from the control group was not administered antibiotic treatment. The length of antibiotic treatment in the study group was 4.0 while in the control group it was 4.5 days.

Pathogenic and symptomatic treatment

Both groups received some maintenance therapy. In 9 patients this included glucose 5%, physiological serum 0.9%, haemodesia and arginine in 1 patient, antipyretics in 15 patients, vitamins (ascorutin) in 29 patients, desensitizers in 15 patients, expectorants in 6 patients, broncholytics in 7, antitussives in 8, respiratory analeptics in 6, vasoconstrictive decongestants in 8, diuretics and corticosteroids in one patient for a day.

Conclusions

Treatment with Tamiflu and BioR in patients with A (H1N1) influenza was beneficial and contributed to:

- Reduction by an average of one day in the length of symptoms that affected the central nervous system, and particularly those reflecting the action of the sympathetic nervous system,
- Reduction of symptoms affecting the respiratory system (3.8 days in the experimental group and 5.3 days in the control one).
- Decreased hospitalization length in patients with influenza A (H1N1) (5.09 days in the experimental group and 6.1 in the control group).
- Decreased hospitalization length of patients with bronchopneumonia in the experimental group (5.2 days vs 7 days)

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Stem Cells in the Future of Dental Care

S. Samson[2*], V. Burlacu[2], E. Zota[1], V. Nacu[1]

[1]Laboratory of Tissue Engineering and Cell Culture, [2]Department of Preventive Dentistry
Nicolae Testemitanu State Medical and Pharmaceutical University
192, Stefan cel Mare Avenue, Chisinau, Republic of Moldova

*Corresponding author: +373 22205242. E-mail: stelutzas@g.mail.com

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Abstract

Tooth loss compromises human oral health. Although several prosthetic methods (such as artificial denture and dental implants) are clinical therapies to tooth loss problems, they are thought to have safety and usage-time issues. Recently, tooth tissue engineering has attracted more and more attention. Stem cell based tissue engineering is thought to be a promising way to replace the missing tooth. Mesenchymal stem cells (MSC) are multipotent stem cells which can differentiate into a variety of cell types. The potential MSC for tooth regeneration mainly include stem cells from human exfoliated deciduous teeth, adult dental pulp stem cells, stem cells from the apical part of the papilla, stem cells from the dental follicle, periodontal ligament stem cells and bone marrow-derived mesenchymal stem cells. This review outlines the recent progress in mesenchymal stem cell research and its use in tooth regeneration and oral and craniofacial applications.

Key words: mesenchymal stem cell, tooth engineering, dental pulp stem cell.

Стволовые клетки в стоматологии будущего

Потеря зубов ставит под угрозу человеческое здоровье. Ткани зуба у взрослых практически не способны к самостоятельной регенерации и дефект эмали, возникающий в результате действия повреждающих факторов, постепенно приводит к потере зуба. Без сомнения, современные технологии протезирования позволяют произвести реконструкцию даже при полном отсутствии зубов. Однако прогресс современной органотипической регенеративной медицины заставляет исследователей искать новые технологии замещения зубов естественными трансплантатами. Последнее время клеточная инженерия тканей зуба привлекает все больше и больше внимания. Стволовые клетки являются многообещающим способом замены недостающего зуба. Мезенхимальные стволовые клетки способны дифференцироваться в клетки костной ткани, что дает возможность использовать их для восстановления зуба. Этот обзор рассматривает современные исследования стволовых клеток и возможность их использования для стимуляции репаративной регенерации тканей зуба.

Ключевые слова: мезенхимальная стволовая клетка, инженерия тканей зуба, стволовая клетка пульпы.

Introduction

What are stem cells? As dentists, why should we be concerned with stem cells? How would stem cells change dental practice? Is it possible to grow a tooth by tissue engineering using stem cells? What should be the carrier material for stem cells? Probably, development of stem cell research will, over time, transform dental practice in a magnitude far greater than did dental implants. Metallic alloys, composites and even titanium implants are not permanent solutions. In contrast, stem cell technology will generate native tissue analogs that are compatible with that of the patient's. Dental implants are not the perfect solution for replacing missing teeth as the healing process extends for many months and rejection of the implant occurs in about 5 percent of patients. Furthermore, dental implants are expected to last for about 15 years [1]. Despite much advancement in implant technology conventional implants do not provide a truly permanent solution for a missing tooth. But the answer could lie in a highly researched new dental technique – dental implants based on stem cell technologies which could be the future of implant dentistry.

