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Effekte von *Porphyromonas gingivalis* Lipopolysacchariden auf humane Progenitorzellen aus dem dentalen Follikel *in vitro*

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Effects of *Porphyromonas gingivalis* Lipopolysaccharide on human dental follicle progenitor cells *in vitro*

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Στον Όδυσσέα

Contents

List of abbreviations

APC	allophycocyanin
αMEM	alpha minimum essential medium
bFGF	basic fibroblast growth factor
BMSC	bone marrow stem cell
BSA	bovine serum albumin
CD	cluster of differentiation
CDD	comment-driven development
CPS	capsular polysaccharide
DAPI	4', 6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DFPC	dental follicle progenitor cell
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	desoxynucleoside triphosphate
dT	deoxythymidine
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EMD	enamel matrix derivative
ERK	extracellular-signal-regulated kinase
ESC	embryonic stem cell
FGF	fibroblast growth factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HSA	human serum albumin
IL	interleukin
iPSC	induced pluripotent stem cell
LPS	lipopolysaccharide

MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
MSCGM	mesenchymal stem cell growth medium
MTT	3- (4, 5- dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OS	oligosaccharide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDL	periodontal ligament
PDLSC	periodontal ligament stem cell
PE	phycoerythrin
PerCP-Cy5.5	peridinin chlorophyll protein-cyanine 5.5
PFA	paraformaldehyde
PI3K	phosphatidylinositide 3-kinase
PMN	polymorphonuclear leukocyte
RNA	ribonucleic acid
SE	standard error
SRP	scaling and root planing
STAP	stimulus-triggered acquisition of pluripotency
TDM	treated dentin matrix
TGF-β	transforming growth factor beta
TLR	toll-like receptor
TMB	3, 3', 5, 5'- tetramethylbenzidine
TNF-α	tumor necrosis factor alpha
USA	United States of Amerika
VEGF	vascular endothelial growth factor
WHO	world health organization

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1. Introduction

1.1 Periodontal regeneration

1.1.1 Tooth Morphogenesis

In the past decades, a wealth of information has become available shedding light on the processes of teeth evolution. It is now widely accepted that odontogenesis begins in the sixth week of embryonic development with the formation of the primary dental laminae (odontogenic bands). These odontogenic bands are thickened U-shaped epithelial ridges along the processes of the developing jaws (Smith 2003). Teeth morphogenesis is regulated by sequential and reciprocal inductive interactions between the oral epithelium and the underlying neural crest-derived mesenchymal cells (ectomesenchyme) of the developing first branchial arch (Martens 2013). These interactions result in teeth formation (Fig. 1.1.1) through a series of different stages (lamina stage, bud stage, cap stage, and bell stage), mediated by numerous growth factors including members of the fibroblast growth factor (FGF) and transforming growth factor beta (TGF-β) superfamilies (Kettunen 2000; Nadiri 2004). It has recently become evident that more than 300 genes, mainly involved in regulation of cellular communication, may be associated with odontogenesis and the differentiation of dental tissues (Thesleff 2006).

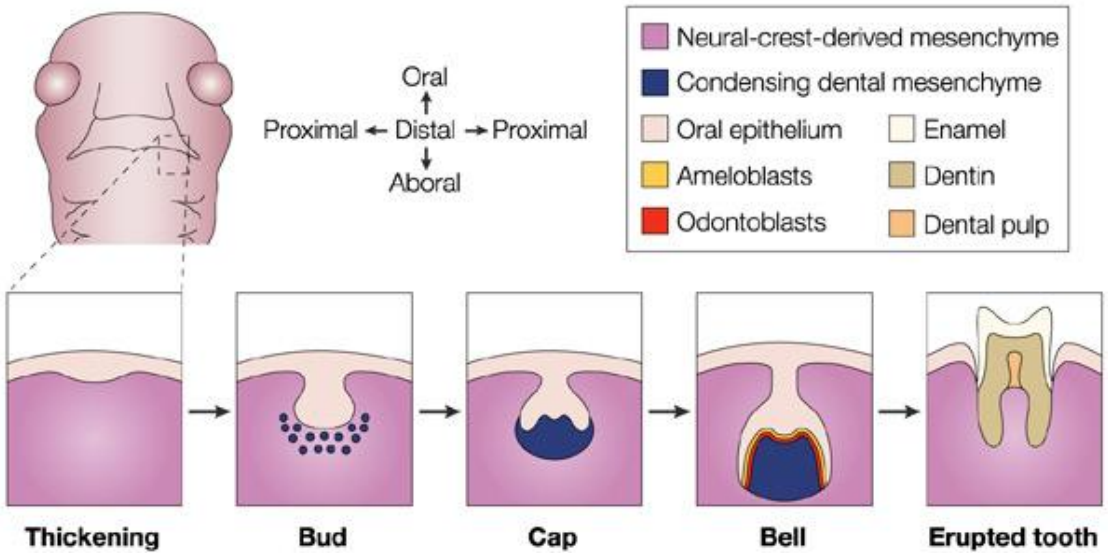


Figure 1.1.1 Schematic illustration showing different stages of human tooth formation (Tucker 2004).

The oral epithelium is speculated to produce the first inductive signals to initiate odontogenesis (Modino 2005). However, the crucial role of cranial neural crest-derived cells in teeth evolution has been demonstrated in animal models, where transplantation of mice neural crest cells into chicken embryos allowed growth of tooth germs *in vivo* (Mitsiadis 2003). Hence, the contributions of each tissue, oral epithelium and ectomesenchyme, remain enigmatic and have been the basis of numerous debates (Hammarström 1996; Ten Cate 1996).

The classical theory suggests that interactions and signals exchanged between epithelial and mesenchymal cells result in the segregation of dental epithelium into an outer layer of enamel (enamel organ) formed by ameloblasts, while dental papilla and follicle are supposed to be ectomesenchymal derivates. Particularly, dental papilla, located in the central chamber of the developing tooth bud, gives rise to dentin-forming odontoblasts and the dental pulp.

Dental follicle is described as a loose ectomesenchymally-derived connective tissue sac surrounding the enamel organ and dental papilla of the tooth germ (Schroeder 1986; Moxham 1995; Ten Cate 1997). Anatomically, dental follicle consists of three layers: the dental follicle proper (associated with the tooth), the perifollicular mesenchyme (associated with the bone) and an intermediate layer of loose connective tissue. Dental follicle plays a central role in tooth eruption, as demonstrated by animal studies showing that removal of dental follicle from teeth prevented their eruption (Cahill 1980). The developmental potential of dental follicle was extensively studied in numerous tooth transplantation experiments (Hoffman 1966; Ten Cate 1970; Palmer 1987). It has been demonstrated that dental follicle harbours progenitor cells able to give rise to all components of the periodontium, including cementoblasts, periodontal ligament fibroblasts and osteoblasts (Diekwisch 2001; Morsczeck 2005; Yao 2008; Dieu 2009). However, the precise role of dental follicle in the differentiation and maturation of these tissues remains unclear because of the proximity to other embryonic tissues, as the Hertwig's epithelial root sheath (Hoffman 1960; MacNeil 1993).

1.1.2 Anatomy of the periodontal ligament

The periodontium is a complex organ consisting of epithelial, connective and mineralized tissues that invest and support the tooth (Pitaru 1994). The structures comprising the periodontium include cementum, periodontal ligament (PDL), alveolar bone and the gingival (Fig. 1.1.2). Structural integrity and interaction between these tissues are required for the proper function of the periodontium (Nanci 2013). Its primary role is the attachment of teeth to the alveolar bone and distribution of forces to surrounding bone by tooth loads. The periodontium serves also as a sensory organ, important for the positioning of the jaws during mastication and occlusion (Trulsson 2006).

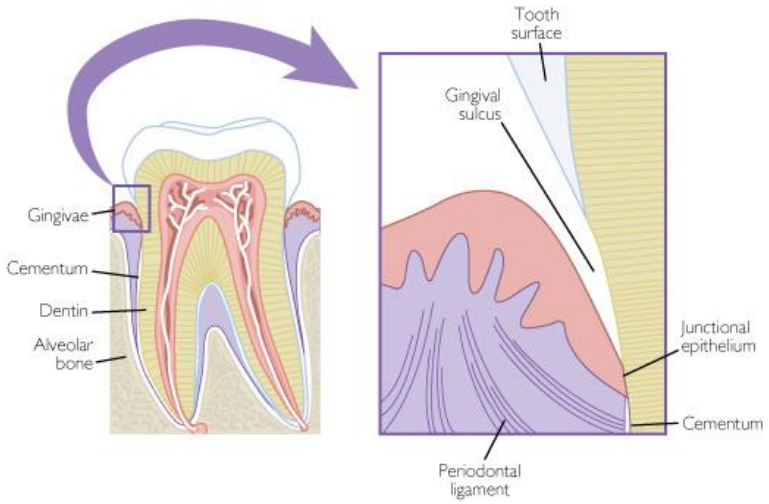


Figure 1.1.2 Schematic illustration showing structures of the periodontium (Bird 2002).

