CHAPTER 3

Restorative Applications for Dental Pulp Therapy

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Summary

Dental pulp therapy of teeth degraded by caries, dental wear lesions (abrasion, attrition, erosion) or trauma is currently limited to conventional restorations such as pulp capping or root canal therapy. Therefore, dental research focusing on strategies for vital pulp therapy might open a promising alternative to the removal of the whole pulp by maintaining the function of the tooth. The implementation of a medical device in dental and oral tissue engineering is based on potential cell sources and biocompatible materials applied as direct pulp capping agents, which can also serve as a carrier of signalling molecules. In addition to nerves and blood vessels, the pulp contains highly proliferative stem/progenitor cells possessing a self-renewal and differentiation capability. The latter have the ability to regenerate degraded dentin in vivo when compromised dentin is located close to the pulp. Hence, the design of a suitable biomaterial is focused on both the healing potential through stimulating regeneration of the pulp tissue, and enhancing tissue formation by released biomolecules. Studies in dental research aim at the development of suitable biomimetic and degradable biomaterials with uniquely functional properties at a nanometer-scale. Because human dental pulp stem/progenitor cells (DPSC) have shown the capacity to differentiate into odontoblasts, this in vitro system can be used to characterize, optimize and evaluate newly developed bioactive materials. The current chapter will summarize selected research methods, current findings in the differentiation of DPSC and also an overview of potential filling materials studied to regenerate dentin-pulp complex.

Keywords: Dentin regeneration, biomaterial, progenitor/stem cells, pulp capping, pulp-dentin biology.

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Introduction

Biological tooth structure

Combined knowledge of the biology of the pulp and dentin as well as the understanding of the materials and techniques applied to tissues, constitute the basis for restorative dentistry and also for biological approaches to dentin repair. Dentin and pulp have a common embryonic origin, the dental papilla, and both stay in a close relationship for the whole life cycle of the vital tooth. This relationship is considered to be the pulp-dentin complex, which includes significant differences in the chemistry between dentin and pulp [1] (Fig. 1).

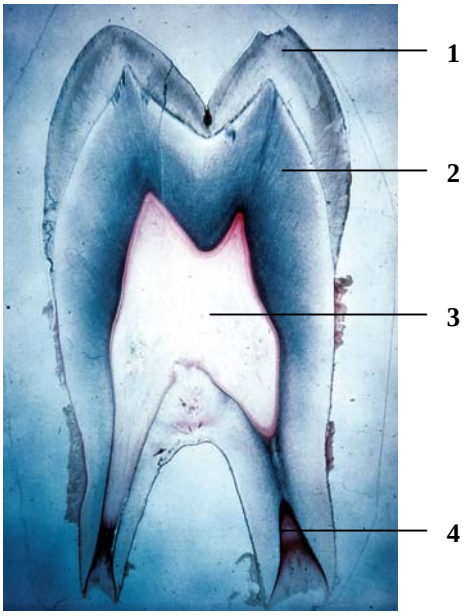

Fig. 1. Histological section through a molar of a young person; roots not completely developed. 1) Enamel, 2) Dentin, 3) Pulp, 4) Apical root still developing. Fuchsin red and light green staining [H. Stich, J.F. Roulet].

Pulp is a specialised connective tissue including blood and lymph vessels, nerves, and the interstitial fluid. Dentin is a tissue that is formed in a highly regulated and well-controlled process displaying several similarities with bone formation. In both processes, a collagenous organic matrix is formed by a layer of cells. In this matrix an inorganic calcium phosphate is deposited as mineral crystals. Contrary to bone, dentin is typically not remodelled and, hence, does not participate in the calcium homeostasis of the organism. As a consequence, dentin is formed during the whole lifetime of a tooth which results in a diminished pulp chamber with increasing age of the tooth. The proportions of components are 70% for the mineral phase, 20% for the organic phase and 10% for water by weight. By volume, the proportion is 50% for the mineral, 30% for the organic material and 20% for water.

Dentin is covered peripherally by enamel on the crown and by cementum on the tooth root surfaces. In case of caries, tooth fracture or gingival recession dentin is exposed and may become sensitive [1]. The organic phase of dentin is mainly composed of collagen which is mineralised by hydroxyapatite. This matrix is penetrated by long narrow parallel channels, the so-called dentinal tubules with diameters between 1 and 3 µm (Fig. 2).

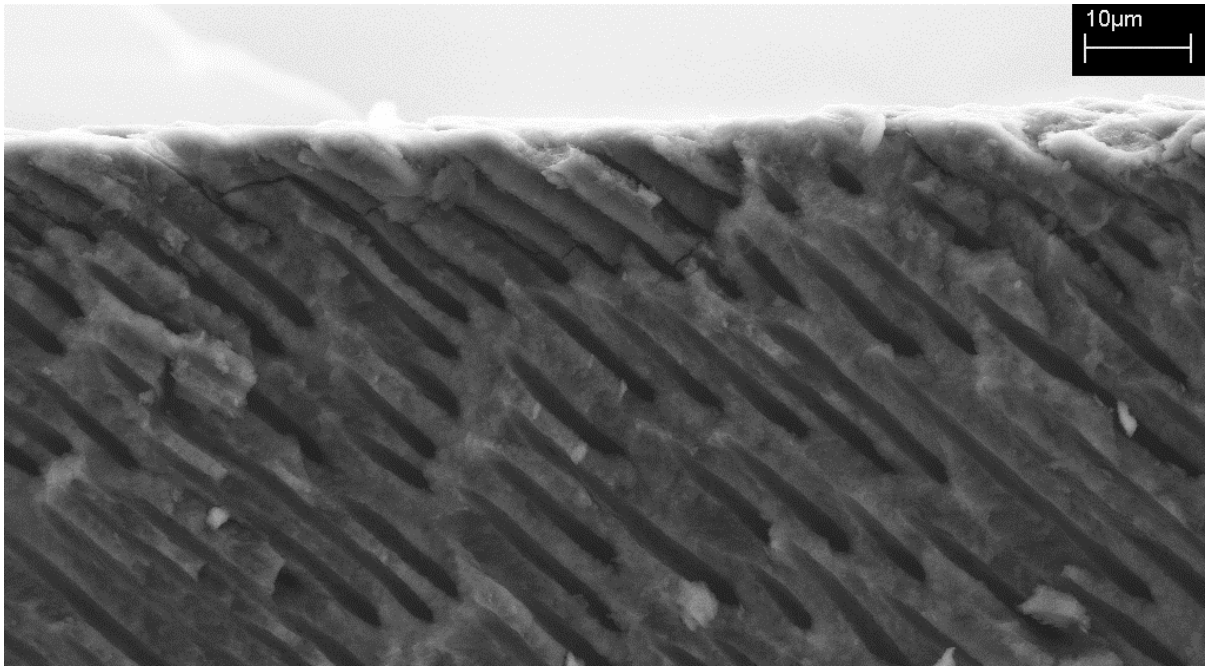

Fig. 2. Dentinal tubules of a bovine dentin matrix recorded by scanning electron microscopy (magnification factor 1,000) [C. Bolis, A. Huwig].

The percentage of the tubules covered surface ranges from 1% beneath the enamel to more than 22% near the pulp [2]. In the outer dentin about 15,000 tubules per square millimeter can be found, 25,000 per square millimeter in the central part and 55,000 per square millimeter close to the pulp [3]. In addition, lateral branches connect different tubules, which altogether result in highly permeable tissue with a porosity which enables invasion by micro-organisms.