Stem cells in dental pulp were discovered in 2000 by Dr. Songtao Shi, a dental researcher at the National Institute of Health (NIH). After verification that these cells were in fact viable stem cells, the NIH announced the discovery in 2003.

The dentists treat patients because of infections, trauma, congenital anomalies or other diseases, such as orofacial cancer and salivary gland disorders. Caries and periodontal disease remain highly prevalent disorders among humans. Whereas native tissue is missing in congenital anomalies, diseases such as caries or tumor resection result in tissue defects. For centuries, dentistry has been devoted to healing defects with durable materials or the patient's own (autologous) tissue. But we now realize that metallic alloys or synthetic materials are not permanent solutions. Amalgam, composites and even titanium dental implants can fail; and all have limited service time (Rahaman A., Mao J., 2005). Why are stem cells better than durable implants such as titanium dental implants? Stem cells lead to the regeneration of teeth with periodontal ligament that can remodel to the host. Why are stem cells superior to autologous tissue grafts? Autologous tissue grafting is based on the concept that a diseased or damaged tissue must be replaced by like tissue that is healthy. Thus, the key drawback of autologous tissue grafting is donor site trauma and morbidity; the harvest of healthy bone from the patient could be taken from the iliac crest, rib bone, chin or retromolar area for bone grafting needs in cleft palate, ridge augmentation, sinus lifting, and maxillary and mandibular reconstruction. In contrast, stem cell-based therapeutic approaches may circumvent the key deficiencies of autologous bone grafting (Rahaman A., and Mao J., 2005). Stem cells from a tiny amount of tissue, such as the dental pulp, can potentially be multiplied or expanded to sufficient numbers for healing large, clinically relevant defects. Stem cells can differentiate into multiple cells lineages, thus providing the possibility that a common (stem) cell source can heal many tissues in the same patient, as opposed to the principle of harvesting healthy tissue to heal like tissue in association with autologous tissue grafting (Moioli E. K., et al., 2007).

Stem cells can be seeded in biocompatible scaffolds in the shape of the anatomical structure that is to be replaced. Stem cells may elaborate and organize tissues in vivo, especially in the presence of vascularisation. Finally, stem cells may regulate local and systemic immune reactions of the host in ways that favor tissue regeneration. Physicians and scientists have recommended that umbilical cord stem cells, placental and amniotic fluid stem cells could be banked for potential application in the treatment of trauma and pathological disorders [19].

The understanding of mesenchymal stem cells in the tissue engineering of dental, oral and craniofacial structures has advanced tremendously (Marion N., Mao J., 2006). We have witnessed tissue engineering of the tooth, temporomandibular joint condyle, cranial sutures, soft tissue grafts, craniofacial bone and other structures in animal models. With all that we have learned about stem cells and tissue engineering of dental, oral and craniofacial structures, we are in a position to bring awareness to our patients regarding the proper storage of their extracted teeth in conditions that will preserve craniofacial stem cells, including tooth-derived stem cells. These include, but are not limited to, extracted wisdom teeth, deciduous teeth and any teeth extracted for orthodontic purposes and any non-infected teeth extracted. Among postnatal tissues that are sources of stem cells that are obtainable without substantial trauma are extracted wisdom teeth, exfoliating or extracted deciduous teeth, teeth extracted for orthodontic treatment, trauma or periodontal disease. Craniofacial stem

cells, including tooth-derived stem cells, have the potential, as do bone marrow-derived stem cells and adipose-derived stem cells, to cure a number of diseases that are relevant to dentistry as well as for medicine: diabetes, Parkinson's disease, cardiac infarct etc.

Stem cells can be defined as self-replicating cells that are able to differentiate into at least two different cell types. Both conditions must be present for a cell to be considered a stem cell. For example, osteoblasts are not stem cells. Although osteoblasts differentiate into osteocytes, they typically do not differentiate into other cell types except osteocytes. Osteocytes are not stem cells; they are end-lineage cells that typically neither self-replicate and not differentiate in to another cells type [1].