The integral part of the periodontium is the periodontal ligament, a soft, specialized connective tissue situated between the cementum and the alveolar bone. Periodontal ligament is adapted to the adjacent tissues by so-called principal fibers. These are collagenous fibers mainly consisting of collagen type I, III and XII being arranged in definite and distinct bundles and anchored in cementum or bone (Sharpey's fibers). The average width of PDL ranges from 0.21 mm at 11 to 16 years of age to 0.15 mm at 51 to 67 years, and has a hourglass shape with the narrowest area at the mid root level (Nanci 2013). The width of the ligament is dependent on vascularity, cell mitotic activity and fiber number and can adapt to forces in cases of decreased occlusal load or hyperfunction (Van der Velden 2004).

The periodontal ligament contains a unique assortment of cells including osteoblasts, cementoblasts, osteoclasts, multipotent stem cells, epithelial cell rests of Malassez, macrophages and fibroblasts, which are the most abundant cell population (Carranza 2003). Periodontal ligament fibrobasts are responsible for the synthesis of collagen and its assembly into collagen fibers and contribute to the continuous remodeling taking place in the ligament. Particularly, periodontal ligament fibrobasts are considered to be mechanoresponsive and transmit mechanical forces to the supporting alveolar bone (Wescott 2007). This load transduction is thought to initiate a process associated with changes in bone architecture needed for tooth movement and adaptation to changing mechanical loads (Diercke 2011). This process is termed bone remodeling and is characterized by continuous bone resorption and new bone formation that is mainly controlled by osteoblasts (Sandy 1993). Moreover, the periodontal ligament includes neurovascular structures that are responsible for the sensory and nutritive function of PDL. Concretely, PDL includes endothelial cells, which line the numerous blood vessels that provide nutrients to cementum and alveolar bone. The periodontal nerve branches contain a mixture of myelinated and non-myelinated axons (Nakamura 1986) transmitting mechanical stimuli able to evoke various oral reflexes (Shi 2005). Finally, it is widely accepted that periodontal ligament cells play a crucial role in homeostasis, healing and regeneration of the periodontium (Shimono 2003; Scanlon 2011; Yu 2013). This potential has been in focus of periodontal research over the past few decades (Melcher 1976; Nyman 1982).

1.1.3 Periodontal disease

Periodontal disease is a bacterially induced inflammatory disease of the periodontium. It is characterized by the progressive destruction of periodontal tissues that eventually leads to the loosening and subsequent loss of teeth (Fig. 1.1.3). It represents one of the major dental diseases that affect human populations worldwide at high prevalence rates. Specifically, it is estimated that over 47% of the adult USA population is affected from periodontitis (Burt 2005), while 28.5% of tooth extractions in Germany are attributed to the disease (Glockmann 2011). Consequently, periodontal disease can significantly affect patients' quality of life and lead to both financial and health related risks (Petersen 2005). Recent cross-sectional epidemiological studies have demonstrated that

at global level improvement of periodontal health can be possible over time (Hugoson 2008). Nevertheless, according to a report of the World Health Organization (WHO), 10 - 15% of world populations still suffer from severe periodontitis, an advanced form of the disease (Petersen 2005). Alarming is, also, the fact that diverse forms of periodontal diseases affect children, adolescents and young adult populations around the world (Albandar 2002).

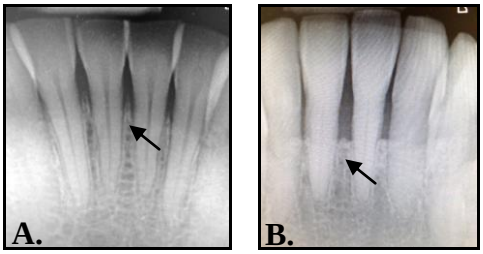


Figure 1.1.3 Radiographs featuring (A) healthy and (B) diseased periodontal tissues. Arrows indicate margins of the alveolar bone.

Periodontal disease was first described by Gottlieb as a degenerative disorder leading to a diffuse atrophy of the alveolar bone (Gottlieb 1928). Until the early 80s, the term periodontosis, coined by Orban and Weinman, was used to denote the non-inflammatory status of the disease (Orban 1942). Advances in dental research have fundamentally changed our understanding of the periodontal diseases (Baer 1971). It has been demonstrated that periodontitis is an inflammatory disease mainly caused by the presence of oral microbial biofilms. Since the first characterization of dental biofilm by van Leeuwenhoek in 1683 a great effort has been made to identify the microbiota at sites of periodontal lesions (Dobell 1958) and to determine the composition of perio-pathogenic biofilms (Holt 2005). A microbial-shift from mostly Gram-positive to mostly Gram-negative species is supposed to lead the transition process from periodontal health to periodontal disease (Darveau 2010). Moreover, it has been speculated that specific bacteria or their consortia may be responsible for the initiation and progression of periodontal disease. Specifically, the ‘red complex’, consisting of *Porphyromonas gingivalis, Treponema denticola*, and *Tannerella forsythia*, has been proposed as a ‘disease-related’ network of pathogenic bacteria, being strongly related to chronic periodontitis (Socransky 1998). Nevertheless, the impact of polymicrobial communities on the development and progression of the disease remains unclear.

Over the last few decades, the key role of immune host response has been in focus of studies on the expression of periodontitis (Darveau 1997). Results of clinical studies challenged the idea that direct effects of bacterial toxins or enzymes were sufficient for the induction of periodontitis (Hirschfeld 1978; McFall 1982). These observations were further confirmed by longitudinal epidemiologic analyses on patients susceptibility to periodontal infections (Hugoson 1982; Lindhe 1983). While the etiology of periodontitis is bacterial, it has become evident that an inadequate host inflammatory-immune response to periodontal pathogens is responsible for the disruption of tissue homeostasis in the pathogenesis of the disease (Van Dyke 2008; Darveau 2010). According to current literature, the destruction of periodontal tissues is neutrophil-mediated and may lead to chronic inflammatory processes (Van Dyke 2003). Thus, genetic polymorphisms in cytokine expression or polymorphonuclear leukocytes (PMNs) dysfunctions have been associated with periodontitis (Kornman 1998; Kinane 2001). Further, periodontal diseases were linked with major systemic diseases such as cardiovascular diseases, rheumatoid arthritis and diabetes (Beck 2000; Garcia 2001; Mealey 2006; Preshaw 2013). Such associations may reflect the presence of common underlying pathogenic mechanisms between periodontitis and general health and remain to be clarified (Armitage 2009).

1.1.4 Periodontal therapy

The ultimate goal of periodontal treatment is the restoration of homeostasis between periodontium and oral microbiota, the long-term maintenance of clinical periodontal attachment levels and the regeneration of lost periodontal supporting tissues (Van Dyke 2008). The conventional periodontal treatment involves the mechanical removal of the pathogenic dental biofilm by scaling and root planing (SRP). This therapeutic concept has proven to be the gold standard approach in the treatment of chronic periodontitis (Sanz 2012). Its efficacy is well documented in a plethora of studies (Badersten 1981, 1984; Lindhe 1984; Kaldahl 1988).

In a large-scale retrospective study, Van der Weijden *et al.* (Van der Weijden 2002) demonstrated successful clinical outcomes such as probing depth reduction and gain of clinical attachment after subgingival debridement. Furthermore, a meta-analysis conducted by Hung *et al.* demonstrated the positive effects of SRP in the reduction of

probing depth and stabilization of attachment loss, after treatment of medium and deep periodontal defects (Hung 2002). Analyses of subgingival plaque samples revealed alterations in the composition of subgingival microflora after SRP (Mousquès 1980; Müller 1986). Particularly, Haffajee *et al.* confirmed a decrease in prevalence of *P. gingivalis* and other periopathogenic bacteria at sites of improved periodontal attachment levels post-therapy (Haffajee 1997) However, histological analyses of healed periodontal tissues reveal in most of the cases the presence of an epithelial lining along the treated root surfaces of the teeth, instead of true periodontal regeneration (Caton 1993). Moreover, it has been showed that clinical outcomes of SRP may vary greatly and are dependent on several parameters such as the extent and severity of disease and patient's compliance with plaque control (Page 1997; Sanz 2012).

Technological advances and a better understanding of the biology of periodontal tissues enabled the introduction of modifications in standard periodontal treatment. New debridement technologies (Tunkel 2002), full-mouth disinfection protocols (Quirynen 1995) antibiotic administration (Haffajee 2003), implantation of autografts, allografts and alloplastic materials (Sculean 2008), chemical root conditioning (Maruyama 2008), growth factors (Raja 2009) and guided tissue regeneration (Sculean 2004) represent some of the therapies or techniques that have been introduced in the conventional periodontal treatment protocol (Fig. 1.1.4). These adjunctive methods used either alone or in combination may result in some cases in histological evidence of bone repair (Sculean 2008). However, the results in clinical applications are marginal and vary greatly, depending on the anatomy of periodontal defects or the amount of resident vital periodontal ligament (Blumenthal 1993). In conclusion, these advanced therapeutic interventions have proved to be insufficient to attain complete and predictable regeneration of the periodontium (Becker 1999; Bartold 2000; Chen 2010).