The dentinal tubules harbour the so-called odontoblastic process or they contain remnants of this process and dentin liquor. The odontoblastic process is in principle the extension of the dentin-forming cells, the odontoblasts, into dentinal tubules. The odontoblasts are the most prominent cells of the dentin-pulp complex [4]. During tooth development, the odontoblasts form the predentin, which is at the very early developmental stage an unmineralized organic matrix consisting mainly of collagen. After tooth eruption, secondary dentin is deposited at much slower rate, for the whole life of the tooth. External

stimuli like attrition, carious decay, and dental restorations may induce the formation of tertiary dentin, also referred to as reactionary, reparative, irritation, and irregular dentin [5-8]. Tertiary dentin that is formed by already-existing odontoblasts by mild stimuli like attrition is usually called reactionary dentin. Alternatively, tertiary dentin which is typically irregular in structure and not tubular, is termed reparative dentin. The cells forming reparative dentin are newly differentiated odontoblasts derived from mesenchymal progenitor/stem cells located in the pulp [9]. These postnatal cells are defined as human dental pulp stem cells (DPSC) and appear as a minor subpopulation within the pulp. A series of studies have demonstrated that DPSC provide characteristic stem cell properties as they are self-renewed, highly proliferative with clonogenic efficiency, and possess the capability for multi-lineage differentiation [10-11].

Furthermore, previous studies have been conducted to demonstrate that DPSC can be induced to differentiate into odontoblast-like cells and generate dentin-like mineral structures *in vivo* and *in vitro* [10-12]. Concerning the remaining questions and gaining insights into mechanisms involved in the expression of an odontoblast-like phenotype, the DPSC demonstrate a promising and useful cell culture system for *in vitro* studies and *in vivo* tissue engineering.

Tissue engineering applied for dentin regeneration

The overall goal of tissue engineering is the functional restoration of tissue structures as well as the maintenance of the natural environment, and thus the viability and function of the damaged tissue due to disease or trauma. In this context, dental replacement in clinical applications depends on the use of a potential material which would be anti-inflammatory, antibacterial and can simultaneously enhance the proliferation and induce the differentiation of present DPSC into odontoblast-like cells leading to dentin formation [13].

Because of the similarities between dentin and bone structures, studies are often performed in dental tissue engineering in dependence on or in comparison to bone formation processes and applied osteoinductive materials. From a tissue engineering point of view it is noteworthy that there are differences between bone formation and a potential dentin formation as well. Different approaches, which are also under investigation for maxillofacial surgery and partly for tooth tissue regeneration, can already be performed for bone reconstruction, such as: 1) An autologous graft from various donor regions comprising bone forming cells and growth factors and therefore being osteogenetic [14]; 2) An allograft and

xenograft, respectively, i.e. a bone sample from other human beings or from animals, which is osteoinductive due to certain proteins like growth factors [15-16]; 3) Various osteoinductive biomaterials acting as carriers for growth factors inducing bone formation [17-19]; 4) Synthetic bone substitutes for bone replacement without or with just partially resorption or for bone repair using osteoconductive porous devices, i.e. scaffolds, providing a mechanical support until the tissue has regenerated and remodelled itself naturally, e.g. calcium phosphates [20-22], poly(D,L)lactide based materials [23-24], collagen-hydroxyapatite composites [25]; bioactive glasses [26-27], methacrylate-based materials [28-29].

The different autogeneic, xenogeneic and alloplastic bone replacement materials can be differentiated according to the functional quality of the new tissue and the dynamics of bone conversion thus induced. Comparing osteoconductive bone substitutes with demineralised, osteoinductive materials and autogenic bone grafts, bone inducing matrices show the largest quantity of new bone formation. In order to extrapolate the findings of bone to dentin repair, it is necessary to understand the dentin-pulp complex in more detail and in particular the challenging situation of the pulp itself especially in case of pulp healing and formation of reparative dentin.

Promising options for dentin regeneration

In this context, the morphology of dentin and the different types of dentin formation has to be considered. The formation of tertiary dentin, reactionary and reparative dentin, represents an important defence mechanism and a regenerative property of the pulp-dentin complex. In this context it is reasonable to assume that there are two different approaches for dentin regeneration by the use of tissue engineering techniques.

The first option includes a device which could be applied as a restorative filling material into a deep tooth cavity on a residual layer of dentin on top of the pulp. This device releases growth factors [30] stimulating the formation of reparative dentin. Such an approach results in a diminished pulp chamber and in an amplified dentin layer. Hence, the natural decrease in size of the pulp chamber is accelerated, but the pulp would be beneficially protected by an enhanced dentin layer thickness. Thus, the formation of a reparative dentin layer would provide an optimal barrier to avoid any bacteria infiltration to the pulp tissue, which is not provided by any artificial restorative materials [31]. For such an objective, one has to consider how to achieve the transport of stimulating factors through cut dentin [32-35].

The second option and more challenging approach is focused upon the application of a scaffold on an open pulp [36-37] enabling odontoblast-like cells to grow into the scaffold and to convert it into dentin. Thus, a deep carious lesion is turned into a rather small dentin wound that could easily be covered by a common restorative material acting as a substitute for enamel. However, it is very difficult to organise the conversion of the scaffold into dentin in a way that the volume of the scaffold is not altered during the regenerative process, to avoid any physical stress inside of the treated tooth. Such a material has to be dentin conductive for odontoblast-like cells. Although the caries-affected dentin is typically removed by excavation, some micro-organisms could survive in particular in the dentinal tubules from where a colonisation of a scaffold would be possible. Such a contamination has to be prevented by antibacterial means, e.g. disinfectants or antibiotics.

In this context, the chapter will give an overview of current studies in the field of dental tissue engineering aimed at the maintenance of a vital dentin-pulp complex after teeth injury. On one hand the ability of DPSC to differentiate into odontoblasts comes to the fore of studies performed by various scientific research groups and the authors' group themselves. On the other hand, the selection of a potential biomaterial being clinically applied to the cavity plays an emerging role due to the stimulating effect on differentiation by itself and carrying active biomolecules (growth and differentiation factors). Therefore, selected methods applied in various laboratories for the isolation of primary dental pulp cells, their cultivation, phenotype characterization as well as potential filling materials in dentistry, are summarized in the following sections giving an idea of the complexity and criteria which have to be implicated in dental repair.

Methods in studies of dental pulp cells in vitro

Isolation procedures

The first and critical step is the isolation of cells providing the potential to differentiate into odontoblast-like cells. A series of experiments have shown that dental pulp cells can be isolated from human impacted third molars (14-29 years of age), which are extracted for clinical reasons under anaesthesia [10, 38-39]. Tooth surface were cleaned by covering with 0.3% chlorhexidine gel [40-41], swabbed with 70% (v/v) alcohol [42] or dipped carefully in 30% hydrogen peroxide for 30 to 120 sec. Pulp was opened by cutting around the cementum-enamel junction using sterilized dental fissure burs to reveal the pulp chamber [10]. Other studies describe that teeth were immediately cracked opened, or opened by means of either a

dentinal excavator or a Gracey curette [39, 41] before removing the coronal pulp tissue gently. After separation of the pulp tissue, cells can be isolated by various methods. Recently, two isolation methods were examined in relation to the growth behaviour of human pulp cells. Pulp cells can be either isolated by (1) digestion [10, 41] or (2) the out-grown method [43].

First, the pulp tissue can be digested in a solution of collagenase type I and dispase as reported in details by Gronthos *et al.* [10, 41]. The cell suspension is then centrifuged and pellets are suspended in Dulbecco's modified Eagle's medium (DMEM). Single-cell suspensions can be obtained by passing the cells through 70 µm strainer and seeding into 6-well plates in DMEM supplemented with 10-20% FCS, 100 µM ascorbic acid 2-phosphate, 2 mM L-glutamine, 100 Units/ml penicillin and 100 µg/ml streptomycin [10, 44-45].

