

# OSTEOGENIC DIFFERENTIATION OF HUMAN DENTAL PULP-DERIVED STEM CELLS UNDER VARIOUS EX-VIVO CULTURE CONDITIONS

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**Summary:** Dental pulp stem cells (DPSCs) can be easily isolated and cultured in low-serum containing medium supplemented with growth factors PDGF-BB and EGF while exhibiting multipotency and immature phenotypic characteristics. In the present study, we investigated their potential to differentiate towards osteogenic lineages using various culture conditions in order to optimize their therapeutic use. DPSCs were cultured either as a cell monolayer or as three-dimensional (3D) micro-mass structures. Monolayers preincubated with bFGF and valproic acid for one week prior their differentiation were cultured in serum containing standard osteodifferentiation medium for four weeks, which resulted in multilayered nodule formation. Micro-mass structures were cultured for same period either in serum containing medium or under serum-free conditions supplemented with TGF- $\beta$ 3 with or without BMP-2. Histochemically, we detected massive collagen I and weak calcium phosphate depositions in multilayered nodules. When culture 3D-aggregates in either standard osteodifferentiation medium or serum-free medium containing TGF- $\beta$ 3, only small amount of collagen I fibres was observed and almost no deposits of calcium phosphate were detected. In contrast, in presence of both TGF- $\beta$ 3 and BMP-2 in the serum-free medium a significant amount of collagen I fibers/bundles and calcification were detected, which is in line with osteogenic effect of BMP-2. Thus, our data indicate that certain environmental cues can enhance differentiation process of DPSCs into osteogenic lineage, which suggest their possible utilization in tissue engineering.

**Key words:** Dental pulp stem cells; Osteogenic differentiation; Valproic acid; BMP-2, Micro-mass culture

## Introduction

Research in cell-based tissue engineering applications remains focusing on identifying an ideal cellular source. Dental pulp entrapped within dental cavity preserved by hard mineralized crown of developing tooth represents a promising alternative source of stem cells due to its relative easy removal ability and immaturity of its cells.

The existence of stem cells within dental pulp of third molars was firstly demonstrated by Gronthos and colleagues (7). Upon isolation and culture in high-serum containing medium, rapidly proliferating dental pulp stem cells (DPSCs) had the capacity to differentiate, not only into odontoblasts, but also into other mesenchymal derivatives such as adipocytes and osteoblasts (6, 7, 17). Afterward, a wider potential of DPSCs was established by differentiating them into cells harbouring chondroblastic, neuronal, endothelial and melanocytic phenotypes (1, 11, 21, 23). Their broad range of differentiation may be related to the cranial neural crest-cell origin of the dental pulp (2). Osteoblastic potential of DPSCs was demonstrated both in

vitro and in vivo, i.e. upon transplantation, highlighting a certain similarity with bone marrow-derived mesenchymal stromal cells (17, 21). Pre-selecting cells on the base of expression of certain markers may increase the yield of DPSCs differentiating into osteoblasts with ability to generate woven bone in vitro and in vivo (12, 13). When used this approach DPSCs were even demonstrated to repair successfully mandible bone defects when co-grafted with collagen sponge in human (5), which suggests DPSCs could be optimal alternative source of cells in bone replacement therapies.

Recently, we demonstrated that isolated DPSCs from impacted third molars could be efficiently expanded in a low-serum (2%) containing medium supplemented with epidermal growth factor (EGF) and platelet-derived growth factor BB (PDGF-BB) over Hayflick's limit while maintaining genetic stability (22). Moreover, their phenotypic analysis showed that they exhibit antigenic properties of both mesenchymal stromal and neural stem cells, and express markers characteristic of pluripotent embryonic stem cells, which reflected their multipotential capacity (Karba-

nova et al., manuscript submitted). Here we present several cultivation approaches having enhancing effect on osteodifferentiation, which is manifesting in increased collagen production and mineralization of extracellular matrix.