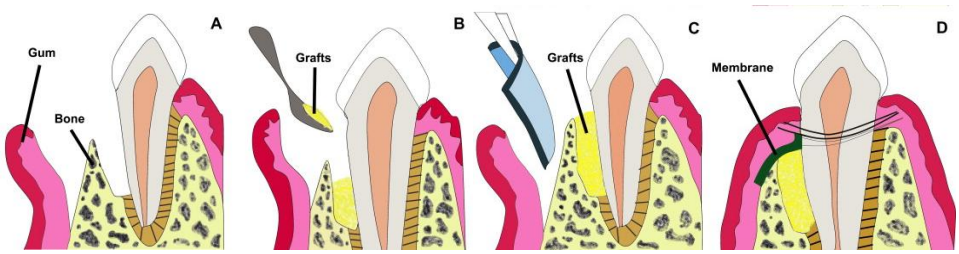


Figure 1.1.4 Schematic illustration of guided tissue regeneration technique (Chen 2010).

1.1.5 Rationale for regenerative periodontal therapy

In recent years, great improvement has been made in understanding the cellular and molecular events involved in the formation and regeneration of periodontal tissues. Interestingly, it has been demonstrated that dental tissues contain populations with characteristics of postnatal stem cells (Hynes 2012). The identification of these multipotent cells has stimulated interest in the potential use of cell-based therapies as prospective alternatives to existing therapeutic approaches for the repair and regeneration of the periodontium (Bartold 2006).

Aim of regenerative periodontal therapy is the predictable restoration of the periodontium, including the formation of periodontal ligament, cementum with inserting periodontal ligament fibres and alveolar bone that have been lost due to periodontal disease or dental trauma (Polimeni 2006). From a biological perspective, one of the critical requirements for successful therapeutic approaches is the repopulation of the periodontal wound by *ex vivo* expanded progenitor populations or the mobilization of endogenous progenitor cells capable of promoting regeneration (Ivanovski 2006).

Dental-derived stem cells are putative candidates for restoration of the complex ultrastructure and the dynamic function relationships between the periodontal components that are important for normal tissue homeostasis. Numerous animal studies have proved the regenerative potency of these cell populations *in vivo* (Trofin 2013).

However, one of the growing concerns in dental research is the exposure of dental-derived progenitor cells to the endotoxin-rich microenvironment of periodontal pockets (Morsczeck 2012). This may affect many cell properties such as self-renewal, differentiation potential, production of cytokines and extracellular matrix (ECM) compounds secretion. Moreover, Sorrell and Caplan demonstrated that multipotent cell grafts might trigger regenerative processes through direct commitment together with paracrine communication with resident cell populations and infiltrating inflammatory or antigen-presenting cells (Sorrell 2010). These interactions provide a regenerative microenvironment for destructed adult tissues to limit the area of damage and to impel a self-regulated regenerative response (Caplan 2007). Hence, a better understanding of cell behavior at sites of inflammation appears to be a key strategy for the development of new approaches for periodontal regeneration.

1.2 Human dental follicle progenitor cells

1.2.1 Stem cells of non-dental origin

In recent years, tissue engineering has emerged as a promising approach that could enable regeneration of diseased tissues, by cell transplantation with or without scaffolds (Langer 1993). The potential use of tissue engineering approaches are endless, and range from preclinical generation of cardiac valve substitutes, to *ex vivo* construction of nasal cartilages, or even to whole organ substitutes such as liver (Stock 2001). Beside the restricted actual clinical feasibility, pilot studies highlight great prospects for future stem cell-based tissue engineering techniques. This progress is mostly attributed to the advances in stem cell biology and recognition of the unique biological properties of stem cells (Eberli 2006).

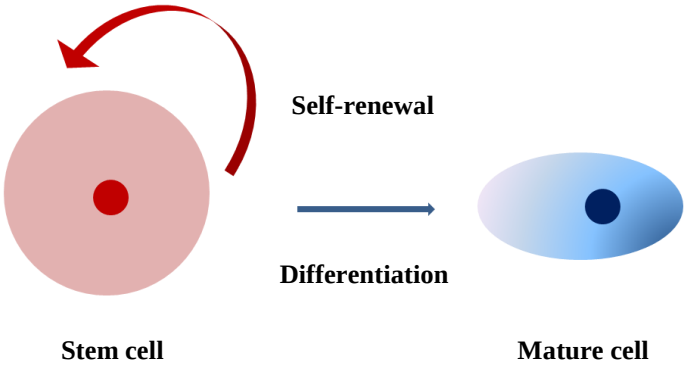


Figure 1.2.1 Graphic illustration of the two main characteristics of stem cells.

Stem cells are defined by their capacity to self-renew and differentiate into multiple cell lineages (Fig. 1.2.1). One of the most studied adult stem cell types are mesenchymal stem cels (MSCs) (Pittenger 1999). Friedenstein *et al.* first described bone marrow stem cells (BMSCs) as a heterogeneous population of multipotent cells derived from bone marrow aspirates with the ability to adhere to plastic surfaces and form colonies of fibroblastic-like cells within the first days of cultivation (Friedenstein 1970, 1976). Although MSCs were originally isolated from bone marrow, similar populations of mesenchymal precursors were isolated from other tissues, including adipose tissue (Zuk 2001), amniotic fluid (Roubelakis 2011), fetal liver (Fiegel 2006) and umbilical cord

blood (Kögler 2004). Recent studies indicated that MSCs are able to differentiate not only into cells of the mesoderm lineage, but also into endoderm and neuroectoderm lineages, including neurons (Sanchez-Ramos 2000), hepatocytes (Schwartz 2002) and endothelia (Janeczek Portalska 2012). MSCs can be easily isolated and have a high expansion potential and genetic stability giving unlimited pool of transplantable cells (Wang 2012). In addition, MSCs are able to migrate to sites of tissue injury and have immunosuppressive properties that can be crucial for successful autologous as well as heterologous transplantations (Le Blanc 2005). MSCs are already utilized in several fields of regenerative medicine. Indeed, MSCs have been successfully integrated in orthopaedic therapeutical interventions facilitating the repair of bone (Mankani 2001) and cartilage (Murphy 2003). Furthermore, BMSCs have been proposed as candidates for cell-based cardiac regeneration therapies (Stamm 2003; Tongers 2011; Donndorf 2013).

Although human BMSCs are not isolated from oral tissues, they have already been extensively studied as putative candidates regarding the regenaration of periodontal tissues. Kawaguchi *et al.* observed in an animal model that BMSCs transplanted into Class III furcation lesions, were able to form new cementum, bone and periodontal ligament in the periodontal osseous defects (Kawaguchi 2004). In an *in vivo* study, Hasegawa *et al.* used BMSCs labeled with green fluorescent protein, and confirmed that after transplantation BMSCs could survive and differentiate into periodontal tissue cells (Hasegawa 2006). It has been proposed that BMSCs are able to sense biological signals, interact with the local microenvironment and contribute to the regeneration of periodontal tissues (Yang 2010). Clinical trials using autologous BMSCs transplantation in periodontal defects combined with platelet-rich plasma showed positive clinical outcomes (Yamada 2006). However, these approaches require an invasive bone marrow aspiration procedure in a secondary clinic and are associated with donor site complications (Chen 2012).

Apart from bone marrow, adipose-derived stem cells (ASCs) have also been investigated in periodontal regeneration studies. ASCs exhibit stable growth kinetics *in vitro* and possess multilineage differentiation ability, while having similar phenotype and genotype to those of BMSCs and DPSCs at the transcriptional level (Hung 2011) (Izadpanah 2006; Zhu 2008). Several animal models have already confirmed the potential of ASCs to promote periodontal regeneration *in situ* (Tobita 2008, 2010).

The use of embryonic stem cells (ESCs) has been newly proposed as an alternative source for regenerative medicine, because of their combined abilities of unlimited expansion and pluripotency. The differentiation capacity of human ESCs towards periodontal ligament cells were recently evaluated *in vitro* (Inanç 2009). However, the clinical application of these unique cell types raises serious ethical and safety concerns.

Finally, the induced pluripotent stem cell (iPSCs) technology represents a major hope associated with the development of personalised cell therapies for treating human disease, as periodontitis (Hynes 2013). This technological advance allows the generation of pluripotent cells by nuclear reprogramming of adult cells via genetic manipulation or introduction of multiple transcription factors (Takahashi 2006; Huangfu 2008). Duan *et al.* have recently demonstrated that iPSCs combined with enamel matrix derivatives (EMD) could be valuable candidates for periodontal tissue engineering approaches (Duan 2011). Alternative approaches, as the newly proposed method of stimulus-triggered acquisition of pluripotency (STAP), could enhance these therapeutic attempts. STAP technology is based on cell nuclear reprogramming triggered by external stimuli such as a transient low-pH stressor (Obokata 2014). Nevertheless, researchers should overcome several biological unknowns, technical hurdles and safety concerns in order to integrate nuclear reprogrammed mammalian cells in clinical therapeutic approaches (Csete 2010).

