BSA containing the lineage-specific antibodies. For odontogenic differentiation, DSPP (LF-148) and dentin matrix protein-1 (DMP-1, LF-151) were selected. PPAR γ 2 (P0744; Sigma) and glucose transporter-4 (GLUT4, E-20; Santa Cruz Biotechnology) were used to check adipogenic differentiation. MyoD1 (554 130; PharMingen, San Diego, CA, USA) and myosin heavy chain (MHC, ALD-58; Developmental Studies Hybridoma Bank) were chosen to analyse the myogenic differentiation. Finally, the slides were incubated with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody, and mounted with a solution containing DAPI to detect nuclei. For mouse-derived primary antibodies, MOM blocking reagent (MKB-2213, Vector Laboratories) was used. Negative controls were assessed with secondary antibody only. All samples were analysed by fluorescence microscopy.

3. Results

3.1. General observations

Visually, the collagen scaffold showed a sponge-like appearance. SEM observation indicated a combination of porous and lamellar structures on the surface of the collagen scaffold, and parallel lamellar structures inside. Visual inspection showed that the pore sizes were in the approximate range 20–100 µm.

3.2. In vitro assays before implantation

Most of the cells retained spindle-like phenotype during the 3 weeks of culture in the different inductive media. Sporadically, small nodular-like structures were seen in the odontogenic culture. After adipogenic induction, the overall cell density seemed reduced compared to all other

cultures. Also, a small number of cells showed a more amorphous morphology with an abundant cytoplasm and a centrally placed nucleus. Microscopically, no specific differences were found in the cultures in myogenic or non-inductive media (data not shown).

RT-PCR showed GAPDH expression in all groups, DSPP expression in the odontogenic culture, PPAR γ 2 was found in both adipogenic and odontogenic cultures, and MyoD1 was only expressed in the culture of the myogenic pathway. Expression of any of the specific markers was always absent in the non-induced cultures (data not shown).

After staining with the Vybrant™ tracer and loading onto the scaffolds, the cells were visible in fluorescence microscopy by a bright green colour. A homogeneous cell distribution was found on the surface of the scaffolds. Cross-sections showed an evident reduction of the cell number from the surface towards the centre of the collagen material; nevertheless, cells had always penetrated to the core of the scaffold.

3.3. Animal experimentation and histological observations

None of the mice showed abnormalities or symptoms of inflammation at the implantation sites throughout the whole test period. After sacrifice, all implants were retrieved with the surrounding soft tissues intact.

In general, microscopy showed that all implants were uniformly encapsulated with three to six layers of cells. The pores of all collagen scaffolds were completely filled with cells and extracellular matrix, which showed a morphology similar to loose connective tissue. Limited infiltration of plasma cells, with a basophilic cytoplasm and a large nucleus, was seen in all implants. Visual inspection showed more cells and extracellular matrix in implants loaded with DPSC, compared to the blank controls. No observable differences in density existed among the samples loaded with DPSC with different pre-treatments. Distinct blood vessels were observed in both peripheral and central regions of all samples loaded