## Material and Methods

### *Cell isolation and culture*

DPSCs were isolated from impacted third molars of healthy young donors (aged 17–23 years;  $n = 9$ ) undergoing tooth extraction from orthodontic reasons (22). [All tissue materials were acquired under the informed consent and processed according to guidelines approved by the Ethical Committee of the Faculty Hospital in Hradec Králové]. DPSCs were isolated as described previously (7, 22). Briefly, the removed pulp tissue was digested with a mixture of collagenase (0.2 mg/ml; Sevapharma, Prague, Czech Republic) and dispase (2 mg/ml; Gibco, Paisley, UK) for 70 min at 37 °C, mechanically dissociated and filtered through a 70- $\mu$ m cell strainer (BD Falcon). Dental pulp cell suspensions were cultured in a basic expansion medium consisting of  $\alpha$ -MEM medium (Gibco), 50 nM dexamethasone (Sigma), 0.2 mM ascorbic acid 2-phosphate (Sigma), 2 mM L-Glutamine (Gibco), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Gibco) 2% fetal calf serum (FCS; PAA Laboratories, Linz, Austria), 10 ng/ml human recombinant epidermal growth factor (EGF; PeproTech, London, UK) and 10 ng/ml human recombinant platelet-derived growth factor (PDGF-BB; PeproTech). The medium was changed after 2 or 3 days. Upon reaching 70% confluence cells were passaged and split 1 to 3.

### *Osteodifferentiation*

Dental pulp-derived cells were initially cultured in basic expansion medium for at least one passage prior differentiation. The latter process was performed on cells growing either as a monolayer (2D) or in the three-dimensional (3D) structure. DPSCs, growing as a monolayer, were preincubated in basic expansion medium supplemented with 2 mM valproic acid (Sigma), 10 ng/ml human recombinant basic fibroblast growth factor (bFGF; PeproTech) and 1% ITS supplement (Sigma) for 1 week. Afterward, they were cultured in standard osteodifferentiation medium [ $\alpha$ -MEM, 10% FCS, 0.2 mM ascorbic acid 2-phosphate, 10 mM  $\beta$ -glycerophosphate (Sigma) and 0.1  $\mu$ M dexamethasone] for 4 weeks.

DPSCs growing as micro-mass high-density cell culture, which was generated by spontaneous cell aggregation ( $10^6$  cells) in rounded bottom 4 ml-culture tube, were differentiated using either standard osteodifferentiation medium or serum-free medium [high-glucose DMEM (Gibco), 100  $\mu$ g/ml sodium pyruvate (Gibco), 40  $\mu$ g/ml proline (Sigma), 0.1  $\mu$ M dexamethasone, 0.2 mM ascorbic acid 2-phosphate, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin, 1% ITS+1 supplement (Sigma) and 10 ng/ml human recombinant transforming growth factor (TGF- $\beta$ 3; R&D systems,

Minneapolis, MN)] containing or not 50 ng/ml human recombinant bone morphogenetic protein (BMP-2; PeproTech). All media were exchanged every 2–3 days.

### *Immunocytochemistry*

Cells growing on polyornithine/fibronectin-coated coverslips were washed three times in PBS, fixed with 4% paraformaldehyde (PFA) in PBS for 10 min at 4 °C followed by methanol/acetone for 5 min at -20 °C. After thorough washing with PBS, cells were blocked in PBS containing donkey serum (Jackson ImmunoResearch Labs, West Grove, PA) and then incubated with mouse monoclonal antibody (Ab) directed against procollagen type I (1:4; clone M-38; Developmental Studies Hybridoma Bank; Iowa City, IA) overnight at 4 °C. As a negative control, primary Ab was omitted. After washing, cells were incubated with Cy3<sup>TM</sup>-conjugated AffiniPure Donkey anti-mouse IgG (H+L; Jackson ImmunoResearch Labs) secondary Ab for 45 min at room temperature. Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI; Sigma). Coverslips were mounted in polyvinylalcohol/glycerol containing the anti-fading agent 1,4-diazobicyclo-[2.2.2]-octane (DABCO). Samples were examined with BX51 Olympus microscope equipped with Olympus DP71 digital camera, and the images were prepared using Adobe<sup>®</sup> Photoshop and Illustrator software.