1.2.2 Dental-derived progenitor cells

During the last decades, rapid progress in dental research has shed light on the molecular and cellular biology of periodontal tissue development. The identification of undifferentiated multipotent cells in the developing, but also in the mature periodontal ligament has inspired researchers to use them in cell-based regenerative approaches (Cho 2000). These multipotent cell populations retain the potential to differentiate into osteoblasts, cementoblasts and fibroblasts (McCulloch 1984). Experimental studies in mice suggest that periodontal ligament stem cells (PDLSCs) niches are perivascular sites in the periodontal ligament and adjacent endosteal spaces (Gould 1977; McCulloch 1987). According to an animal study in rats, Roberts *et al.* suggested that PDLSCs undergo a vascularly oriented differentiation during migration to the bone and cemental surfaces (Roberts 1987). Apart from resident PDLSCs, *ex vivo*-explanted

PDLSCs are, also, able to promote regeneration of typical cementum/ periodontal ligament-like structures (Seo 2004).

Until now, numerous animal models have confirmed the capacity of PDLSCs to regenerate PDL tissues *in vivo* (Liu 2008b; Ding 2010). Specifically, Lang *et al*. demonstrated the formation of periodontal ligament-like connective tissues with orientated fiber bundles attached to both host bone and root, after replantation of PDLSCs-covered roots in a large animal model (Lang 1995). Kim *et al.* conducted *in vivo* studies providing further insides in the sequential histological changes during periodontal tissue regeneration by hPDLSCs (Kim 2012). Based on these promising preclinical results, PDLSCs were first candidates for tissue engineering techniques.

At present, PDLSCs, as well as BMSCs, are used as main cell sources for periodontal regeneration (Feng 2010; Hoogduijn 2013). Nevertheless, the results of such therapeutical interventions remain marginal and unpredictable. Interestingly, recent findings suggest that PDLSCs proliferation and differentiation potential may be influenced by donor age and microenvironment factors (Zheng 2009). Such observations highlight the need of cautious interpretation of study results. Further elucidation of the molecular mechanisms governing stem cell differentiation could improve cell-mediated therapeutical approaches against periodontal disease (Chen 2012).

Newly identified dental-derived stem cells have been proposed as alternative cell sources. In particular, multipotent cells have been successfully isolated from several dental tissues as dental pulp (Gronthos 2000), dental follicle (Morsczeck 2005), exfoliated deciduous teeth (Miura 2003) and root apical papilla (Sonoyama 2006). A plethora of *in vitro* and *in vivo* studies on dental-derived stem cells provide evidence of their multi-differentiation capacity and their potential role in periodontal regeneration (Chen 2012).

1.2.3 Dental follicle progenitor cells

Advances in the field of periodontal regeneration facilitated the development of cell-based periodontal therapies. Recently identified dental-derived stem cells have been proposed as putative candidates for such therapeutical approaches. Specifically, dental follicle stem cells represent a population of precursor cells originating from the loose ectomesenchyme-derived connective tissue sac that surrounds the developing tooth germ prior to eruption (Ten Cate 1997). After the extraction of impacted third molars, dental follicles are commonly discarded as medical waste. However, DFPCs can be isolated from extracted dental follicles for research purposes, thus representing an easily accessible cell source.

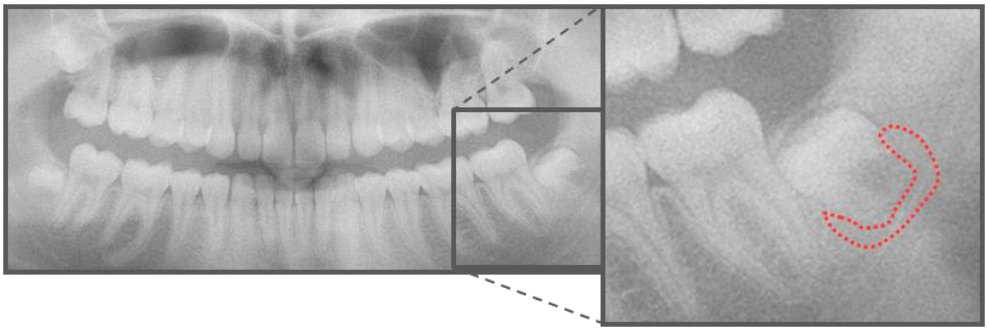


Figure 1.2.3 Panoramic radiograph of a 17 years old patient showing wisdom tooth follicle.

Dental follicle is considered as a source of multipotent cells with the ability to generate all periodontal tissues, namely cementum, bone and PDL (Honda 2010). These precursor cells are present in dental follicle at various stages of differentiation (Luan 2006; Yao 2008). Handa *et al.* first reported the presence of progenitor cells in animal dental follicle. In their study, cultured bovine dental follicle progenitor cells (DFPCs) were able to form cementum tissues and fibroblasts *in vivo* (Handa 2002). In 2005, Morsczeck *et al.* were able to isolate multipotent cells from dental follicles of human impacted third molars and described their stem cell characteristics (Morsczeck 2005). Subsequent investigations on human and animal DFPCs shed light to their regenerative potential. In particular, several studies demonstrated that DFPCs have a fibroblast-like morphology, are plastic-adherent and show excellent proliferation rates (Guo 2012). Until now there is no rigid expression pattern of stemness-related genes (Kemp 2005). Nevertheless, a broad set of markers has been proposed to define stem cell cultures.

Similar to other stem cell populations, DFPCs express CD10, CD13, CD29, CD44, CD53, CD59, CD73, CD90 and CD105, and do not express CD34, CD45, or HLA-DR (Lindroos 2008; Huang 2009). Demonstration of self-renewal ability and multilineage differentiation capacity are additional indications of stem cell phenotype. Indeed, it has been proven that DFPCs are able to form single cell-derived colonies and differentiate into several lineages, when induced by special media *in vitro* (Yao 2008).

Moreover, the regeneration potential of DFPCs has been supported by a series of animal experiments. Recently, Yokoi *et al.* demonstrated in an animal model the ability of DFPCs to regenerate the periodontal ligament *in vivo* (Yokoi 2007). Further, Guo *et al.* investigated the potential of dental follicle stem cells to contribute to the formation of the tooth root (Guo 2012). Interestingly, DFPCs combined with treated dentin matrix (TDM) scaffolds contributed to the formation of root-like tissues with a pulp-dentin complex and a periodontal ligament connecting a cementum-like layer to host alveolar bone (Guo 2012). Recent data indicate that dental follicle cells induced by Hertwig's epithelial root sheath cells may form periodontal tissues *in vivo* through epithelial-mesenchymal interactions (Bai 2011). However, the mechanisms governing DFPCs regeneration potency remain to be elucidated.

1.3 *Porphyromonas gingivalis*

1.3.1 General characteristics

Porphyromonas gingivalis (formerly *Bacteroides gingivalis*) is a Gram-negative, non-motile, asaccharolytic bacterium. It requires anaerobic conditions for growth, exhibits both cocco-bacillary to short rod morphologies and forms smooth to rough colony morphotypes (Reynolds 1989; Haffajee 1994). The species belongs to the black-pigmented *Bacteroides* group and can form brown to black colonies when cultured in blood agar plates (Oliver 1921). The black pigmentation of *P. gingivalis* colonies is correlated to the accumulation of hemin (oxidized form of heme) on its cell surface (Liu 2004; Smalley 2008). Furthermore, it is postulated that heme has a profound effect on virulence of *P. gingivalis*, as when grown under hemin-limited conditions, it becomes less virulent (McKee 1986; Lewis 1999).

When considering its role in the establishment of multispecies subgingival biofilm communities, *P. gingivalis* is classified as a late colonizer, capable to adhere to oral surfaces and interact synergistically with antecedent biofilm inhabitants (Capestany 2008; Kolenbrander 2011). The species is intensively invasive, as demonstrated in several *in vitro* studies (Sandros 1993; Weinberg 1997; Houalet-Jeanne 2001). Particularly, *P. gingivalis* is able to invade and remain viable in host cells (Andrian 2006; Amano 2007), while suppressing host cell apoptosis (Yilmaz 2008; Yao 2010).

The major ecological habitat of *P. gingivalis* is the gingival sulcus. Moreover, the organism can be detected in tongue coat, tonsils, oral mucous membranes and saliva samples (Zambon 1981; Danser 1996; Tanaka 2004). In addition to the oral niches, *P. gingivalis* can also spread systemically and be found in distant sites as demonstrated for atheromatous plaques (Kozarov 2006), osteomyelitis lesions (Welkerling 2006), amniotic cavity (León 2007), respiratory tract (Scannapieco 1999; Mojon 2002) and cerebrospinal fluid (Iida 2004).