Mesenchymal stem cells (MSC)

(MSC) can be isolated from different sources. First described in bone marrow, MSC have been extensively characterized *in vitro* by the expression of markers such as STRO-1, CD146 or CD44. STRO-1 is a cell surface antigen used to identify osteogenic precursors in bone marrow, CD146 a pericyte marker, and CD44 a mesenchymal stem cell marker. MSC possess a high self-renewal capacity and the potential to differentiate into mesoderm lineages thus forming cartilage, bone, adipose tissue, skeletal muscle and the stroma of connective tissues. The potential of dental MSC for tooth regeneration and repair has been extensively studied in the last years. Below, we will present the mesenchymal progenitors that have been assessed for tooth engineering purposes, such as progenitors derived from teeth (adult dental pulp, apical part of papilla, dental follicle, periodontal ligament) (fig. 1) and bone marrow [2].

Stem cells from human exfoliated deciduous teeth (SHED)

The isolation of post-natal stem cells from an easily accessible source is indispensable for tissue engineering and clinical applications. Recent findings demonstrated the isolation of mesenchymal progenitors from the pulp of human deciduous incisors. These cells were named SHED (Stem cells from Human Exfoliated Deciduous teeth) and exhibited a high plasticity since they could differentiate into neurons, adipocytes, osteoblasts and odontoblasts. *In vivo* SHED cells can induce bone or dentin formation but, in contrast to dental pulp, DPSC failed to produce a dentin-pulp complex [3].

Adult dental pulp stem cells (DPSC)

The possibility that tooth pulp might contain mesenchymal stem cells was first suggested by the observation that severe tooth damage that penetrates both enamel and dentine into the pulp stimulates a limited natural repair process, by which new odontoblasts are formed, which produce new dentine to repair the lesion (Smith A. J., Lesot H., 2001). Putative stem cells from the tooth pulp and several other dental tissues have now been identified. The first stem cells isolated from adult human dental pulp were termed dental pulp stem cells (DPSCs) [1]. They were isolated from permanent third molars, and exhibited high proliferation and high frequency of colony formation that produced sporadic, but densely calcified nodules. Additionally, *in vivo* transplantation into immunocompromised mice demonstrated the ability of DPSCs to generate functional dental tissue in the form of dentine/pulp-like complexes [2]. Further characterization revealed that DPSCs were also capable of differentiating into other mesenchymal cell derivatives *in vitro* such as odontoblasts, adipocytes, chondrocytes and osteoblasts (Koyama N., et al., 2009). DPSCs differentiate into functionally active neurons, and implanted DPSCs induce endogenous axon guidance, suggesting their potential as cellular therapy for neuronal disorders (Arthur A. et al., 2009).

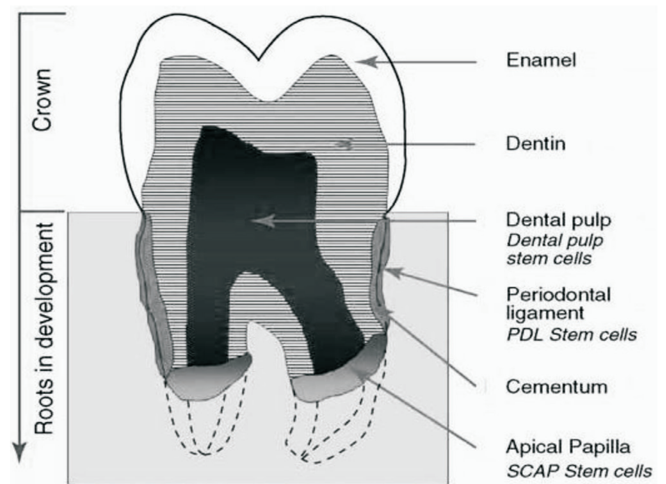

Fig. 1. Diagram of a human third molar as a source of dental stem cells. Because the tooth was in the process of erupting, root growth is incomplete, and the apical papilla is visible.