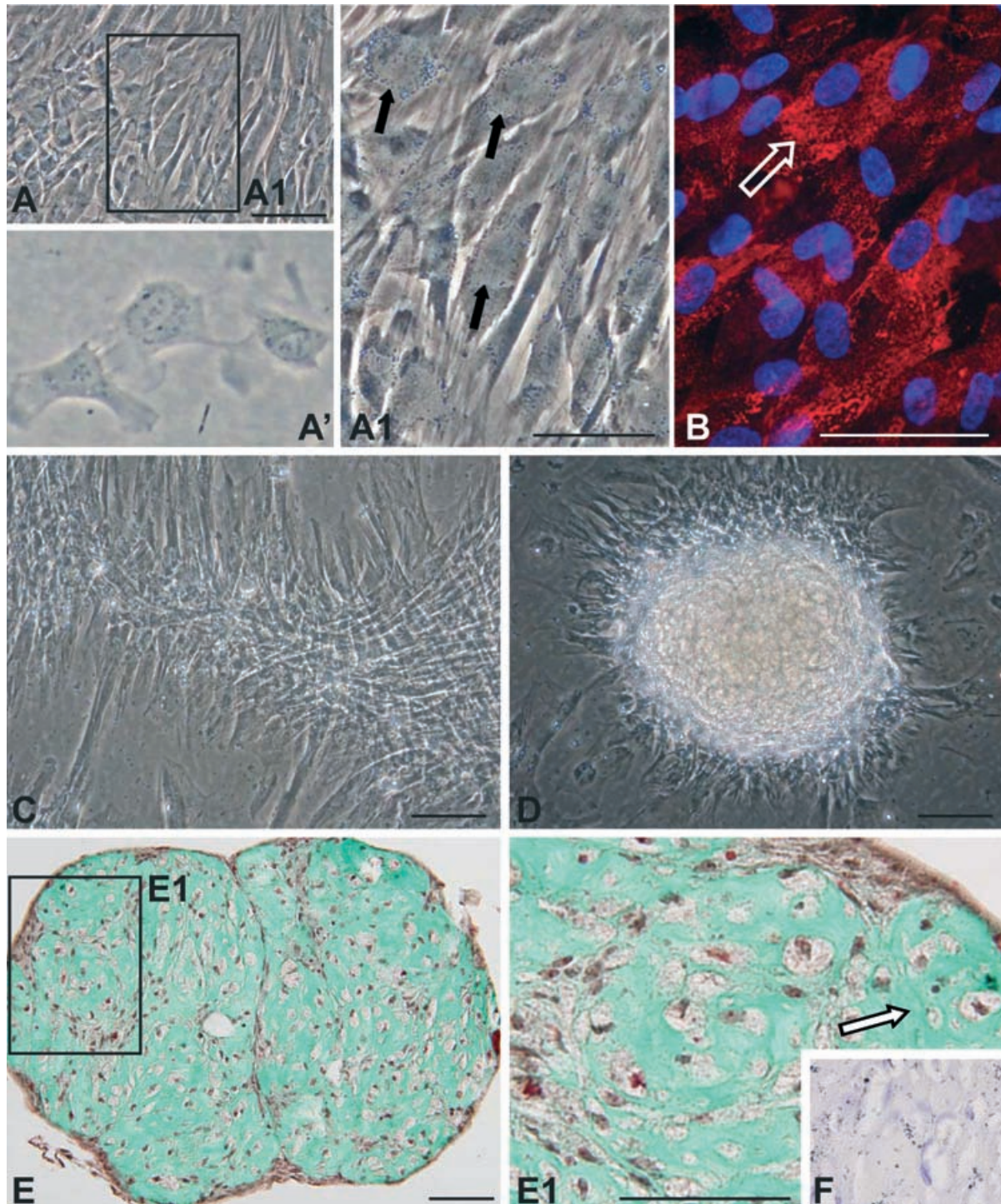
### *Histochemistry*

Cells growing either as micro-mass cultures or multilayered nodules, which were generated upon 30 days of cultivation as monolayer, were fixed with 10% formalin and embedded in paraffin. Serial sections (5–6  $\mu$ m) were cut, mounted on glass slides pretreated with chrome alum-gelatin, and dried overnight at room temperature. Paraffin-embedded sections were deparaffinized by xylol treatment, hydrated with decreasing concentrations of ethanol (96, 80 and 70%), and then rinsed twice with distilled water. Collagen I fibers were detected using Goldner's trichrome staining whereas calcium phosphate deposits with von Kossa staining. Tissues were then dehydrated and mounted in DPX (Fluka; Darmstadt, Germany).