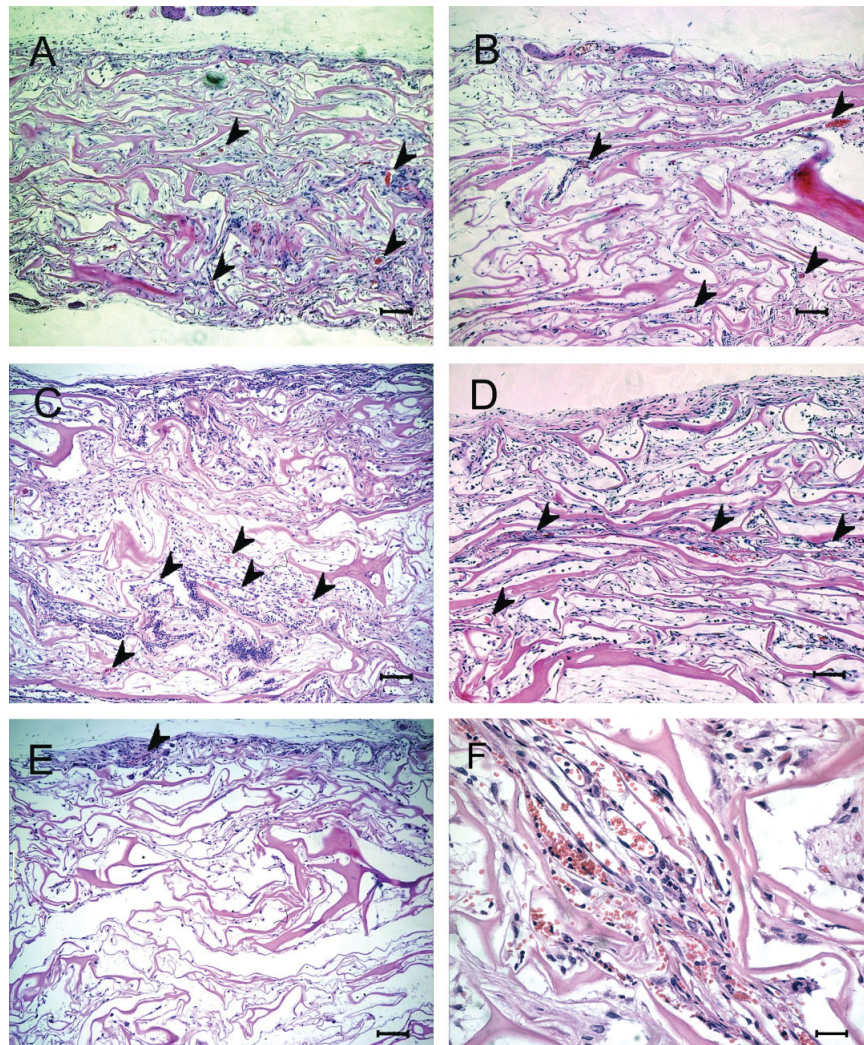


Figure 1. HE staining of implants after implantation in nude mice for 3 weeks; arrows indicate blood vessels. (A) Implant cells were treated with odontogenic medium before implantation; (B) pre-treated with adipogenic medium; (C) pre-treated with myogenic medium; (D) non-pre-treated; (E) blank scaffold without DPSC; (F) higher magnification of sample pre-treated with myogenic medium, evidencing newly formed blood vessels (bars: A–E, 100 μ m; F, 50 μ m)

with DPSC. In contrast, non-loaded blanks showed only limited angiogenesis in the marginal regions (Figure 1). Although it was not possible to measure the relationship between labelled cells and position of the observed vessels, in general no labelling was found in or directly adjacent to the vessels. Statistical analysis indicated that the number of blood vessels was on average $1.5 \pm 0.6/\text{field}$ in the non-loaded samples, whereas it was $12.3 \pm 2.2/\text{field}$ in the loaded samples. Statistical analysis with a Student's *t*-test showed this difference was significant ($p < 0.001$). However, no differences existed among the samples with differently pre-treated DPSC.

In total, approximately 5% of cells showed the cell-tracer in fluorescence microscopy. Such labelled cells were always found grouped together. These groups of cells were dispersed within the porosity of the collagen scaffold, as well as on the margin of the fibrous tissue capsules parallel to the scaffold surface (Figure 2A, B).

The Von Kossa staining showed no indication of a calcified extracellular matrix in any of the implants, even in the samples with odontogenic pre-treatment DPSC.

Oil red O staining showed abundant red-stained lipid droplets formed in the implants loaded with adipogenic pre-treated DPSC. Large amounts of lipid droplets were also found when the loaded cells received odontogenic pre-treatment, and even without pre-induction. The formation of lipid droplets was mostly occurring together with the cell tracers-positive cells (Figure 3). Still, limited amounts of lipid droplets were found around the implants with myogenic cells or without DPSC. Such observations were confirmed by the quantitative destaining measurement of oil red O staining. In such measurement, the adipogenic group showed significantly higher values than all the other groups. Odontogenic and non-pre-treated groups were similar, and significantly higher compared to both the myogenic and non-cell loaded blanks (Figure 3).

For the myogenic pre-treatment, myofibre-like structures were occasionally found within the scaffolds. In one out of three implants, distinct muscle fibres were found, which were located both at the dorsal and ventral sides of the implant (Figure 4). Cross-sections indicated that these myofibres had various diameters in the range 3–30 μm .