Stem cells from the apical part of the papilla (SCAP)

Recently another type of MSCs was discovered in the apical papilla of human immature permanent teeth termed stem cells from apical papilla (SCAP) (Wataru Sonoyama, Yi Liu, Takayoshi Yamaza, 2008). We found that apical papilla is distinctive to pulp in terms of containing less cellular and vascular components than those in pulp. Cells in apical papilla proliferated 2- to 3-fold greater than those in pulp in organ cultures. Both SCAP and DPSCs were as potent in osteo/dentinogenic differentiation as MSCs from bone marrows while weaker in adipogenic potential. The immunophenotype of SCAP is similar to that of DPSCs on the osteo/dentinogenic and growth factor receptor gene profiles. Double staining experiments showed that STRO-1 co-expressed with dentinogenic markers such as bone sialophosphoprotein (BSP), osteocalcin (OCN) and growth factors FGFR1 and TGFβRI in cultured SCAP. Stem cells from the apical part of the human dental papilla (SCAP) have been isolated and their potential to differentiate into odontoblasts was compared to that of the periodontal ligament stem cells (PDLSC). SCAP exhibit a higher proliferative rate and appears more effective than PDLSC for tooth formation. Importantly, SCAP are easily accessible since they can be isolated from human third molars.

Stem cells from the dental follicle (DFSC)

DFSC have been isolated from follicle of human third molars and express the stem cell markers Notch1, STRO-1

and nestin. The dental follicle is a loose of ectomesenchyme-derived connective tissue sac surrounding the enamel organ and the dental papilla of the developing tooth germ before eruption (Ten Cate, 1998). It is believed to contain progenitors for cementoblasts, PDL and osteoblasts. Dental follicle cells (DFC) form the PDL by differentiating into PDL fibroblasts that secrete collagen and interact with fibres on the surfaces of adjacent bone and cementum. DFC can form cementoblast-like cells after transplantation into SCID mice (Handa K. et al., 2002). Dental follicle progenitor cells isolated from human third molars are characterized by their rapid attachment in culture, expression of the putative stem cell markers Nestin and Notch-1, and ability to form compact calcifiednodules *in vitro* (Lin N. H. et al., 2008). DFC were transplanted into immunocompromised mice, however, there was little indication of cementum or bone formation (Lin N. H. et al., 2008). DFC, in common with SCAP, represents cells from a developing tissue and might thus exhibit a greater plasticity than other dental stem cells. However, also similar to SCAP, further research needs to be carried out on the properties and potential uses of these cells.

Periodontal ligament stem cells (PDLSC)

The PDL is a specialized tissue located between the cementum and the alveolar bone and has the maintenance and support of the teeth as a role. Its continuous regeneration is thought to involve mesenchymal progenitors arising from the dental follicle. PDL contains STRO-1 positive cells that maintain certain plasticity since they can adopt adipogenic, osteogenic and chondrogenic phenotypes *in vitro*. It is thus obvious that PDL itself contains progenitors, which can be activated to self-renew and regenerate other tissues such as cementum and alveolar bone. It was shown that cultured PDLSCs proliferate in higher rate on the rough surface especially at the 75μm Al_2O_3 particle treated surface than other surfaces. Also, osteocalcin was highly expressed on the rough surfaces treated with 75μm and 125μm Al_2O_3 particles (Heo Y. Y., Um S., Kim S. K., Park J. M., 2011).

Bone marrow derived mesenchymal stem cells (BMSC)

BMSC have been tested for their ability to recreate periodontal tissue. These cells are able to form *in vivo* cementum, PDL and alveolar bone after implantation into defective periodontal tissues. Thus, bone marrow provides an alternative source of MSC for the treatment of periodontal diseases (Kawaguchi H., 2004). BMSC share numerous characteristics with DPSC and are both able to form bone-like or tooth-like structures. However, BMSC display a lower odontogenic potential than DPSC (Yu J. et al., 2007), indicating that MSC from different embryonic origins are not equivalent. Indeed, DPSC derive from neural crest cells, whereas BMSC originate from the mesoderm. Furthermore, the comparison of the osteogenic and adipogenic potential of MSC from different origins shows that, even if cells carry common genetic markers, they are not equivalent and are already committed toward a specific differentiation pathway (Musina R. A. et al., 2006). Commitment could arise from conditioning of stem cells by their specific microenvironment or stem cell niche.