Secondly, pulp tissue explants (4 mm^3) were placed in 6-well plates and designated as human pulp cells/out-grown method (HDPC-o). These cells were cultivated to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented wit 10% FBS and antibiotics [46]. Further, human pulp primary cultures (HPPc) could be obtained by mincing tissue fragments of extracted pulps into small pieces (< 1 mm^3), which were than placed in well plates containing RPMI 1640 medium-glutamax supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml amphotericin-B and 10% FCS [38-39]. All cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C and medium change should be performed every two days.

Cell proliferation and investigation of stem/progenitor cells

When cells reach confluence, they can be harvested by trypsinization and subcultured. Previous studies showed that cells were mainly cultured until the 3^{rd} – 5^{th} passage for experimental use [39, 45-46]. Further, Iohara *et al.* demonstrated two cell culture systems in order to investigate the proliferation *in vitro* and differentiation potential of DPSC *in vivo*. Here, cells were either seeded onto cell culture dishes at a density of 10^4 cells per cm^2, or $2 \cdot 10^5$ cells per ml were centrifuged in 15 ml conical polypropylene tubes at 1000 rpm for 5 min to obtain a pellet. The monolayer and three-dimensional pellet culture were proliferated in DMEM + 10% FBS + penicillin/streptomycin as described before [47].

Analytical methods

Proliferation rate. To investigate the effect of various culture conditions on the proliferation of dental pulp cells the proliferation rate can be determined by bromodeoxyuridine (BrDU) incorporation [10, 46] or through incorporation of [^{3}H] thymidine to quantify DNA synthesis

[39]. It is also described that colony efficiency and proliferation potential can be evaluated by toluidine blue staining 80.1% (w/v) in 1% paraformaldehyde (PFA) and counting the numbers of clones. For cell counting cells are dispersed by trypsin and counted using the trypan blue exclusion method.

Immunocytochemistry/fluorescence-activated cell sorting. Next, it has to be observed whether the isolated cell cultures represent an undifferentiated pre-odontogenic phenotype expressing the characteristic cell surface marker STRO-1 and/or CD 146 as perivascular cell marker, both early mesenchymal stem cell markers found to be expressed in DPSC [11, 48]. Both markers can be examined by immunofluorescence labelling or fluorescence-activated cell sorting (FACS). Therefore, cells are harvested after defined cultivation time by treatment with trypsin-EDTA and counted. Cells are fixed in acetone or PFA. Staining is carried out by incubation with supernatant containing STRO-1 monoclonal antibody or purified antibody, then adding the secondary antibody (e.g. FITC-conjugated goat-anti mouse IgM). Next, cells are counterstained in PBS containing ethidium bromide to observe the fluorescence staining by light microscopy [39]. Additionally, cells can be stained with horseradish peroxidase-conjugated avidin-biotin complex (ABS staining) and incubated with DAB solution. Haematoxylin can be applied to counterstain the nuclei and then observe by light microscopy [49]. Applying the FACS flow cytometer method, STRO-1 and CD 146 (IgM) primary mouse antibody are added to 2-2.5x 10^5 cells in suspension, followed by washing steps and incubation with FITC-conjugated goat anti-mouse IgM antibody [11-12].

Cell differentiation. Since it was shown by various studies that DPSC derived from pulp tissue have the potential to differentiate into either odontoblast-like cells or fibroblasts, calcified matrix deposition can be induced by supplementation of 10 nM dexamethasone, 50 µg/ml ascorbic-acid and 10 mM glycerophosphate or by signalling factors such as BMP-2 (100-200 ng/ml) to proliferation medium (DMEM + 10% FBS + penicillin/streptomycin) [47].

Characterization of an odontoblast-like phenotype: The formation of dentin matrix by differentiated odontoblasts involves the secretion of specific mineralized matrix proteins such as alkaline phosphatase (ALPase), collagen type-I (Col-I), dentin matrix protein 1 (DMP-1), dentin sialophospho-protein (DSPP), osteonectin (ON), and osteocalcin (OC) at protein and

gene expression level [48]. Therefore, it is of importance to study these markers performing molecular biological and biochemical methods as follows:

ALPase activity. For measuring the ALPase activity, cultured cells are scraped into lysis buffer containing 10 mM TRIS-HCL, 5 mM $MgSO_4$, 0.1% Triton X-100 and 0.1% $NaNO_3$. Samples can be frozen at -20°C, then thawed 2-3 times and additional sonicated to disrupt cell membranes [39]. Another method would be the incubation on ice before intensive pipetting. ALPase activity can be determined by the hydrolysis of p-nitrophenyl phosphate in 2-amino-2-methyl-1 propanol buffer at 37°C for 30 minutes. Absorbance can be measured at 405 nm using a spectrophotometer.

Protein content. Total protein content is commonly measured by the Bradford method or colorimetric micro-BCA method with bovine serum albumin as standard [39, 50].

Quantification of Col-I. Col-I synthesis can be detected in cell culture supernatant. Collagen is synthesized as procollagen proteins with large propeptide domains. The cleavage of these domains leads to the secretion into the supernatant in a 1:1 ratio to type I collagen incorporation into growing collagen fibrils. Hence, collagen type I synthesis can be calculated from the C-terminal propeptide concentration measured in duplicate samples by enzyme-linked immunoabsorbance assay (ELISA) [51].

Mineralization. For the detection of mineralized nodule formation cells can be fixed with 4% PFA overnight or 70% ethanol for one hour. Calcium deposits are stained with 2% Alizarin Red S (pH 4.2) or von Kossa stain ($AgNO_3$) [10, 41, 47, 52]. Additionally, the mineral phase can be investigated in detail by micro-Raman spectroscopy indicating the formation of hydroxyapatite [52]. For the quantification of mineralized nodules the mineral calcium content can be measured. Therefore, cell cultures are washed with PBS without Ca^{2+} and Mg^{2+} and decalcified with 0.6 N HCl. The calcium concentration in the supernatant is detected by o-cresolphthalein complexone method applying the Calcium C-Test Kit as described by Nakao *et al.* [50].

Immunocytochemistry. Cell cultures are prepared and immunocytochemistry performed as described in numerous studies. Briefly, the cells are washed and fixed in 4% PFA prior to blocking with goat serum (GS) or bovine serum albumin (BSA). Pretreatment of samples with 0.3% H_2O_2 is necessary to inhibit endogenous peroxidase activity followed by washing

procedures with PBS. The incubation with primary antibodies is performed overnight at 4°C in a humidity chamber. The use of the following antibodies proving the components of the extracellular matrix formed by odontoblast-like cells has been described: anti-DSPP [12], anti-DSP (a fragment of DSPP) [11, 53], anti-DMP-1 [54-55], anti-ON and anti-OC [10, 42, 53], anti-Col-I [10, 42]. Cells are washed and primary antibody reactivity can be revealed by incubation of an appropriate biotinylated secondary antibody followed by streptavidin-horseradish peroxidase. After additional washing, cell culture is further exposed to substrate solution producing a brownish precipitate, which can be analyzed by light microscopy.

Polymerase chain reaction (PCR). Most studies monitored the proliferation as well as differentiation of dental pulp cells into an odontoblastic phenotype by using semi-quantitative reverse-transcription (RT)-PCR [10, 39, 45-46, 49, 53, 56]. A few studies also describe in detail the real-time PCR method applying either the TaqMan® MGB probe [50, 52] or SYBR Green I system [47].