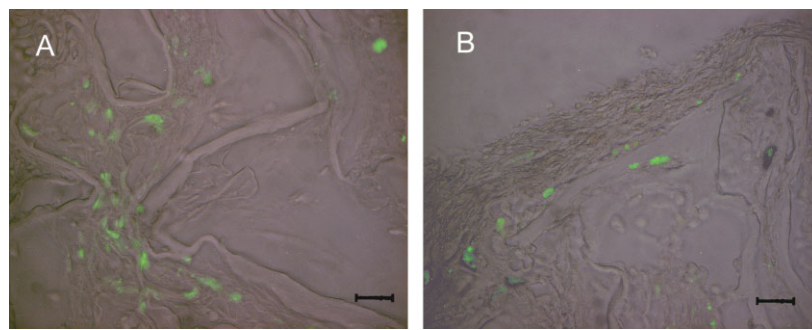


Figure 2. Cell distribution in implants after 3 weeks of implantation. Each figure was made by merging a fluorescent micrograph with an optical micrograph. Implanted cells were green. (A) Cell distribution in porosity; (B) cell distribution in the fibrous tissue capsules (bar: 25 μm)

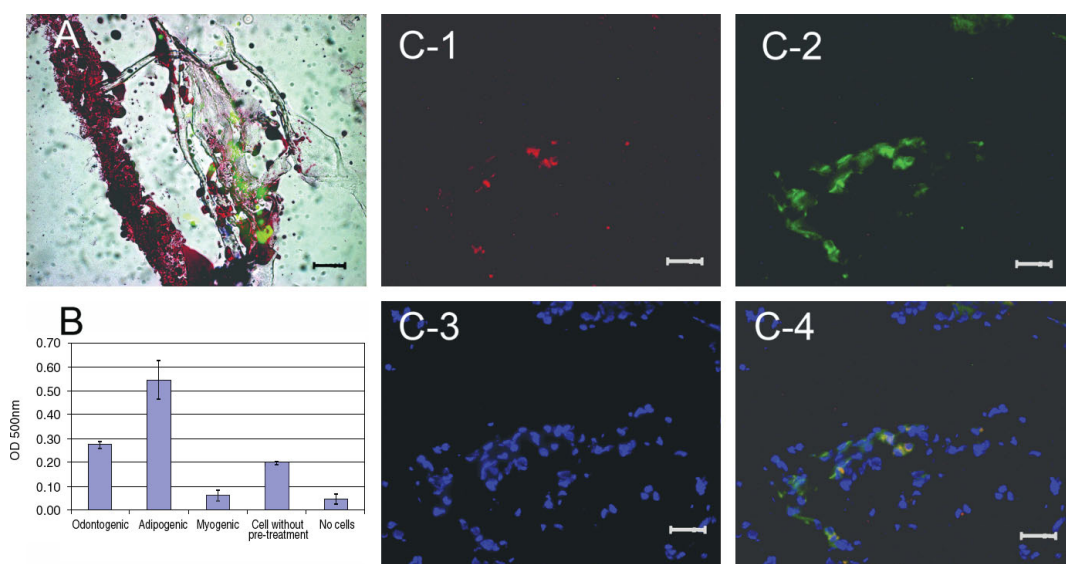


Figure 3. Adipogenic differentiation. (A) Oil red O staining of implant with adipogenic pre-treatment (bar: 25 μm). (B) Quantitative assessment of oil red O staining. C1, immunofluorescent staining of PPAR γ 2 (positive cells red); C2, cell-tracer (positive cells green); C3, routine DAPI staining (cell nuclei blue); C4, merged image of C1–3 (bar: 25 μm)