Therefore, the biological significance of the species has been in focus of research in the last decades. The potential effects of *P. gingivalis* on several host cell types, including not only oral epithelial cells and fibroblasts, but also dendritic cells, macrophages, neutrophils, endothelial cells have been extensively discussed in previous reviews (Kantarci 2002; Amano 2003; Bélanger 2006; Cutler 2006; Hajishengallis 2007; Kinane 2008; Sheets 2008; Yilmaz 2008). The impact of several perio-pathogenic bacteria, as *P. gingivalis*, on BMSCs and dental-derived stem cells has been recently demonstrated, thus revealing new insights into the interactions between live bacteria and multipotent stem cells (Kriebel 2013).

1.3.2 Association with periodontal disease

The oral cavity is habitat for a plethora of bacterial species. Specifically, it is estimated that the oral microflora consists of more than 700 different bacterial species, which normally coexist in commensal harmony with the host (Moore 1994; Aas 2005). Despite the enormous diversity of the oral microbiome, only some of these species are considered to have an impact on the initiation and progression of periodontitis (Paster 2006).

In recent years, the use of advanced techniques for identification of microorganisms, in combination with the relative non-invasive nature of sampling, facilitated the conduction of thorough analyses of the oral microbiota in both health and disease (Haffajee 2008). These studies led to the identification of *P. gingivalis* as a major contributor to periodontal disease and member of the 'red complex' pathogens, comprising *T. forsythia* and *T. denticola* (Socransky 1992; Lamont 1998; Holt 2005). Specifically, several studies based on *in vivo* experimental models demonstrated that *P. gingivalis* may induce dysbiosis, or a microbial shift in the commensal composition, ultimately leading to periodontitis (Baker 2000; Page 2007; Hasturk 2007; Hajishengallis 2011). Increased incidence of *P. gingivalis* is positively correlated to destructive forms of periodontal disease (Van Winkelhoff 2002; Kawada 2004). Besides, reduced levels of *P. gingivalis* are associated with clinical improvement at diseased sites after periodontal treatment (Haffajee 1997; Takamatsu 1999; Ximénez-Fyvie 2000; Fujise 2002).

Even though a large body of evidence on the pathogenic role of *P. gingivalis* in disease formation exists, the pathogenesis of infection is still not fully understood (Yilmaz 2008). Hence, the proposed idea of using *P. gingivalis* as a prognostic marker for periodontitis is open to dispute (Leonhardt 2011). Remarkably, studies on the composition of subgingival species in or on the sulcular gingival epithelial cells showed no significant differences in the levels of *P. gingivalis* in the epithelial samples obtained from healthy or diseased subjects (Colombo 2006, 2007). Indeed, it has been recently proposed that the oral epithelium of healthy subjects, may exhibit a natural tolerance towards oral microbiota (Jump 2004; Rudney 2006). Parallely, the notion that *P. gingivalis* may act as an opportunist, that under certain circumstances is able to subvert host defence, is now well established (Bostanci 2012). In conclusion, the sophisticated mechanisms behind the reciprocal interactions between perio-pathogenic bacteria and host cells appear to be highly complex and remain to be elucidated (Curtis 2005; Yilmaz 2008).

1.3.3 *P. gingivalis* LPS

It is widely accepted that *P. gingivalis* produces a range of potential virulence factors. These exhibit multiple functions and are part of the proposed survival strategies of *P. gingivalis* into the host (Holt 2005). Specifically, a major virulence factor of *P. gingivali* is its capsular polysaccharide (CPS), also known as K-antigen (Brunner 2010). It is demonstrated to be involved in the modulation of host immune mechanisms by circumvention of phagocytosis (Singh 2011). Further, gingipains of *P. gingivalis* are extracellular cysteine proteases, also present in soluble form (Bostanci 2012). Gingipains are demonstrated to possess proteolytic and adhesion domains and are involved not only in the degradation or cleavage of host cell proteins, but also the attachment of *P.* gingivalis to the tissues (Potempa 2000; Curtis 2005; Sheets 2008). Adhesins such as fimbriae, haemagglutinins, a putative invasin (haloacid dehalogenase family phosphoserine phosphatase) and a variety of toxic by-products (e.g. ammonia) are also included in the panel of the organism virulence factors (Yilmaz 2008).

The major component of the outer membrane of *P. gingivalis* is LPS, an endotoxin and common characteristic of all Gram-negative bacteria. The long polysaccharide chains of LPS are able to stimulate the complement system and provoke the release of pro-inflammatory molecules, thus initiating host immune responses (Siqueira 2007). Moreover, LPS plays a crucial role in maintenance of bacterial structural integrity and establishment of selective permeability barrier to noxious compounds or nutrient molecules (Shoji 2002; Nikaido 2003). Therefore, LPS is supposed to be essential for the survival of *P. gingivalis* within host cells (Jain 2010).

Structurally, LPS comprises three domains: an O-antigen, a core oligosaccharide (OS) and lipid A. The O-antigen may be a long polysaccharide that comprises the outermost domain of the LPS molecule and forms the external surface of the bacterium. The structure of O-antigen is highly variable and immunogenic. Thus, O-serotyping is used to distinguish between sub-species of bacteria according to O-antigen composition (Sims 2001). The core OS contains a vast variety of glycoforms and is typically composed of two domains, the outer and inner core being directly attached to the O-antigen and lipid A, respectively (Paramonov 2009). The inner-most component of LPS, the hydrophobic lipid A, is embedded in the bacterial outer membrane of Gram-negative cell wall and serves as an anchor for LPS. It is suggested that *P. gingivalis*

lipid A may be responsible for the strong innate immune response at sites of infection (Wang 2002). Interestingly, *P. gingivalis* is able to synthesize a variety of lipid A structures, as result of its exposure to several environmental conditions. Thus, it is demonstrated that high concentrations of hemin, at sites of severe periodontal inflammation may provoke the production of antagonistic lipid A that is able to activate host cell receptors (Jain 2010). Hence, the heterogenous structure of lipid A could explain the binding of *P. gingivalis* LPS to several cognate TLR receptors and the activation of differential immune signalling pathways (Bostanci 2012).

1.3.4 Target cell receptors of *P. gingivalis* LPS

In general, cells recognize the presence of bacteria and bacterial components via so-called pattern recognition receptors (PRRs). Specifically, PRRs sense typical patterns of microbial molecules, known as pathogen-associated molecular patterns (PAMPs) (Janeway 1989). Toll-like receptors (TLRs) are the first identified and best-characterized group among the human PRRs. The family of TLRs contains to date 10 members in humans (Staquet 2011). TLRs are type I transmembrane proteins and are able to recognize a wide range of PAMPs (Kawai 2011). Upon PAMP engagement, TLRs are able to trigger transcriptional or post-translational cellular responses (Vance 2009). In fact, *P. gingivalis* LPS may induce the production pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-6, and IL-8 in host cells (Wang 2002; Zhou 2005; Hamedi 2009). Especially, LPS sensing via TLRs is considered to play a critical role in signal transduction at sites of periodontal inflammation (Wang 2002).

Two members of the Toll-like receptor family, TLR2 and TLR4, have been identified as possible signaling receptors of *P.gingivalis* LPS (Darveau 2004). These receptors have been detected in gingival epithelium and connective tissue samples (Beklen 2008). Moreover, TLR2 and TLR4 are demonstrated on many cell types including immune cells, macrophages and dendritic cells (Schuman 1990; Wright 1990; Flo 2001; Kaisho 2002). According to a large body of evidence, TLR4 is documented as a specific receptor for most bacterial LPS (Noreen 2012). Particularly, several *in vitro* and *in vivo* experimental studies have demonstrated *P. gingivalis* LPS as potent agonist of TLR4 (Ogawa 2002; Sawada 2007; Kumada 2008; Zhang 2008). Further, TLR2 has been

identified as a predominant receptor for *P. gingivalis* LPS, even though it is mainly involved in the recognition of lipoproteins, lipoteichoic acid and peptidoglycans (Siqueira 2007). Interestingly, *in vivo* studies in TLR2 or TLR4 knock-out mice demonstrated the induction of bone loss after *P. gingivalis* treatment via TLR2, but not TLR4 (Hirschfeld 2001; Burns 2006). On the contrary, other experimental studies suggested the activation of both TLR2 and TLR4 which, nevertheless, can lead to activation of different signalling cellular cascades (Bainbridge 2001; Zhou 2005). These contradicting results could be explained by the ability of *P. gingivalis* to alter its lipid A structure. This LPS modification could modulate its binding affinity to TLRs resulting in opposing host innate immune responses and increased survival chance for the species (Bostanci 2012).