Total cellular RNA extraction is often performed using either RNaesy Mini Kit offered by Quiagen [56] or Trizol reagent [39] according to the manufacturer's instructions. The latter isolation procedure is followed by a chloroform/phenol extraction and RNA precipitation by the addition of isopropanol. Afterwards, pellets are washed with ethanol and suspended in RNase-free water. A DNase procedure is necessary in order to obtain a high purity grade without DNA contamination. Next, RT-PCR is performed according to standard protocols. Isolated mRNA is reverse-transcribed using oligo-dT primer and resulting cDNA is amplified in a typical PCR sample as for example described by Liu *et al.* [56] applying specific forward and reverse primers as shown in Table 1. The resulting PCR samples can then be analyzed by 1% agarose gel electrophoresis.

Table 1. PCR primer sequences for specific gene expression markers of human odontoblast-like cells described by various research groups. GAPDH: gyceraldehyde 3-phosphate dehydrogenase used as internal control for the normalization of all target gene expressions, OC: osteocalcin, ON: osteonectin, DSPP: dentin sialophosphoprotein, Col-I: collagen type I, ALP: alkaline phosphatase.

Protein	Sequence (5´-3´) amplicon	Reference
GAPDH	Forward: GAGGATAAAGGACAACATGG Reverse: AAGAAGCATCTCCTCGGC	39
OC	Forward: CCATGGAGAAGGCTGGG Reverse: CAAAGTTGTCATGGATGACC	53
ON	Forward: ATGAGGGCCTGGATCTTCTT Reverse: CTGCTTCTCAGTCAGAAGGT	120, 45
DSPP	Forward: GGCAGTGACTCAAAAGGAGC Reverse: TGCTGTCACTGTCACTGCTG	10
Col-I	Forward: TCAGAGAGGAGAGAGAGGCT Reverse: ATTCAGGGGAACCTTCGGCA	46
ALP	Forward: GACCCGTCACTCTCCGAGATG Reverse: CTGCGCCTGGTAGTTGTTGTG	56

Part I: Dental pulp cell cultivation

In order to develop a potential biomaterial for dental pulp regeneration and reconstitution of a complete dentin-pulp-complex, the understanding of the proliferation as well as differentiation processes is indispensable. Hence, studying processes in dental regeneration using an *in vitro* dental pulp cell culture system can provide an insight into biological processes which lead to odontoblast-like cell differentiation and induced dentin matrix mineralization. Just based on a complete knowledge about *in vitro* dental pulp cell (DPC) behaviour and following *in vivo* experiments, conclusions can be drawn upon the requirements on the development of a highly suitable filling material. The following section demonstrates whether it is possible to isolate a potential cell population comprising DPSC, and furthermore the proliferation and differentiation ability has to be proven.

Isolation, identification and proliferation

Therefore, the first and critical step in order to investigate the proliferation and differentiation ability of these cells is the *isolation* of a suitable cell population. DPC and DPSC, respectively, have already been isolated from adult human teeth (14-29 years of age) [10, 12, 38-39, 41-42, 44-46, 52], pork [47] and rat dental pulp [50]. A further cell culture system was obtained from human exfoliated deciduous teeth (SHED) (6-10 years of age) [12, 41]. Miura *et al.* reasoned from his findings that SHED are distinct from DPSC because of a higher proliferation rate, increased cell-population doubling, and stem cell typical formation of spherical cell clusters and osteoinductive potential *in vivo*. However, these cells do not maintain the capacity to reconstitute a dentin-pulp-complex for which reason there remain only mentioned [12].

Currently, two isolation methods are performed in various reports to isolate DPSC by either enzyme digestion, or the out-grown method, as described before. Huang *et al.* investigated whether cell isolation methods yield in the same pool of cell population. Although the out-grown method is more convenient and not as technically extensive as the enzymatic digestion, cells migrate out of the tissue fragments growing slower than human DPC obtained by digestion method until becoming confluent in 2-3 weeks [46]. Even enzymatic digestion may cause a cell damage it allows different types of cells to form compact and loose types of colonies within 1-2 weeks, which can separately be characterized [10, 46]. All cell cultures display a wide range of cell morphology such as fibroblast-like cells, endothelial-like or epithelial-like cell populations. Gronthos *et al.* and Batouli *et al.* have applied the enzyme digestion method and were able to demonstrate that dental pulp cells differentiated into odontoblast-like cells, which also formed dentin matrix *in vivo* [10, 57]. The out-grown method showed that cells are potentially capable to differentiate into odontoblasts or forming mineralized nodules *in vitro* [42, 44, 53, 58]. Concerning the growth behaviour and characterization ability of single cell colonies the digestion method seems to be more reasonable. Both methods demonstrated the ability to isolate cells containing a minor population of odontoblast precursor cells with typical criteria for postnatal somatic stem cells [59], such as their high rate of proliferation, clonogenic nature [10], and co-expression of specific markers.

Identification studies showed that DSPC express the cell surface antigen STRO-1, which is known to immunoselect osteogenic precursors in bone marrow stromal cells [10, 12, 39, 48-49]. Alliot-Licht *et al.* investigated the effect of dexamethasone contained in the

differentiation medium resulting in a significant increase of STRO-1 positive cell population in human DPSC [39]. Previous studies have demonstrated that isolated SHED cells proliferated *in vitro* contain approximately 9% of STRO-1 positive cell population [12]. These observations agree to that of Shi and Gronthos [48] and Zhang *et al.* [49], demonstrating a similar percentage of about 5-6% of the total pulp cell population. Further analysis revealed that DPSC express the perivascular cell marker CD146 [48, 60], but does not react with the hematopoietic markers CD14 (monocyte/macrophage), CD45 (leucocyte) or CD34 (hematopoietic stem cells/endothelium) [10, 61]. To date there is no investigation published that demonstrates the effect of the applied isolation method on the yield of precursor cells in DPC.

After providing the evidence to isolate stem/progenitor cells out of the dental pulp, *proliferation* studies have been described in various reports and exhibit a high proliferation rate (mean 72% BrDU-positive DPSC after passage 1) [10]. The growth potential was beyond 100 population doublings and cell populations formed clonogenic cell clusters [10, 61]. Studies have also demonstrated that cultures can be maintained after extensive subculturing of up to 20 passages after seeding isolated DPSC [10, 39]. After subculturing they are able to adhere quickly to conventional plastic dishes showing a typical fibroblastic, spindle-shape to polygonal morphology [42, 49], as shown in figure 3.

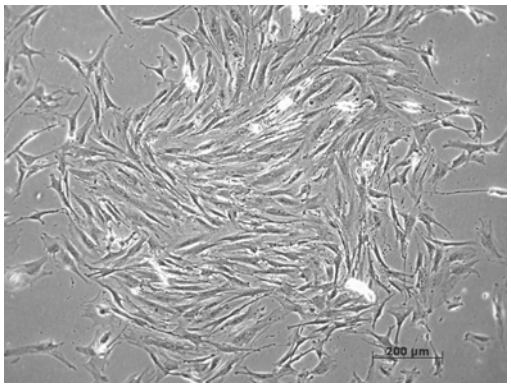

Fig. 3. Isolated human dental pulp cells plated on culture dishes and forming colonies after 7 days (bar = 200 µm). [C. Mauth, U. Graf-Hausner]

Concerning phenotypic changes occurring during passages or by the choice of isolation method, it has been investigated that the ALPase activity decreases after several passages beyond 13-16 passages in human DPC. Studies from Shiba *et al.* have shown that the age of donor also has an effect on the lifespan of cell cultures. Hence, human pulp cells from young donors could be cultivated over a longer cultivation period than those from older

donors. Also, growth rates and the ALPase activity of human pulp cells decreased with increasing donor age. Although further studies are required, these findings suggest that impaired repair of pulp and dentin in aged patients is partly due to a decrease in the proliferative ability and the ALPase activity in aged pulp cells [62].