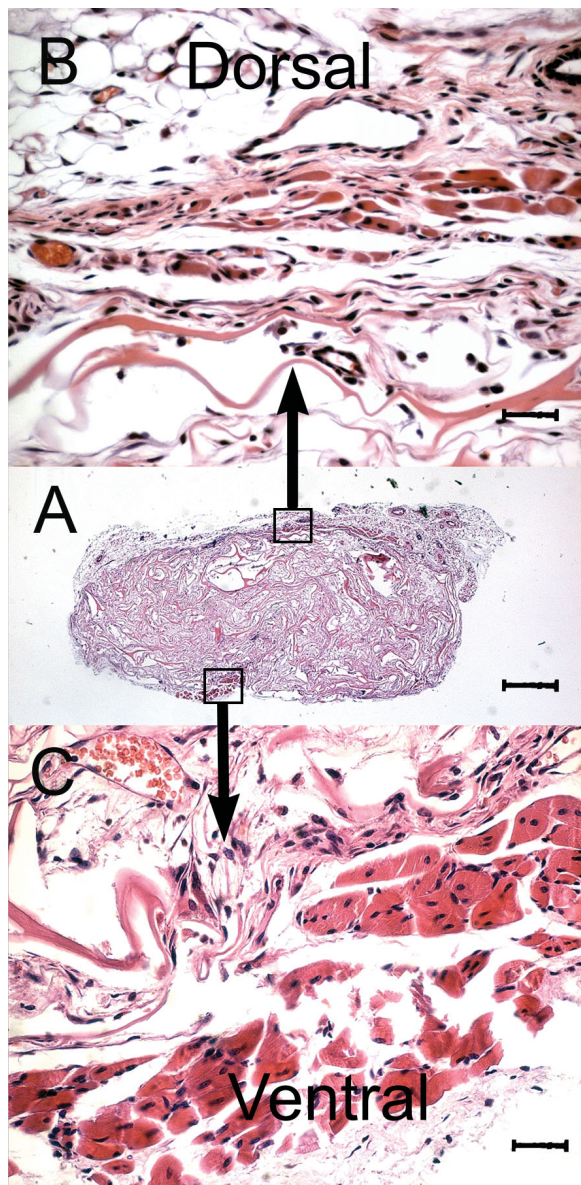


Figure 4. HE staining showing the formation of muscle fibres. (A) Overview (bar: 625 μm); (B) higher magnification of dorsal side (bar: 25 μm); (C) higher magnification of ventral side (bar: 25 μm)

Some of the myofibres were striated with subsarcolemmal nuclei, although the majority exhibited a central nucleus.

3.4. Immunofluorescent staining

In general, the expression of the specific immunological markers coincided frequently, but not always with the Vybrant™ labelling. After odontogenic induction, the expression of DSPP (Figure 5) and DMP-1 (data not shown) was identified. Limited staining was also found in samples without pre-induction. However, none of the positive cells showed the typical columnar shape of naturally occurring odontoblasts.

In the implants with DPSC, which received adipogenic pre-treatment, PPAR γ 2 (Figure 3) and GLUT4 (data

not shown) were found. Still, corroborating the *in vitro* RT-PCR results described above, positive staining of both adipogenic markers was also found to lesser extent in the odontogenic pre-treatment group, as well as in the DPSC without pretreatment.

Also, in the DPSC with myogenic pre-induction MyoD1 and MHC were detected. The positive cells had formed myotube-like structures with multinucleated cells (Figure 6).

Finally, immunofluorescent staining was done on cell-free implanted scaffolds. No positive staining for DSPP, DMP, PPAR γ 2, MyoD1 or MHC was found. We did see a small amount of positive staining for GLUT4 in some samples, believed to be caused by cross-reaction of the GLUT4 antibody with mice tissue.

4. Discussion

Previously, the multilineage differentiation potential of adult stem cells derived from human dental pulp was demonstrated by incubating such cells in culture media with specific additives and subsequently identifying lineage-specific changes according to morphological and phenotypical criteria (Zhang *et al.*, 2006a). However, the *in vitro* and *in vivo* situations are unquestionably different environments. In addition, the RT-PCR or immuno-methods as used in these studies to monitor the changes of cell phenotype can only distinguish the expression of specific markers but are unable to prove functional changes. For these reasons, the current study analysed the *in vivo* differentiation potential of human dental pulp stem cells stimulated towards odontogenesis, adipogenesis or myogenesis. Based on our previous study (Zhang *et al.*, 2006a), a single patient's tooth pulp was chosen as a cell source for this study. Hence, we cannot consider the possible variations which can occur from patient to patient. Still, the *in vitro* part of our study again confirmed that specific markers were detectable by RT-PCR, suggesting differentiation of the cells towards particular lineages. Moreover, the expression of PPAR γ 2 in the odontogenic culture even suggested that the DPSC could differentiate into adipocyte-like cells under the influence of an odontogenic medium. Nevertheless, no typical morphological changes towards typical odontoblast, adipocyte or myoblast phenotypes were observed, which was in accordance with previous experiments.