1.4 Hypothesis statement

The rapid advancements in the field of dental research over the last few years could deliver the promise of tissue regeneration through stem cells. The demand for novel therapies against inflammatory diseases, like periodontitis, has created the need for a better understanding of the behaviour of progenitor cells in sites of bacterial infection.

Specially, *P. gingivalis* has been considered as protagonist in the initiation and development of inflammatory processes leading to periodontitis (Hamada 1994; Tobias 1997). Particularly, *P. gingivalis* LPS is supposed to able to stimulate inflammatory cytokine production and bone resorption. Most studies on cellular effects of periodontopathic bacteria focused on gingival fibroblasts (Wang 2002; Tardif 2004; Herath 2011) and periodontal ligament cells (Seo 2012), which are mostly involved in the remodelling of the periodontium. Also osteoclasts, a key cell population involved in bacteria-induced bone destruction, have been in focus of research (Chen 2001; Scheres 2011). Nevertheless, only few studies have been done so far investigating the influence of bacterial components on osteoblast progenitors (Loomer 1994, 1995; Kadono 1999). Further, Chang *et al.* investigated dental pulp stem cells treated with LPS and detected activation of NF-κB (Chang 2005), while Yamagishi *et al.* analyzed the effects of *P.gingivalis* LPS on dental pulp stem cell differentiation ability (Yamagishi 2011). Till now most studies on DFPCs have focused on cell characterization and tissue regeneration potential (Bai 2011; Jung 2011; Guo 2012; Yang 2012). Nevertheless, little is known about the impact of bacteria on DFPCs properties.

Here we hypothesized that DFPCs are able to sense and respond to *P. gingivalis* LPS. Further, DFPCs responses were speculated to be differential in comparison to other populations of multipotent cells. Aim of the study was to investigate the possible influence of LPS on cell proliferation, gene expression, wound healing capability and cytokine production of DFPCs. In this context, DFPCs were compared with early passages of bone marrow-derived stem cells (BMSCs), a well-studied class of adult stem cells.

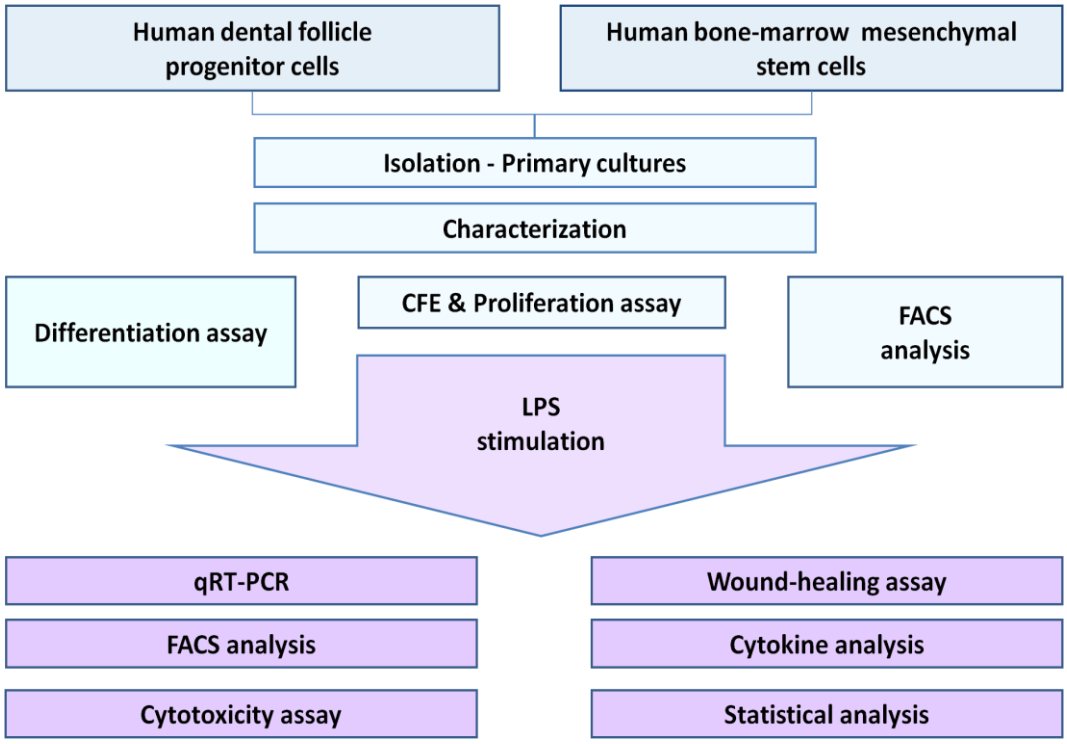


Figure 1.4 Experimental design of the study.

2. Materials and Methods

2.1 Cell isolation and culture

2.1.1 Isolation and culture of human DFPCs and BMSCs

Human impacted third molars (n = 6) were surgically removed and collected from patients (aged 17 to 23 years) at the Rostock university school of dental medicine. Patient inclusion and exclusion criteria are shown in Table 2.1.1. Patients must have met all the criteria to be eligible for participation in the study. Written informed consent was obtained from all patients or their legally authorized representatives, following approved guidelines set by the commission of ethics of Rostock university school of medicine (Reg.Nr: A 2010 87).

Inclusion criteria
- patients had to be between fifteen (15) and twenty five (25) years old
- patients required surgical extraction of impacted third molars because of medical reasons independent of this research project
Exclusion criteria
- patients with wisdom teeth under periodontal or endodontic treatment
- patients with developmental dental anomalies (e.g. tooth fusion or genimation)
- patients having any systemic diseases (e.g. diabetes mellitus)

Table 2.1.1 Patient inclusion and exclusion criteria.

Teeth extractions were conducted under local anesthesia by a team of two cooperating oral surgeons. After elevation of a full-thickness flap, maxillar/ mandibular bone over the impacted teeth was excised by round diamond burs (head dia 2.3 to 2.9 mm) under irrigation with sterile saline, to prevent tissue damage. During sample collection, operators avoided contact with oral mucosa thus minimizing bacterial contamination risks. The freshly extracted tissues were immediately placed into sterile plastic tubes containing ice-cold basic culture medium (aMEM) supplemented with 1% of penicillin streptomycin and transferred to the laboratory within 30 minutes in a sealed plastic box. On arrival at the laboratory the specimens were placed in 60 mm tissue culture dishes for further manipulations under aseptic conditions provided by a laminar flow cabinet.

Dental follicles were separated from teeth by the use of sterile curettes and a pair of fine-tipped forceps. After extensive washes with PBS, follicle tissues were minced into 0.5 to 2 mm pieces with a single-use scalpel and digested in culture medium supplemented with 0.1 U/ mL collagenase and 0.8 U/ mL dispase for 1 hour at 37°C. Explants were then transferred to T-25 cell culture flasks and cultivated in a mesenchymal stem cell growth medium (MSCGM) at 37°C in a 5% CO_2 humidified atmosphere. Single cells attached to the plastic surface within 24 hours and non-adherent cells were removed by gently washing the plates three times with PBS. Finally, fresh culture medium was added and cells were left for incubation (Fig. 2.1.1).

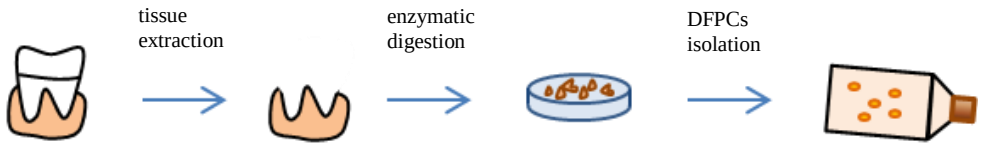


Figure 2.1.1 Schematic overview of DFPCs isolation procedure.

Human mesenchymal stem cells processed from bone marrow aspirates of human adult volunteers (n = 8) were generously provided by the Reference and Translation Center for Cardiac Stem Cell Therapy (RTC) at Rostock University. Informed consent was obtained from all patients according to the Declaration of Helsinki. BMSCs were isolated according to the standard isolation protocol of the RTC laboratory (Gaebel 2011). After isolation, BMSCs were cultivated in MSCGM under standard culture conditions and served as an experimental control group.