One has also to take into consideration that the cultivation model (monolayer or pellet) can have an influence on the proliferation and lately the differentiation of DPSC, as indicated by the experiments of Iohara *et al.* with porcine premolar pulp cell cultures. It was believed that pellet cultures obtained higher ALPase activity, and were surrounded by newly formed osteodentin after 21 days, despite of their minor proliferation capacity compared to monolayer cultures [47].

Another influencing factor on the proliferation and the differentiation capacity of DPSC presents the ability to cryopreserve isolated cells. If this source of human adult stem cells could be stored without any loss of the differentiation potential, it would be a useful and reliable source for later recovering with respect to delayed therapies in tissue regeneration. The study from Papaciccio *et al.* using DPC isolated from adults (21-45 years of age) show that it is absolutely possible to freeze these cell populations and store them over 2 years prior to re-starting proliferation and successful differentiation into osteoblasts [40]. The findings are confirmed by studies of Zhang *et al.*, who recovered rat as well as human DPC from liquid nitrogen before seeding onto three-dimensional scaffolds [37, 49].

Differentiation capacity of DPSC

Several previously performed studies have shown that dental pulp cells have the ability to differentiate into odontoblasts in order to repair the dental damage caused by trauma, caries or dentin erosion [10]. Therefore, *in vitro* cell culture systems are useful to study intracellular processes leading to odontoblast-like cell differentiation demonstrating that cultured human pulp cells containing stem/progenitor cells are able to differentiate *in vitro* into odontoblast-like cells and form dentin matrix [42]. The dentin formation of human tooth demonstrates a highly regulated and controlled pathway of cellular and extracellular reactions. In order to characterize the *in vitro* cell cultures of differentiated DPSC grown on cell culture dishes, in pellet cultures and onto three-dimensional constructs various analysis have to be performed and interpreted.

Characterisation of the intracellular marker ATPase

First, ALPase is considered to play an important role in the initiation of connective tissue mineralization. Therefore, ALPase is often used as a marker expressed during odontoblast-like differentiation *in vitro* and *in vivo* localized in the pre-odontoblast and odontoblast-layers [63]. The increase in ALPase activity is an often demonstrated feature of odontoblastic differentiation [44, 56]. Lopez-Cazaux *et al.* recently demonstrated the effect of culture media resulting in significant stimulation of ALPase activity in human dental pulp cells after 14 days cultured in MEM media compared to cultures in RPMI media [45]. It has also been demonstrated that dexamethasone, a glycocorticoid class hormone and known to induce osteoblast-like differentiation, can markedly stimulate ALPase activity after 14 days in culture [39, 64]. The level of ALPase can be detected to remain constant during cell growth and increased during differentiation, whereas a down-regulation can be observed after 3 weeks of confluence when the formation of mineralized nodules becomes visible [39, 52, 56]. An explanation for the typical down-regulation of ALPase activity with induced mineralization could be that crystal formation can take part without ALPase after initiation [65]. In addition, as a gene involved in the collagen metabolism the ALPase mRNA expression confirms these findings by an up-regulation of gene expression over 3 weeks after confluence [56].

Characterisation of extracellular markers

In general, the formation of extracellular matrix plays an important role in tissue regeneration. DPC are able to synthesize a specific matrix resembling dentin [42, 44, 49, 58, 66]. During *in vivo* formation, which is believed to be a complex process of several reactions, collagen fibres convert from noncalcified matrix to a carbonate-rich apatite mineral phase ($Ca_{10}(PO_4)_6(OH)_2$) [67] within and around the collagen fibres providing a rigid and strong quality. The predominant collagen in this matrix is Col-I, which was detected *in vitro* by a series of studies on gene expression or protein synthesis. Shiba *et al.* found that human pulp cells synthesize besides laminin and fibronectin a large amount of Col-I per day in culture, which has been measured by detection of procollagen type-I C-terminal peptide (PIP). The cultivation time did not influence the Col-I synthesis over 22 days [44]. These results agreed with those of Tjäderhane *et al.*, who also showed a rather constant concentration of Col-I synthesis in odontoblast cultures over 10 days [68]. Immunocytochemical staining demonstrating the Col-I protein synthesis at specific time points due to intensive positive

staining, indicated that dental pulp cells have the potential to form collagen fibres [41]. Further reports described the Col-1 mRNA gene expression pattern under similar culture conditions performed by semi-quantitative PCR or quantitative real time PCR [49, 52, 64]. The studies showed the up-regulation of Col-1 expression during the first 14 days in monolayer cultures, but expression remaining constant for the following cultivation time of up to 28 days [49, 52]. However, when formation of mineralized nodules was induced at day 14 in differentiation medium, the expression of Col-1 was slightly down-regulated shown by Liu *et al.* [52].

Further, noncollagenous proteins in dentin secreted by odontoblast-like cells are osteonectin (ON), osteocalcin (OC), dentin sialoprotein (DSP), dentin phosphoprotein (DPP), as well as dentin matrix protein (DMP-1). DSP and DPP are products of a single transcript called dentin sialophosphoprotein (DSPP) encoding both proteins and considered as being an important phenotypic marker of odontoblasts [53-54]. Most of the performed studies of various research teams showed the production of these proteins at the gene expression level, and others at protein level by quantification of calcium content, western blot analysis or immunocytochemistry. In summary, it has been reported that dental pulp cultures dramatically increase the gene as well as protein expression of DSP and DSPP, respectively, after 14 days during differentiation [39, 45, 47, 49, 52, 56, 58]. Zhang *et al.* demonstrated clearly the interaction and stage dependent gene expression level of Col-1, DSPP and OC [49]. The OC expression increases after Col-1 expression reached a maximum indicating that the protein matrix is formed [64]. Furthermore, DSPP up-regulation after 14 days in culture can be associated with the collagen-rich mineralized matrix at day 21. Although DMP-1 is quite rarely demonstrated as dentin specific marker to characterize the differentiation of DPSC *in vitro*, it has been shown to be increased at gene expression level in pellets, but not in monolayer cultures [47]. Additional studies showed the direct induction of the differentiation of DPSC by the supplementation of ascorbic acid, β-glycerophosphate and dexamethasone and also by medium selection, whereby MEM seems to induce DSPP expression better than RPMI 1640 [39, 52].

After demonstrating that cells obtained from the dental pulp can form specific matrix proteins, these cell cultures can also form mineralized nodules, as established by Tsukamato *et al.* [70] and shown in figure 4 [69]. Mineralization detected by Alizarin Red S staining has been demonstrated to appear after 2 to 8 weeks in differentiated dental pulp cultures [10, 38, 42, 49]. The time variable observations concerning the induced mineralization of various references reflects the effect of the isolation method used, as well as seeding density and

culture conditions, which are applied for individual studies. Further, the influence of the donor age might have an effect on the culture population and content of stem/progenitor cells.