In further *in vivo* experimentation, these pre-treated cells were transplanted into a nude mouse model. For this, first a suitable scaffold had to be chosen. Collagen type I is widely used for repair and regeneration of different tissues, such as bone (Xiao *et al.*, 2003), muscle (Luameechanporn *et al.*, 2006), cartilage (Buma *et al.*, 2003) and liver (Hansen *et al.*, 2006). Furthermore, earlier results indicated that using a cross-linked collagen type-I scaffold not only supported the growth and differentiation of DPSC (Zhang *et al.*, 2006b), but also

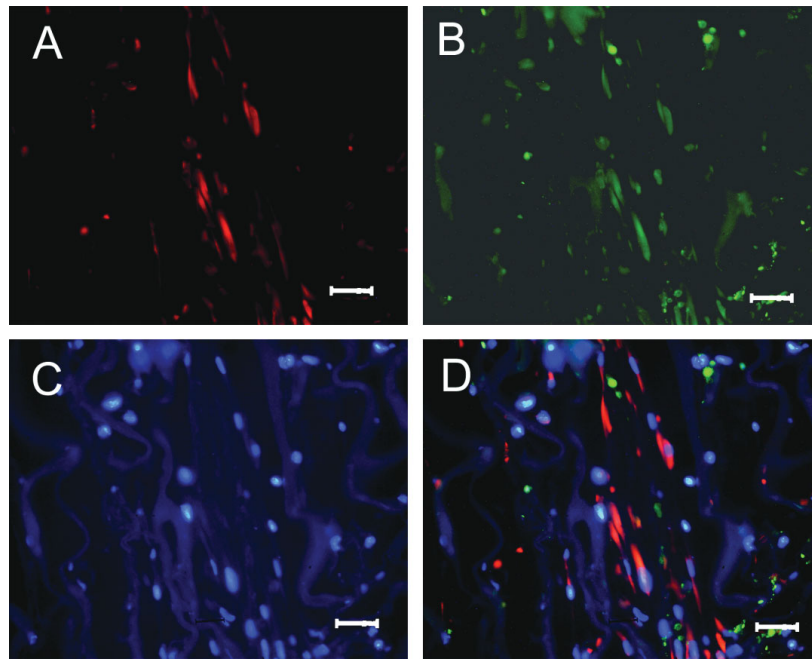


Figure 5. Osteogenic differentiation. (A) Immunofluorescent staining of DSPP (positive cells red); (B) cell tracer (positive cells green); (C) routine DAPI staining (cell nuclei blue); (D) merged image of A–C (bar: 25 μ m)