Material description	Type	Company
articain solution	Ultracain 2% DS	Sanofi-Aventis
round diamond burs	2.3 to 2.9 mm dia	Komet Dental
scalpel blade	Nr 10, 15	Henry Schein Dental
scalpel handle	12.5 cm length	Henry Schein Dental
root elevator	2mm round-tip	Henry Schein Dental

Material description	Type	Company
periosteal elevator	6.5 mm round-tip	Henry Schein Dental
gracey curette	1/2	Hu-Friedy
collagenase / dispase	0.1 U/mL / 0.8 U/mL	Roche
cell culture flasks	Cellstar, T-25	Greiner Bio-One
petri dishes	Cellstar, 60 mm dia	Greiner Bio-One
penicillin	100 U/ mL	PAA
streptomycin	100 µg/ mL	PAA
αMEM	with L-Glutamine	PAN-Biotech
MSCGM	culture medium	Lonza
PBS	with Ca and Mg	PAN-Biotech

2.1.2 Cell culture of human DFPCs and BMSCs

Cell culture was performed under sterile conditions in a 5% CO_2 incubator at 37 °C. All cell culture manipulations were done under a laminar flow hood. Work surfaces were wiped down with 70% ethanol before and after each use. Inside the hood all work was done with sterile pipette tips. All supplies and reagents that came into contact with the cell cultures were sterile. Solutions were divided into small aliquot tubes whenever possible in order to reduce the possibility of microbial contamination. Cultures were inspected every 2 to 3 days under a phase contrast microscope (magnification 400x) for signs of contamination. Any contaminated cell cultures were immediately discarded.

Cells seeded in culture flasks grew in mesenchymal stem cell growth medium supplemented with 1% of penicillin and streptavidin. Culture medium was replaced every 2 to 3 days until cells reached 70 - 80% optical confluence. In order to produce large number of cells for the subsequent experiments, cell passaging was required. Thus, culture media were removed from flasks and cells were washed twice with PBS. Pre-warmed 0.05%/ 0.02% (w/ v) trypsin/ EDTA solution was added and cells were incubated for 7 minutes to detach. After incubation, a 9fold volume of pre-warmed culture medium was added to stop trypsin reaction and cells were carefully resuspended to a single cell suspension. Then cell suspension was transferred to a 15 mL conical

tube and centrifuged at 300 g for 10 minutes. After centrifugation, supernatants were removed and cells were aspirated and resuspended in fresh culture medium. Cell number was determined with a hemocytometer. For that 10 µL of cell suspension was stained with trypan blue (0.4% w/ v in PBS) and introduced into the sink of the chamber to be counted under the microscope (100x magnification). Cell number was calculated according to the following formula: $C = N \times D \times 10^4$, where C stands for the number of viable cells/ mL, N is the average number of viable cells counted in 10 subgrids (1.0 mm^3), D is the dilution factor, and 10^4 is the hemocytometer correction factor. After counting cells were placed into new flasks at a density of 200 cells/ cm^2. Cells from passages 1 to 3 were used for the subsequent *in vitro* experiments.

Material description	**Type**	**Company**
laminar flow hood	Herasafe	Thermo Scientific
centrifuge	Heraeus Multifuge 1 SR	Thermo Scientific
hemocytometer	T728.1	Carl Roth
Trypan bleu	solution, 0.4%	Sigma-Aldrich
mikroscope	DMLB	Leica
cell culture flasks	Cellstar, T-75/ -125 flasks	Greiner Bio-One
cell culture tubes	polystyrene, 15/ 50 mL	Greiner Bio-One
aliquot tubes	safe - lock, 0.5/ 1.5/ 2 mL	Eppendorf
trypsin/ EDTA	0.05% / 0.02% (w/ v)	PAA
carbon dioxide incubator	APT.Line CB	Binder
mikroscope	Axiovert 40 CFL	Carl Zeiss
water bath	W6	Medingen
gloves	KC500 purple nitrile	Kimberly-Clark
pipettes	Research, 0.5 - 1000 µL	Eppendorf
pipettor	Easypet	Eppendorf
pipette tips	Cellstar, 10 µL – 25 mL	Greiner Bio-One
ethanol	solution, 70 %	University Central Pharmacy

2.1.3 Cryopreservation of cells

Cryopreservation was used for creating cell stocks ready for use in the subsequent experiments. Prior to cryopreservation cells were trypsinized and cell suspensions were processed for cell counting. After determining viable cell density, cells were transferred to 15 mL tubes and were pelleted by centrifugation at 300 g for 7 minutes at 24°C. Then cell pellets were resuspended in freezing solution consisting of 10% DMSO and transferred to 2 mL labeled cryogenic vials at a concentration of 10^6 cells per mL. The aspiration of cells in DMSO supplemented solution before freezing is critical for the prevention of cell damages due to ice crystal formation. According to a slow rate cooling protocol cryovials were placed upright for 4 hours in a -20°C freezer before being transferred to a -80°C freezer. After at least 24 hours in the -80°C freezer, cells were finally transferred to liquid nitrogen for long-term storage.

To thaw frozen cell suspensions, cryovials containing cells were rapidly thawed by submersion in a 37°C water bath for 1 - 2 minutes with constant agitation. Rapid thawing was critical because it prevented the recrystallization of ice crystals as the temperature rose, thus enhancing cell viability after cryopreservation (Seki 2008). Thawed cell solutions were transferred to sterile 15 mL tubes containing 9 mL of pre-warmed culture medium, pelleted at 300 g for 10 minutes at 37°C and resuspended in 5 mL of fresh culture medium.Then cell suspensions were transferred to T-75 cell culture flasks containing 10 mL of medium and were cultured under standard culture conditions.

Material description	Type	Company
cryogenic tubes	Nunc CryoTubes	Sigma-Aldrich
cell freezing medium	Gibco's Recovery	Invitrogen
laboratory freezer	set at –20°C	Kirsch
laboratory freezer	set at –80°C	Kirsch
water bath	W6	Medingen

2.2 *In vitro* cell characterization

After isolation by plastic adherence, DFPCs and BMSCs were characterized *in vitro* based on morphology, clonogenic potency, proliferative activity, multilineage differentiation capacity and expression of a specific set of cell surface marker proteins (Fig. 2.2).

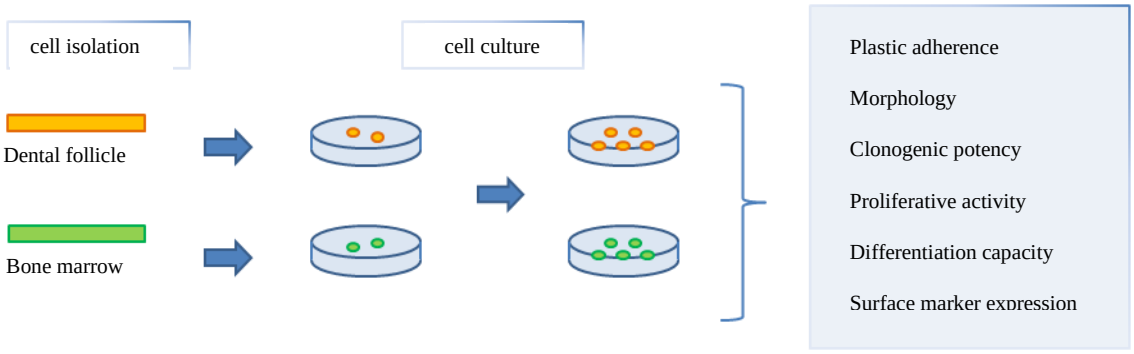


Figure 2.2 Overview of *in vitro* cell characterization.

2.2.1 Colony forming assay

Human DFPCs and BMSCs at passage 1 were cultured to sub-confluence. After counting, single-cell suspensions were seeded at low densities (30 cells per cm^2) into 6-well plates. Cells were cultured in MSCGM to form colonies. Culture medium was replaced every 3 to 4 days and cells were monitored microscopically for overgrowth. After 12 days of incubation, culture medium was removed; cells were fixed with 4% PFA for 10 minutes at room temperature and subsequently washed with distilled water. The total number of colonies was determined microscopically, by scoring aggregates of more than 50 cells. Percentages of colony-forming efficiency (CFE) were calculated as follows:

CFE (%) = (no. of colonies formed/ no. of cells seeded) x 100%

Material description	Type	Company
cell culture flasks	Cellstar, T-75/ -125 flasks	Greiner Bio-One
MSCGM	Culture medium	Lonza
PFA	powder	Sigma-Aldrich
mikroscope	Axiovert 40 CFL	Carl Zeiss

2.2.2 3-2, 5-Diphenyltetrazolium bromide (MTT) dye reduction assay

Cell metabolic activity was determined by MTT assays. Single-cells were seeded in 96-well plates at a density of 1 x 10^3 cells per well in MSCGM. Wells containing only culture medium served as blank controls for non-specific dye reduction. For measurement, 200 µL MTT solution was added to each well to a final concentration of 0.5 mg/ mL. After 4 hours of incubation at 37°C, culture medium was removed and purple formazan crystals were dissolved in 100 µL DMSO. Absorbance was measured at 550 nm (test wavelength) and 655 nm (reference wavelength) using a microplate reader. Raw data were expressed as percentages of viability according to the following formula:

Cell viability (%) = (OD550 – OD655, sample/ OD550 – OD655, control) x 100%

Material description	Type	Company
multiwell cell culture plates	96 wells	Greiner Bio-One
MSCGM	culture medium	Lonza
microplate reader	Model 680	Bio-Rad Lab
MTT	powder	Sigma-Aldrich
DMSO	solution	Carl Roth

2.2.3 *In vitro* functional differentiation assay

The ability of human DFPCs and BMSCs to differentiate into multiple mesenchymal lineages was determined using a human mesenchymal stem cell functional identification kit according to the instructions of the manufacturer. Observation of cell morphology changes and cytological staining were used to verify cell differentiation capacity toward three mesodermal lineages.