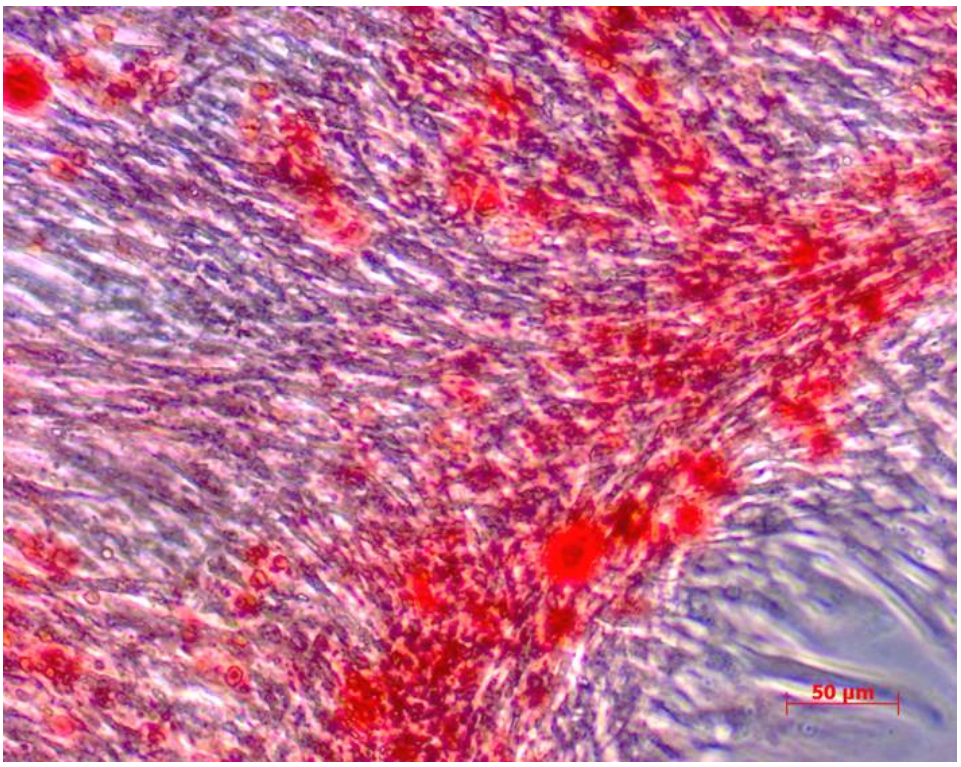

Fig. 4. Mineralized cell nodule formation detected by Alizarin Red S staining in dental pulp cell cultures after for 25 days in culture and induced differentiation (bar = 50 µm) [69].

Part II: Scaffold materials for DPSC cultivation

Tissue regenerative treatment of the dental-pulp complex will require a second key element besides the responding DPSC: a suitable inductive carrier. The selection of an appropriate scaffold material is of decisive importance to induce and confer the optimal formation of new dentin matrix. Thereby, the suitability of the scaffold depends on both chemical and physical material properties, as well as geometric structure, which are all subjects of current research.

Biomaterials for potential clinical applications in regenerative dentinogenesis have to provide optimal conditions for cell adhesion, migration, proliferation and differentiation. Essentially, the biomaterial has to fulfil the following demands: 1) biocompatible and non-toxic, respectively 2) biomechanical features including tensile, compressive and flexural strength, 3) conductive for odontoblast-like cells, 4) bioresorbable, and 5) bioactive. The mechanical properties of course carry a crucial role due to the extensive stress of up to 20 MPa several times per day [72]. Further, after implantation the material will be replaced by regenerated tissue matrix contemporaneous to the scaffold degradation without any volume

loss. Sufficient nutrient supply as well as removal of metabolic waste-products, guided by the porosity, interconnectivity and pore size is of great interest to ensure the viability and migration of dental cells [13, 31]. In order to mediate the interaction between material and pulp cells influencing the differentiation of DPSC, the precise surface geometry and chemical properties at nanoscale functions have to be controlled [73]. One of the most interesting and well studied function of biomaterials and their compositions is the delivery of stimulating growth and differentiation factors as well as peptides.

A set of biomaterials has been already tested for a regulated regeneration of bone, predominantly, but also of dentin. Applied bioactive materials are available as potential scaffolds composed of natural or synthetic polymers and can be divided in three groups: (1) natural organic materials such as collagen [37], (2) synthetic organic materials such as alginate [74], polylactic acid (PLA), polyglycolic acid (PGA) and their copolymers [75-77], (3) hydroxyapatite (HA), tricalcium phosphate (TCP), and compositions of silicate, phosphate glasses and bioglasses [10, 22, 37, 49, 72]. The most promising materials in dental regeneration will be described in the following abstracts.

Collagen scaffolds

Collagen, which belongs to the natural polymers, is an important protein within the human body composed of insoluble fibres. It is a predominant component of dentin matrix and supports the initiation of calcification, not inducing the mineralization by itself. Collagen has the advantage to be cytocompatible and bioactive. In addition, as a synthetic polymer a controlled production is possible, whereas a determined structure, porosity, degradation rate and mechanical properties can be precise varied and regulated [31]. Because collagen exhibits a high tensile strength it can be brought into the desired form by knitting, weaving and twisting. Collagen implants are degradable within the body and occasionally can cause mild inflammatory reaction [78]. After implantation of the material it seems that the matrix stimulates the adhesion as well as growth of cells accelerating and enhancing the wound healing process. It is assumed that collagen fibres support the migration and adhesion of pulp cells and subsequent dentin formation *in vivo* [79]. Because of the potential as extracellular matrix for seeded or in-growing cells, a series of studies were focused on the design of collagen scaffolds [80-81]. Currently, Zhang *et al.* performed experiments where DPSC were seeded onto a collagen matrix. Hence, the group arrived at the result that DPSC attach well at the surface, grow into the material, fill the pores with loose connective-like tissue, and that

cell proliferation is distinctly enhanced by collagen *in vivo*. However it could not be demonstrated that the collagen-based biomaterial stimulated DPSC to synthesize mineralised hard tissue [37]. Showing the supportive effect of collagen, other studies indicate that the presence of an insoluble dentin matrix such as calcium hydroxide seems to be also important for the inductive differentiation process of DPSC to an odontoblast-like cell phenotype during regenerative dentinogenesis *in vivo* [82-83].

Biodegradable polymers

Synthetic polymers, such as PLA and PGA and their copolymer are becoming more and more attractive for tissue engineering applications and already approved by the Food and Drug Administration (FDA). Since the 1960s PLA and PGA are applied in medicine as controlled drug delivery systems, mostly injectable as microspheres form, orthopaedic fixation devices such as pins and screws and also as scaffold to mimic the extracellular matrix for cells [76, 84-87].

Depending on the lactic and glycolic portion of the polylactide-co-glycolide (PLGA) copolymer various physical and mechanical properties can be produced and the degradation rate can be specifically regulated [78]. PLA shows a high crystallinity and is attractive as a biodegradable polymer because degradation products naturally occur in the body and are resorbed through the metabolic pathway. The common and most applied processing techniques for PGA preparation is the ring-opening polymerization [84]. PLA presented in various isomeric forms (D(-) and L(+)) can also be prepared by catalysed ring-opening polymerization, but also by fermentation of molasses or glucose [78]. Both polymers demonstrate a similar degradation rate caused by random hydrolysis of their ester linkage producing either lactic acid from PLA or glycolic acid from PGA, which can be excreted by urine. Thereby, it has to be mentioned that the degradation rate of the material depends on various criteria besides the copolymer ratio, like configurational structure, crystallinity, morphology, stress, the amount of residual monomer, porosity and site of the implantation [84]. The desired mechanical properties are also based on the polymer ratio but also on the molecular weight and crystallinity of the scaffold.