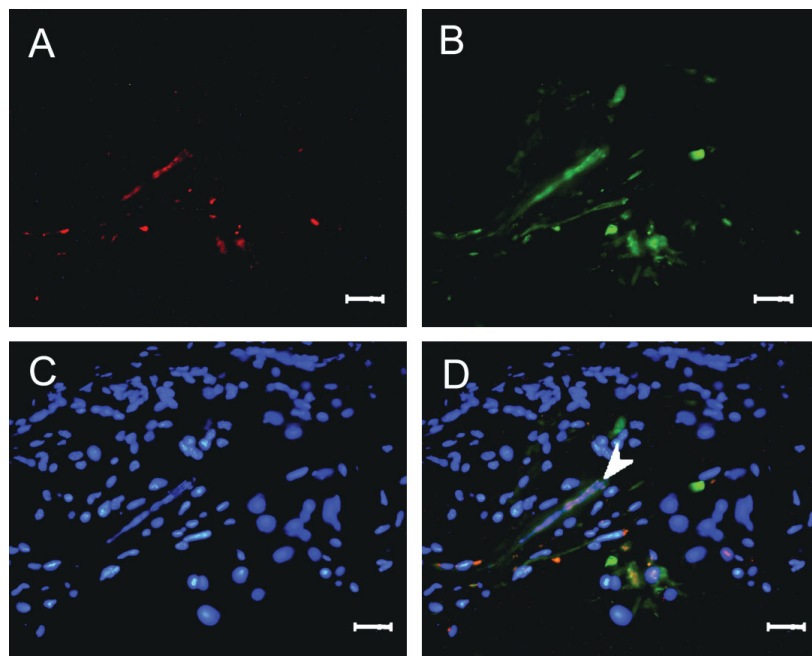


Figure 6. Myogenic differentiation. (A) Immunofluorescent staining of MHC (positive cells red); (B) cell tracer (positive cells green); (C) routine DAPI staining (cell nuclei blue); (D) merged image of A–C (bar: 25 μ m) Arrow indicates a myotube-like structure

allowed the ingrowth of blood vessels *in vivo* (Pieper *et al.*, 2000). Therefore, in the current experiment again a cross-linked collagen type I scaffold was used.

Another issue of technical nature was the selection of the implantation site. The subcutaneous area is rich in fatty tissue, so adipogenic differentiation is to be expected here. Similarly it might have been argued to place the other constructs in their particular environments tissues, too. For instance, previous studies reported that stem cells derived from bone marrow were

able to differentiate into and function like hepatocytes (Lagasse *et al.*, 2000) or kidney cells (Kale *et al.*, 2003) after transplantation into the respective target organs. However, such observations will always be controversial, since other investigations suggested that such newly formed functional cells might have arisen from cell fusion between the transplanted stem cells and available progenitors within the host tissue (Wang *et al.*, 2003). In order to avoid any influence from host cells, the subcutaneous area is regarded as an appropriate recipient

site for ectopic tissue regeneration. It has been shown previously that subcutaneous implantation is a suitable model environment for testing hard tissue regeneration by stem cells (Ohgushi *et al.*, 1993). Furthermore, satellite cells isolated from muscles could form skeletal muscles when they were placed in subcutaneous tissue (Irintchev *et al.*, 1998), which suggested that the subcutaneous location is also an appropriate site for myogenesis.

A final technical decision was the selection of the differentiation pathways that were to be included in this study. In our previous *in vitro* studies, osteogenic/odontogenic, adipogenic, myogenic, neurogenic and chondrogenic pathways were examined. It was evident that neurogenic differentiation occurred in an essentially different time path than the other types of differentiations (hours vs. weeks). Furthermore, it is already known that DPSC showed differentiation towards the neurogenic pathway when injected into the dentate gyrus of the hippocampus of immunocompromised mice (Miura *et al.*, 2003). For these reasons, we excluded the investigation towards the neurogenic pathway in this study.

The chondrogenic pathway initially was also part of the current study. First, an *in vitro* pellet culture method was used as described previously (Johnstone *et al.*, 1998). In order to be able to retrieve the pellets, they were inserted in a porous collagen disk and then implanted. However, upon retrieval no remaining pellets could be found. Since it was not clear whether the pellets had degraded or we were unable to locate their exact position, we chose not to present these efforts in our study description.

In our study, we labelled cells with the Vybrant™ CFDA SE cell tracer. This cell tracer is suitable for both *in vitro* and *in vivo* studies; since the label can be inherited by daughter cells after cell division and cell fusion. However, it cannot be transferred to adjacent cells (Bronner-Fraser, 1985). Those properties made it a useful tool to follow specific cells over time. In many cases we saw colocalization of the cell tracer's green fluorescence together with the specific labels, thus leading us to the general conclusion that the DPSC are capable of differentiation to the pathways described. However, this is the only conclusion that can be made, and whether a contribution of host cell differentiation exists cannot be documented for practical reasons with the applied technique. The intensity of the fluorescence will decrease over time (due to regression of the label, or to cell division), thus making it impossible to prove that a non-fluorescent cell is actually a host cell, and thus to quantify host vs. donor cells. As mentioned, however, the dye is retained by the cells throughout development but is not transferred to adjacent cells in a population. This means that, although we cannot prove that non-labelled cells are from donor or host origin as mentioned above, we can still be 100% certain that labelled cells are donor cells.