## Results

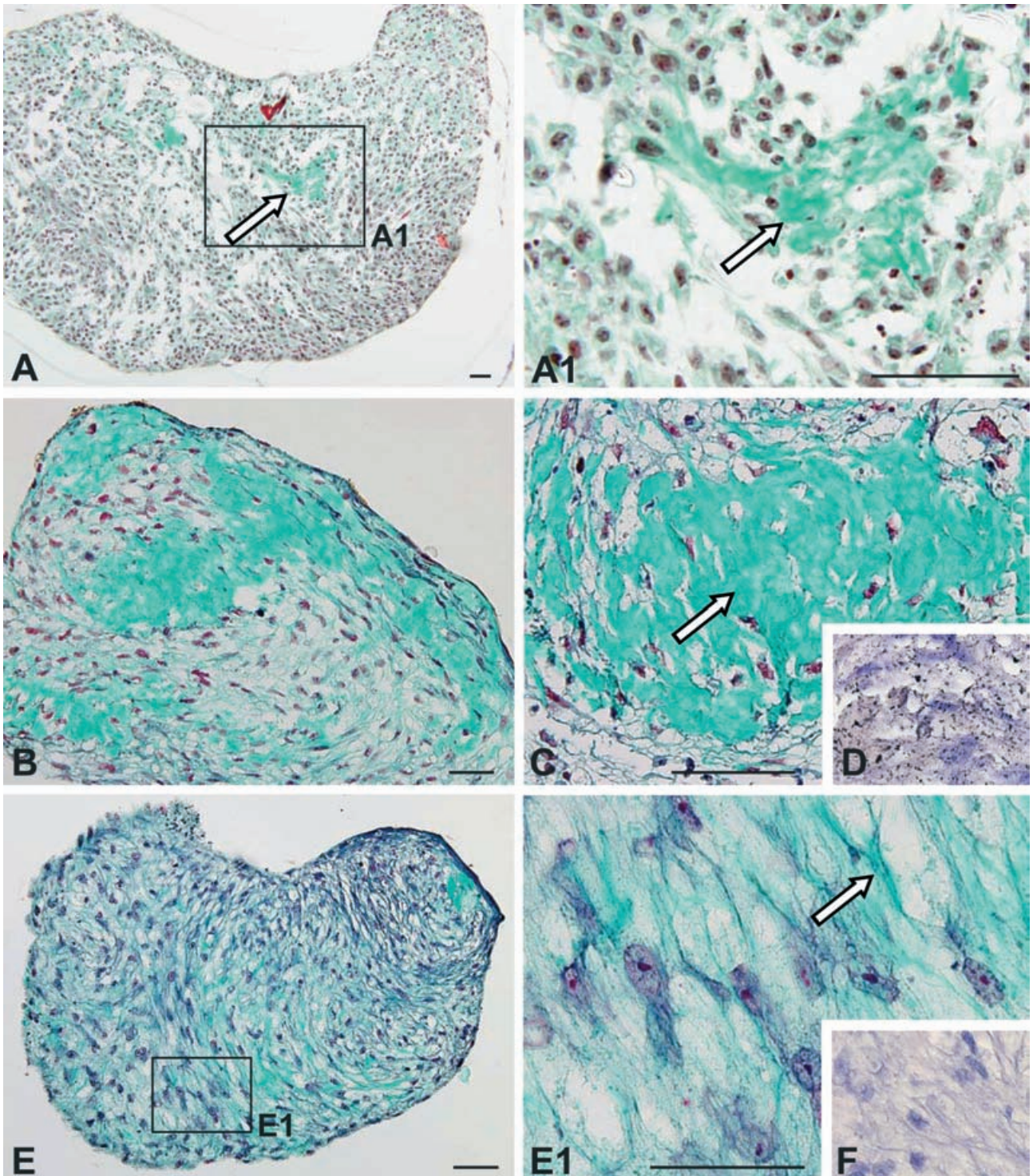
Dental-pulp-derived stem cells were maintained and expanded in low-serum containing medium as described (J.K. et al., manuscript submitted). Here, we evaluated and more importantly dissected their properties to differentiate into osteoblasts when cultivated in 2D or 3D cultures. First, for the monolayer cell culture, they were preincubated with valproic acid and bFGF, which have a beneficial effect on osteodifferentiation (4, 16). Interestingly, non-confluent cells cultured under the latter conditions continued in proliferation and generated a fully confluent monolayer. After 10 days in osteodifferentiation medium, drastic morphological alterations of cells were observed, e.g. their cell bodies