Various *in vitro* and *in vivo* studies have approved the biocompatibility, and also the biodegradability of these polymers. Thereupon, mild inflammatory reactions have been observed after a massive release of acidic degradation *in vivo* depending on the amount and degradation rate of the material [88-93]. However, the use of synthetic biocompatible

material has the enormous advantage to perform a reproducible synthesis, to control the mechanical and chemical properties including the structure, size, viscosity, and porosity, as well as degradation rate of the desired scaffold. Furthermore, incorporated bioactive molecules can locally applied by controlled release of the biodegradable PLA, PGA or PLGA system and so influencing the cell phenotype expression.

Ceramic scaffolds

Calcium phosphate (Ca/P), bioactive glasses and glass ceramics have been widely applied for hard tissue regeneration such as bone substitutes and also in dental tissue repair as pulp-capping agent. Ca/P scaffolds consist of either β-tricalcium phosphate (β-TCP) or hydroxyapatite (HA). The intention to produce a TCP and/or HA scaffold is based on the fact that both occur in the mineral matrix of the bone as well as tooth. Because of the similarity to the naturally mineralized tissue they are very suitable as biocompatible and osteoinductive materials. Furthermore, they can be produced synthetically and are today's state-of-the-art in medical applications.

TCP and HA are both applied not only in bone regeneration, but also in therapeutic healing processes of bone defects in dental regeneration and maxillofacial surgery. A series of techniques has been reviewed and discussed to prepare HA powder and dense or porous ceramics divided in wet methods and solid state reactions. Briefly, the fabrication can be carried out by precipitation, hydrothermal technique and hydrolysis of Ca/P obtaining various morphology, stoichiometry, and levels of crystallinity. The material seems to be a quite appropriate application for artificial tooth and dental regeneration. Even so it is limited to unloaded implants or filling material as coating or powder because of the poor fracture toughness also depending on the preparation techniques (processing, forming, and densification) and porosity [72]. Though, the clinical applications of HA or TCP, respectively, are based on the following advantages: enhancing the cell attachment of surrounded hard tissue; formation of a strong connection between implant and tissue with high strength and shorter healing periods compared to metallic implants.

Previous studies by Gronthos *et al.* demonstrated the *in vivo* behaviour of DPSC in conjunction with granular ceramic powder (TCP/HA) due to the transplantation into immunocompromised mice. The results showed the generation of a dentin-like matrix layer on the surface of HA/TCP particles indicating its potential as bioactive material for dentin-pulp complex repair [10-11, 37, 49].

Bioactive glass ceramics

A further invention for clinical application in tissue maintenance and regeneration was made by Hench and his team in 1969, who prepared bioactive glasses due to a specific chemical composition [94]. Thereby, the main advantage of bioactive glass is the induction of quick and direct interfacial bonding to the hard tissue due to biological equivalence of inorganic components of the mineralized tissue and the growing HA on the bioactive material surface. In general, bioactive glasses are appropriate scaffolds and in particular rhenanite glass ceramics. These systems are based on SiO_2–Na_2O–CaO–P_2O_5 components displaying good conditions of crystallization and crystal growth forming a hydroxyl carbonate apatite (HCA) layer in simulated body fluid, a buffer system with similar ion concentration to human blood plasma [95]. By preparing bioactive glass materials as an open porous scaffold the reactivity is even higher to form apatite because of the enlarged surface exposed to the solution for ion exchange and reaction. Additionally, a controlled dissolution rate (solubility) is required to design a scaffold or even granulated powder, which is reliable without any volume loss, such as a filling/capping material in dental medicine. This way the material has to be stable und supportive before new tissue regeneration has occurred. It has been demonstrated that the effect of bioactive glass can occur due to chemical changes in the extracellular environment. Osteoblast activity is, therefore, enhanced via the released dissolution products such as calcium phosphate [96-97]. Nowadays, the application of bioactive glass is provided as middle ear implant and in dental surgery as pin or bone filling material [98-100]. Based on previous studies and successful implantations the material could be a potential material to regenerate dentin-pulp complex due to enhanced odontoblast activity on the particular surface (Fig. 5).

Composites

The production and application of composites from synthetic bioresorbable polymers and bioactive Ca/P such as HA, TCP or selected bioactive glasses became increasingly important taking advantage of the bioactive and bioresorbable properties to guide tissue formation processes. Thereby, the design is focused on the minimisation of the disadvantages and the utilization of the profitable properties of the single components to develop an optimised tissue engineering scaffold with defined properties including the degradation rate *in vivo* balanced to the formation of new tissue [101-105]. A further advantage of the composites is

the increased toughness of the scaffold since bioactive ceramics are relatively stiff. Resorbable polymers are on the other hand easier to fabricate but too weak for the requirements of dental tissue regeneration. Moreover, a study of Blaker *et al.* indicated that cell adhesion, spreading and viability of cells grown onto polymer-bioglass-composites could be improved, and confirmed the high bioactivity and biocompatibility of the material for hard tissue repair [106]. Last-mentioned, basic resorption products of HA and TCP could buffer the decreased pH caused by acidic degradation of the polymer matrix [91].

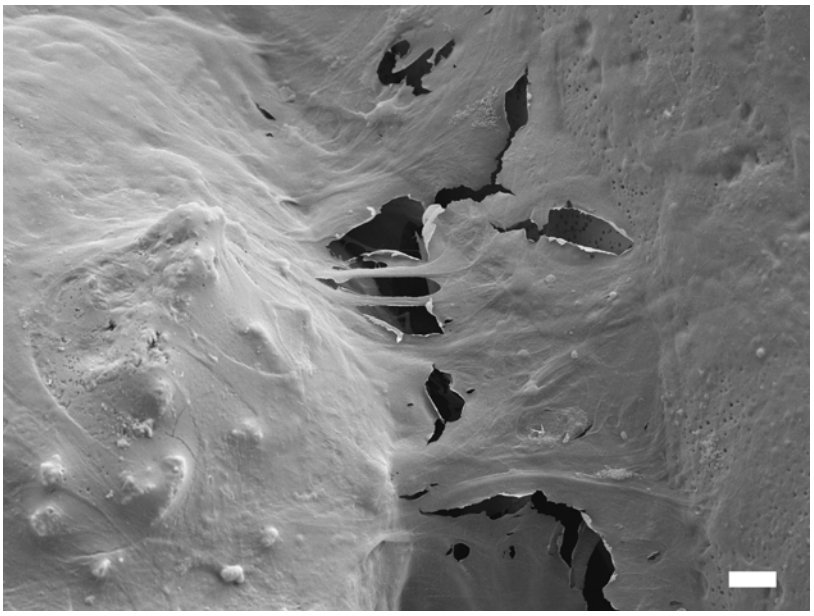

Fig. 5. Human dental pulp cells seeded onto novel bioactive glass-ceramic granules pretreated with simulated body fluid (SBF) and analyzed by SEM (bar = 10 µm) [C. Mauth, R. Grimm, A. Huwig, U. Graf-Hausner].

Part III: Delivery and effect of growth factors

The third key element of the tissue engineering triad is the regulation of cell proliferation and differentiation in the presence of bioactive molecules governing the neo-tissue formation and organization [107]. It is of increased importance to release the growth factor in a regulated manner from the extracellular matrix such as the scaffold. Indeed, it has been demonstrated in previous studies that the release of growth factors from the scaffold can have an extensive effect on the tissue formation [108]. Growth factors need to be incorporated in the material studying predominantly polymers as potential carriers for a localized and controlled delivery [109-110]. It is also of importance to design the delivery systems for a gradient availability of the signalling factor throughout the cell migration, proliferation and tissue formation, consequently. On the other hand it is necessary to adjust the growth factor release to the

degradation rate and metabolic removal. Nowadays, the design of suitable growth factor delivery system meeting all requirements and mimicking a natural biological environment still remains as one of the most important subjects in tissue engineering. In this context studies are focused on different strategies. First, a widely applied strategy is the direct incorporation of the growth factor limited by the enzymatic degradation when exposed to the surrounding *in vivo* tissue [111]. Another promising strategy would be the use of biodegradable microspheres embedding the growth factor and thus preventing both rapid release out of the scaffold and the factors degradation [112].