In general, immunofluorescent staining did not always correlate exactly with the green colour of the cell tracer. Some cells only showed positivity for lineage specific

markers, but were negative for the cell tracer; whilst not all the cell tracer-positive cells showed the lineage-specific differentiation markers. These results suggested that some cells might have lost the cell tracer after several cell division cycles, and of course also that not all loaded cells will differentiate towards the indicated lineage.

Regarding the study outcomes on odontogenic induction, the expression of DSPP and DMP-1 indicated that DPSC retained odontogenic differentiation *in vivo*. Interestingly, DSPP and DMP-1 were also found to lesser extent in implants loaded with the non-induced cells, which demonstrated that DPSC exhibited the potential of spontaneous odontogenesis. Still, none of the cells which expressed the markers showed the typical columnar morphology of an odontoblast. Furthermore, no obvious mineralization of the extracellular matrix was observed in any of the implants. These study results corroborate our earlier results (Zhang *et al.*, 2006b). Still, Gronthos *et al.* (2002) showed that DPSC could produce a dentine–pulp complex-like tissue *in vivo*. The main difference with that study set-up is they used a calcium phosphate particle/cell pellet model. In our study, the DPSC were seeded in porous materials. Proof of actual mineralization of newly formed tissue by DPSC still has to be established, conceivably using direct pulp-capping experiments (Tziafas *et al.*, 2001) or through the external addition of growth factors, such as BMP2 in the *in vitro* culture phase (Iohara *et al.*, 2004).

With regard to the results for the adipogenic differentiation, again it was evident that the PPAR γ 2 and GLUT4 adipoblast markers were also detectable in implants of the odontogenic, and not pre-induced, cultures. This suggests that DPSC intends to differentiate towards adipogenic direction *in vivo*. Expression of the markers in these cases usually coincided with evident morphological changes. Comparatively, more oil red O-positive lipid droplets were found in the implants with adipogenic pre-induction. All the other experiments confirmed that DPSC could differentiate into adipocyte-like cells. The presence of oil red O staining in adipogenic pre-induced and non-pre-induced cells is likely to be indicative of the subcutaneous environment inducing and maintaining differentiation of the DPSC. The presence of oil red O staining in the odontogenic pre-induced cells again likely highlights the subcutaneous environment causing redifferentiation of the cells.

For the myogenic differentiation pathway, premature as well as matured myofibres were found in one of the samples directly adjacent to the collagen scaffold. It might be argued that such fibres arose from the musculature adjacent to the implant site, and that they were inadvertently retrieved during the explantation procedure. On the other hand, such myofibres were only observed in the samples with myogenic medium pre-treated DPSC and none of the explants in the four other groups. Second, most of the myofibres contained central nuclei, which indicated that they are newly developing structures (Carlson and Faulkner, 1983). Also, fibres were found inside the more central

regions of the scaffold, albeit in limited quantity. The immunofluorescent staining verified the presence of DPSC inside the multinuclear myotube-like structures. Moreover, expression of myogenic markers for muscle formation was identified exclusively in the cultures with myogenic pre-treatment.

Finally, the implanted DPSC enhanced angiogenesis, as statistically more blood vessels were found in all the samples loaded with DPSC. Still, it is not clear whether this enhanced blood vessel ingrowth or formation is a specific property of the DPSC, or could be induced also when other types of cells would have been loaded onto the scaffold. Further investigation should be performed to clarify this.

5. Conclusions

In conclusion, it can be stated that the dental pulp contains a population of cells, which have multilineage differentiation potential, even without cell selection and after cryopreservation. After culture in inductive media, and transplantation into immunocompromised mice, stem cells derived from human dental pulp tissue in some measure were able to further differentiate or mature towards odontogenic, adipogenic and myogenic specific lineages. Without pre-induction cells also exhibited a (limited) potential to differentiate toward odontogenic and adipogenic pathways. Dental pulp seems a suitable source of multipotent stem cells, applicable for autologous tissue regeneration and cell-based therapies. Still, additional analysis should be performed, since only limited amounts of typically matured morphological changes were identified.

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