**Fig. 1: Osteodifferentiation of DPSCs in monolayer culture.** DPSCs pre-incubated with bFGF and valproic acid were further cultured in standard osteodifferentiation medium. Cells were observed by phase contrast microscopy either before differentiation (A') or after 10 (A), 20 (C) and 30 (D) days or immunolabeled for procollagen I after 10 days and observed by fluorescent microscopy (B). Collagen I fibers and calcium deposits generated in 30-day-old multilayered nodules (D) were detected using Goldner's trichrome (E, green) and von Kossa staining (F, black), respectively. Insets in panels A and E demarcate regions shown at higher magnification in A1 and E1, respectively. Black arrows indicate opaque cell regions (A1), open arrow points out the procollagen I immunoreactivity (B), and white arrow indicates collagen I fibers/bundles (E1). Scale bar: 50  $\mu$ m.





**Fig. 2. Osteodifferentiation of DPSCs growing in micromass cultures.** DPSCs were cultured for 4 weeks as micro-mass bodies either in standard osteodifferentiation medium (A) or in serum-free media supplemented with either TGF- $\beta$ 3 and BMP-2 (B-D) or TGF- $\beta$ 3 only (E, F) and then processed for paraffin-embedded sections. Collagen I fibers were detected using Goldner's trichrome (A-C, E; green, white arrow) and calcium phosphate deposits by von Kossa staining (D, F; black). Sections were counterstained with hematoxylin. Note that in the presence of TGF- $\beta$ 3 alone no deposition of calcium phosphate was detected. Insets in panels A and E demarcate regions shown at higher magnification in A1 and E1, respectively. Scale bar: 50  $\mu$ m.

became more polygonal and opaque (Fig. 1A, black arrow; for comparison see non-induced (control) cells panel A'). Furthermore, we could demonstrate by immunocytochemistry that the central areas exhibited procollagen I deposits (Fig. 1B, empty arrow). After 20 days, cells started to congest, retract and they exhibited characteristic hill- and valley-growth pattern (Fig. 1C). After 30 days, multilayered nodules were formed (Fig. 1D). Histological examination showed massive deposition of collagen I that was organized in thick bundles and well-formed lacunae around cell bodies (Fig. 1E). Only a weak deposition of calcium phosphate was detected (Fig. 1F).

Second, we evaluated osteodifferentiation properties of DPSCs in 3D-structures, i.e. growing as micro-mass high-density cell culture arising by their self-aggregation in the presence or absence of specific additives. Under all culture conditions, cells generated rounded or oval structures. Their histological examination showed differences either in amount of collagen I secreted, either in layout of cells versus collagen fibers. In standard osteodifferentiation medium only small amount of collagen I fibers was observed, localized mainly in the central part of the micro-mass bodies (Fig. 2A, 2A1, white arrow). Collagen I fibers were randomly distributed between cells with no specific lacunae containing cells. In these regions, only weak calcifications were found. When cultured in serum-free media supplemented with TGF- $\beta$ 3 and BMP-2, a massive deposition of collagen I was found throughout the whole bodies (Fig. 2B). Collagen I fibers were grouped to thick bundles (Fig. 2C, white arrow) surrounding lacunae with cells, resembling intramembranous ossification. Interestingly, significant deposits of calcium phosphate were detected (Fig. 2D). In contrast, when medium was supplemented solely with TGF- $\beta$ 3, collagen I was detected only regionally as fine collagen I fibers distributed along or between cell bodies (Fig. 2E, E1, white arrow). No calcification was detected (Fig. 2F).