Given that in dental tissue engineering the growth factor has to transcend a residual layer of dentin due to diffusion through dentinal tubules it seems quite challenging to design an appropriate drug and/or growth factor delivery system, respectively. Therefore, polymeric and microsphere systems could be a potential and reproducible application in dentin regeneration to effect odontoblastic differentiation followed by dentin formation.

To achieve this goal of an effective delivery system, it is self-evident to use a growth factor showing an enhancing effect on dental-pulp complex formation. Therefore, it is known that certain signalling molecules have a specific regulatory function involved in tooth development as well as regeneration (Table 2).

Table 2. Signalling factors implicated as mediators in tooth development

Growth Factors		Reference
BMPs	Bone morphogenetic protein	8, 31, 47, 113-114, 121
TGF-β1	Transforming growth factor-β	44, 115, 118, 121
bFGF-2	Basic fibroblast growth factor	50, 113-114
IGF-I	Insulin growth factor-I	122

cells of the dental papilla to differentiate into odontoblasts. TGF-β1 as well as BMP and IGF-1, which are components of the extracellular matrix of dental pulp, are thought to be involved in differentiation process of odontoblasts and in dentin and predentin formation in adult tooth. Whereas TGF-β1 induces differentiation of odontoblast-like cells and stimulates primary odontoblasts secretion, BMPs have been implicated in tooth development and associated with the differentiation of odontoblasts and ameloblasts responsible for dentin and enamel secretion respectively [13, 31, 47]. Iohara *et al.* showed that BMP-2 at various concentrations had no effect on cell proliferation, but did increase the alkaline phosphatase activity in a dose dependent manner in cultures of porcine pulp cells [47]. Further, this growth factor induced the mRNA expression of DSPP after the implantation onto the dental papilla in organ culture. Additionally, the FGF family members have been thought to contribute to the mineralization of the dentin matrix [113-114] without increasing the alkaline phosphatase activity in human pulp cell cultures [44]. These cytokines may also be important during cellular response after injury occurs from caries. Smith *et al.* described the possibility that growth factors synthesized by odontoblasts and contained within the dentin matrix can be released due to caries demineralization processes caused by plaque bacterial acids. Hence, these soluble and diffusible signalling molecules may be released into the pulp environment, as schematically shown in figure 6 [115-116].

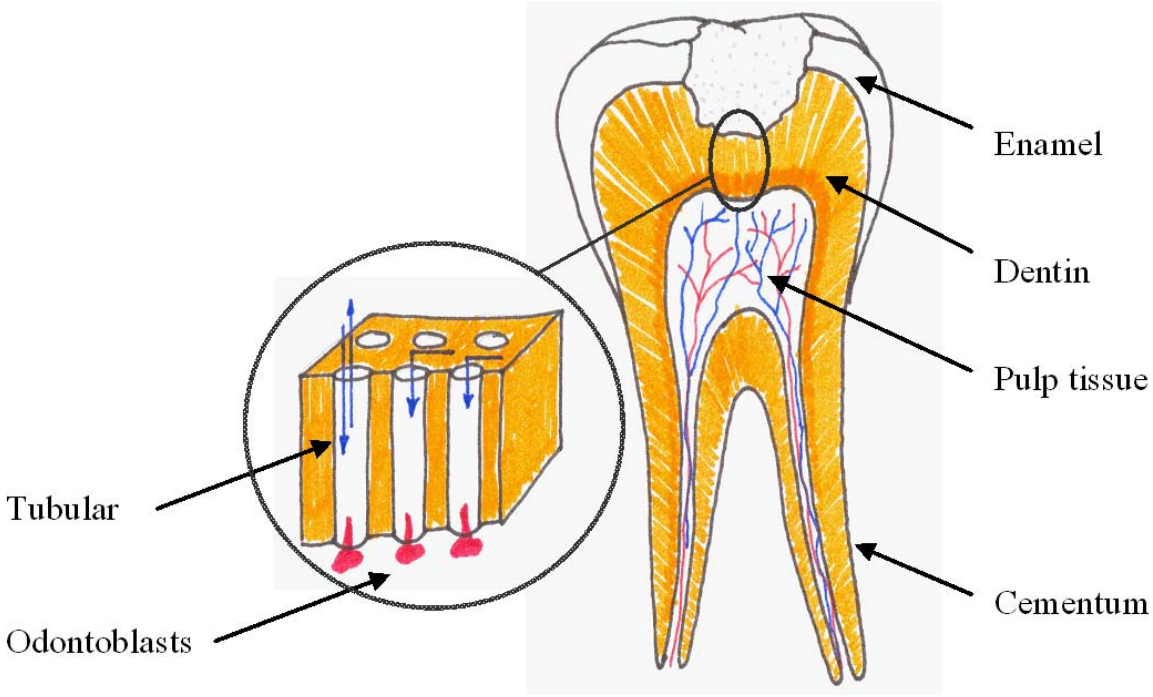

Fig. 6. A scheme of the carious tooth as well as the tubular structure of dentin (see magnification to the left) having channels for the diffusion of solvents across the dentin layer. Teeth degraded by caries undergo a demineralization process and matrix-bound protein signalling molecules (growth factors) may be released and diffuse along the dentin tubules (marked by blue arrows) [C. Mauth].

Given that during dentinogenesis pulp cells adhere to the osteodentin matrix before their differentiation into odontoblasts forming tubular dentin, it is suggesting itself that growth factors are sequestered in the dentin matrix mediating the cellular response [115]. Interestingly, studies from Tziafas *et al.* and Smith *et al.* could demonstrate that dentin matrix components directly influence dentinal repair activity. Therefore, EDTA-soluble dentine matrix fractions were prepared and implanted into a cavity using an experimental animal model (dog molar teeth) and showing that the matrix provides necessary inductive signals for dentinogenic response [115, 117]. Previous experiments also indicated that these effects can be mimicked by the application of morphogens such as TGF-β1 and BMPs inducing tubular matrix deposition around the implant [118-119].

Conclusion

Despite the rapid findings and wealth of data provided by *in vitro* and *in vivo* approaches in the field of dental regeneration, further research studies are required before pulp regeneration and even tooth restoration can be applied in dentistry. However, all data also confirm a realistic feasibility of dental tissue repair in the near future. It is obvious that our knowledge in dental tissue engineering expands rapidly. In this context it has been demonstrated that present dental pulp stem/progenitor cells have the ability to differentiate *in vitro* as well as *in vivo* into odontoblast-like cells. Furthermore, the application of bioactive glasses incorporated into a biodegradable polymer matrix also seems to be a suitable material as a regenerating dental substitute. In particular, the material would provide stability and a stimulation effect on hard tissue formation, as described before. The next step has to be the design of a “smart” and appropriate growth factors release system for diffusion through a residues dentin matrix after cavity preparation. Referring to previous findings, future experiments should be focused on the design of a highly sophisticated biological based scaffold system, which would greatly improve tooth viability and health maintenance in dentistry. Therefore, the development of a 3D-material as pulp capping agents meeting afore mentioned prerequisites, and aiming at the dentin-pulp complex regeneration, are currently a high priority in research investigations but also of high interest at the Ivoclar Vivadent AG.

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