Tissue engineering

There are several areas of research for which dental stem cells are currently considered to offer potential for tissue regeneration. These include the obvious uses of cells to repair damaged tooth tissues such as dentine, periodontal ligament and dental pulp [16]. Even enamel tissue engineering has been suggested (Honda M. J. et al., 2009), as well as the use of dental stem cells as sources of cells to facilitate repair of non-dental tissues such as bone and nerves (Graziano A. et al., 2008).

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 (Nakashima M., Reddi A. H., 2003). 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 (Kneser U., Schaefer D. J., Polykandriotis E., Horch R. E., 2006); 2) An allograft and xenograft, respectively, i. e. a bone sample from other human beingsor from animals, which is osteoinductive due to certain proteins like growth factors (Richardson C. R., Mellonig J. T., Brunsvold M. A.); 3) Various osteoinductive biomaterials acting as carriers for growth factors inducing bone formation (Spiro R. C., Liu L. S., Heidaran M. A., Thompson A. Y., 2000); 4) Synthetic bone substitutes for bone replacement without or with just partially resorption or for bone repair using osteoconductive porous devices.

The different autogeneic, xenogenetic 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.

***In vitro* studies, isolation and identification procedures of dental pulp cells**

The proper isolation of cells provides the potential to differentiate into odontoblast-like cells. A lot 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 [5, 6, 7]. Tooth surface were cleaned by covering with 0.3% chlorhexidine gel [8, 9], swabbed with 70% (v/v) alcohol [10] 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 [5]. Other studies describe that teeth were cracked opened, or opened by a dentinal excavator or a Gracey curette [7, 9]. After separation of the pulp tissue, cells can be isolated by various methods. Pulp cells can be either isolated by digestion or the out-grown method [5, 9]. First, the pulp tissue can be digested in a solution of collagenase type I and dispase as reported in details by Gronthos S., et al. 2002 [5, 9]. 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-phosphates, 2 mM L-glutamine, 100 Units/ml penicillin and 100 µg/ml streptomycin [5, 11, 12]. Secondly, pulp tissue explants (4 mm) 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 with 10% FBS and antibiotics [13]. Further, human pulp primary cultures (HPPc) could be obtained by mincing tissue fragments of extracted pulps into small pieces (< 1 mm), which were then 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 [6, 7]. All cultures were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37°C and medium change should be performed every two days.

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.

Proliferation and differentiation

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) [5, 7, 9, 10, 11, 12, 13, 16], pork [14] and rat dental pulp [15]. A further cell culture system was obtained from human exfoliated deciduous teeth (SHED) (6-10 years of age) [16, 9]. Miura M., et al. 2003 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 [16].

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 [13]. 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 [5, 13]. All cell cultures display a wide range of cell morphology such as fibroblast-like cells, endothelial-like or epithelial-like cell populations. Gronthos S., 2002 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* [5, 18]. The out-grown method showed that cells are potentially capable to differentiate into odontoblasts or forming mineralized nodules *in vitro* [10, 11, 17]. Concerning the growth behavior 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, such as their high rate of proliferation, clonogenic nature [5], 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 [5, 16, 7]. 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 [7]. Previous studies have demonstrated that isolated SHED cells proliferated *in vitro* contain approximately 9% of STRO-1 positive cell population [16].

These observations agree to that of Gronthos S., 2002 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, but does not react with the hematopoietic markers CD14 (monocyte/ macrophage), CD45 (leucocyte) or CD34 (hematopoietic stem cells/endothelium). 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. The growth potential was beyond 100 population doublings and cell populations formed clonogenic cell clusters [5].

Studies have also demonstrated that cultures can be maintained after extensive subculturing of up to 20 passages after seeding isolated DPSC [5, 7]. After subculturing they are able to adhere quickly to conventional plastic dishes showing a typical fibroblastic, spindle-shape to polygonal morphology [10].

Conclusions

It is obvious that our knowledge in dental tissue engineering is expanding rapidly, and existing data confirm a realistic feasibility of dental tissue repair in the near future. 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. 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.

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 including nanotechnologies, in particular, the material would provide stability and a stimulation effect on bone tissue formation.

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