## Discussion

In the present study, we demonstrate that the osteogenic differentiation of DPSCs cultured under low-serum culture conditions could be induced in both micro-mass and monolayer cultures when defined and proper additives were present. Specifically, most efficient differentiation was observed in the monolayer culture system where secreted and deposited collagen I occupied more than 90 % extracellular matrix. For micro-mass cultures the best outcomes were obtained upon supplementation of TGF- $\beta$ 3 and BMP-2. In a more general note, both culture system approaches meet some advantages or disadvantages. For instance, cultures in 3D-objects, micro-mass bodies, allow proper cell-cell interaction, closely imitating *in vivo* situation. However, the availability of nutrients in centrally positioned cells decreases proportionally with the distance to the surface resulting in starving of cells and rarely cell death. In monolayer cul-

ture, although all cells have an equal access to osteoinductive cues and nourishment from media they can hardly reach proper morphological organization of the bone.

It has been previously demonstrated that valproic acid, an inhibitor of histone deacetylase, induces, for instance, differentiation of neural progenitors to neurons (9) or stimulates osteogenic differentiation of mesenchymal stromal cells (4, 20). Interestingly, the pre-incubation with valproic acid induced morphological and growth changes in DPSCs resulting in retraction and spontaneous formation of multilayered nodules. Such cell dynamic process was not observed without pre-treatment nevertheless similar growing pattern was previously reported for DPSCs cultured under high-serum conditions (14) or cultures of perivascular pericytes (19). In addition bFGF, which is known for its effect potentiating osteogenesis in mesenchymal stem cells (16, 18), seems to have synergic effect with valproic acid on osteodifferentiation. Furthermore, the sequential retraction of cells and collaring of new cells to nodule surface allow subsequent cell differentiation and production of collagen and that together might make differentiation successful.

We also demonstrated that osteogenic differentiation could be induced as well in serum-free medium supplemented with TGF- $\beta$ 3 and BMP-2, two factors of the TGF- $\beta$  superfamily playing a role in osteo- and chondrogenesis during development (3). TGF- $\beta$ 3 is generally known to activate transcription, synthesis and secretion of extracellular proteins including collagen, fibronectin and proteoglycans, and to stimulate their incorporation in extracellular matrix (10) whereas BMP-2 was shown to have an osteogenic differentiation potential on bone marrow stromal stem cells (8). For DPSCs, BMP-2 seems to be responsible for the osteogenic effect due to extensive collagen I production and characteristic cell arrangement. On the other hand, samples incubated with TGF- $\beta$ 3 only generated minimal amount of collagen I and lacked any calcification. Indeed, TGF- $\beta$ 3 was reported to have a major chondrogenic effect on bone marrow stromal stem cells (15). In the case of DPSCs, we could observe (in some regions) a chondroblast-like phenotype with characteristic acid mucin-rich extracellular matrix (Karbanova et al, manuscript submitted).

Dental pulp was previously reported to originate from ectomesenchyme of cranial neural crest (2). It is supposed that DPSCs isolated from this tissue will maintain neural crest properties. As the cranial neural crest during development gives rise predominantly to facial bones and cartilages, it is not surprising, that DPSCs successfully differentiate into osteoblasts *in vitro* (as shown in this work) or *in vivo* after cell transplantation (17).

It is important to note that DPSCs can differentiate also into odontoblasts. Generally, there are only minimal differences in differentiation process of stem cells into osteo- and odontoblasts concerning composition of extracellular components, however, morphologically there is huge difference in the organization of cells and produced extracellular matrix. Successful differentiation of DPSCs with proper

cell/matrix arrangement was reached only in vivo after cell transplantation together with hydroxyapatite/tricalcium phosphate powder (7). It is highly probable that providing similar spatial environment to DPSCs allowing them to recede after dentin formation might trigger odontoblast differentiation even in vitro.

## Conclusion

Taken together we showed that DPSCs isolated and cultured under low-serum conditions in the presence of appropriate differentiation cues can generate osteoblasts in vitro in both 2D and 3D conditions. Due to the relatively easy accessibility of its source in the tooth DPSCs could become suitable source of cells for tissue engineering.

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