According to the adipogenesis protocol sterile coverslips were inserted into wells of a 24-well plate. Then 3.7 x 10^4 cells were seeded in each well and were cultured in αMEM (0.5 mL/ well). When 100% confluency was reached, adipogenic differentiation was induced by culturing cells for 14 days in αMEM with 10% HSA, 1% penicillin-streptomycin, and adipogenic supplement containing hydrocortisone,

isobutylmethylxanthine, and indomethacin in 95% ethanol. Adipogenic differentiation medium was freshly prepared and replaced (0.5 mL/ well) every 3 - 4 days. The appearance of lipid vacuoles could be monitored by microscopic examination 7 - 9 days after adipogenic induction. On 14th day adipocytes were fixed with 4% PFA (0.5 mL/ well) for 10 minutes at room temperature and subsequently washed twice with PBS (1 mL/ well).

Oil droplets in the cultures were identified by staining cells with Oil Red O. For lipid staining, Oil red O was diluted in 60% isopropanol for 15 minutes at 37°C and were filtered through a 0.2 μm syringe driven filter. Cell samples were incubated in dye solution (0.5 mL/ well) for 45 minutes at room temperature and then rinsed twice with 1 mL PBS to remove redundant Oil red O, before nuclear staining with DAPI (5 minutes, room temperature). Then coverslips were inverted onto a microscope slide containing 10 μL of mounting media. Excess mounting media was removed with fiber-free tissue wipers, without disturbing the coverslip. The edges of each coverslip were sealed with regular transparent nail polish and were left to dry for 10 minutes. Cells were then ready for microscopic viewing. Images were obtained using a light microscope with a video camera attachment.

For osteogenic differentiation, 7.4 x 10^3 cells were seeded on coverslips inserted into wells of a 24-well plate. Cells were cultured in αMEM (0.5 mL/ well) until 50 - 70% confluence was reached. Then, osteogenic differentiation was induced by culturing cells for 14 days in αMEM with 10% HSA, 1% penicillin-streptomycin, and osteogenic supplement containing dexamethasone, ascorbate-phosphate, and β-glycerolphosphate. Osteogenic differentiation medium was freshly prepared and replaced (0.5 mL/ well) every 3 - 4 days. On 14th day, osteocytes were fixed with 4% PFA (0.5 mL/ well) for 10 minutes at room temperature and were subsequently washed twice with PBS (1 mL/ well).

Calcium containing precipitates were visualized after staining with 2% aqueous Alizarin red S adjusted to a pH of 4.2 with ammonium hydroxide. Cell samples were incubated in dye solution (0.5 mL/ well) for 15 minutes at room temperature in the dark and then rinsed twice with 1 mL PBS to remove redundant Alizarin red S, before nuclear staining with DAPI (5 minutes, room temperature). Then coverslips were mounted on microscope slides as described above, and were examined by microscopy.

For chondrogenic differentiation, 25 x 10^4 cells were transferred in a 15 mL conical tube for centrifugation at 200 g for 5 minutes at room temperature. Then cells were resuspended in 1 mL DMEM/ F-12 and centrifugated at 200 g for 5 minutes at room temperature. After discarding the medium, cells were resuspended in 0.5 mL of chondrogenic differentiation medium consisting of DMEM/ F-12 basal medium with 1% penicillin-streptomycin, 1% ITS supplement and chondrogenic supplement containing dexamethasone, ascorbate-phosphate, proline, pyruvate, and TGF-β3 and centifugated once again at 200 g for 5 minutes at room temperature. After loosing the cap of the tube to allow gas exchange, pelleted cells were cultured for 21 days at 37°C in a 5% CO_2 humidified atmosphere protected from light. Chondrogenic differentiation medium was freshly prepared and replaced (0.5 mL/ tube) every 2 - 3 days. After 21 days, chondrocyte pellets were fixed with 4% PFA for 20 minutes at room temperature and were subsequently washed with 1 mL of PBS for 5 minutes. For frozen sectioning, cell pellets were cryo-embedded in tissue freezing medium and stored at -80°C. Cell pellets were cut by a cryostat-microtome and frozen sections (5 µm thick) were mounted onto microscope slides and placed in sealed slide boxes at -20°C until needed.

Sections were stained with 0.1% aqueous Safranin O (0.5 mL/ well) to visualize sulfated proteoglycans. Cell samples were incubated in dye solution (0.5 mL/ well) for 5 minutes at room temperature in the dark and then rinsed twice with 1 mL PBS to remove redundant Safranin O. Coverslips were then mounted on microscope slides as described above, and examined by microscopy for chondrogenic differentiation.

Material description	**Type**	**Company**
human mesenchymal stem cell functional identification kit		R & D Systems
multiwell cell culture plates	24 wells	Greiner Bio-One
coverslips	13mm dia	Menzel
cell culture tubes	polystyrene, 15 mL	Greiner Bio-One
αMEM	with L-Glutamine	PAN-Biotech
DMEM/ F-12	with L-Glutamiine	PAN-Biotech
HSA	solution, 20%, low salt	CSL Behring
Oil Red O	powder	Sigma-Aldrich

Material description	Type	Company
Alizarin red S	powder	Sigma-Aldrich
Safranin O	powder	Sigma-Aldrich
ammonium hydroxide	solution	Avantor Performance Materials
isopropanol	solution	Avantor Performance Materials
fine dosage syringe	Omnifix-F	B. Braun
syringe driven filters	Millex PVDF, 0.2 µm	Millipore
needles	23 Gauge	BD Microlance
Ph-meter	MP220	Mettler Toledo
mounting medium	Glycergel, aqueous	Dako
tissue freezing medium	Tissue-Tek	Sakura Finetek
cryostat-microtome	CM 1900	Leica
microscope slides	plain	Marienfeld-Superior
tissue wipers	Kimtech science	Kimberly-Clark
microscope	SP2 Confocal Microscope	Leica
digital camera	DC 200	Leica

2.2.4 Cell staining for immunofluorescence

The differentiation capacity toward different cell lineages was verified further by immunostaining for specific markers that are fatty acid binding protein (FABP-4) for adipocytes, osteocalcin for osteocytes and aggrecan for chondrocytes.

Antibody	Description	Company
Primary antibody	goat anti-mouse FABP-4	R& D Systems
	mouse anti-human osteocalcin	R & D Systems
	goat anti-human aggrecan	R & D Systems
Secondary antibody	anti-mouse AlexaFluor 488	Invitrogen
	anti-goat AlexaFluor 488	Invitrogen
Nuclear stain	DAPI	Invitrogen

Table 2.2.4 Antibodies for immunofluorescence microscopy.

Prior staining, cells were fixed with 4% PFA (0.5 mL/ well) for 10 minutes at room temperature and were subsequently washed twice with PBS (1 mL/ well). Then cells were washed three times with 0.5 mL of 1% HSA in PBS for 5 minutes. Prior antibody incubation cells were permeabilized and blocked cells with 0.5 mL of 0.3% TritonX-100, 1% HSA, 10% horse serum in PBS at room temperature for 45 minutes. After blocking, cells were incubated with 300 µL/ well of antibody working solution overnight at 5°C. Primary antibodies were diluted in PBS containing 1% HSA and 10% horse serum to a final concentration of 10 µg/ mL. Negative controls were performed using PBS containing 1% HSA and 10% horse serum with no primary antibody added. Followingly, cells were washed three times with 0.5 mL of PBS containing 1% HSA for 5 minutes and incubated with secondary antibodies (300 µL/ well) diluted in PBS with 1% HSA at in the dark for 60 minutes at room temperature. For background staining control samples were incubated with secondary antibody only. After incubation cells were washed with 0.5 mL of PBS containing 1% HSA for 5 minutes to remove redundant antibodies. For nuclear staining cells were stained with DAPI (5 minutes, room temperature). Then coverslips were mounted on microscope slides, as described above, and were examined by a microscope equipped with fluorescence optics. The primary and secondary antibodies used in the study are listed in Table 2.2.4.

Material description	**Type**	**Company**
PFA	powder	Sigma-Aldrich
HSA	solution, 20%, low salt	CSL Behring
donor horse serum	heat-inactivated, 10%	PAA
TritonX-100	solution, 0.3%	Carl Roth
microscope	DMLB	Leica
digital camera	DC 200	Leica

2.2.5 Fluorescence-activated cell sorter analysis

DFPCs and BMSCs were analyzed for surface marker expression by fluorescence-activated cell sorter (FACS) analysis. Cells were detached from culture flasks, counted, suspended in ice-cold buffer (1x PBS supplemented with 0.5%BSA and 2mM EDTA) and transferred in 1.5 mL aliquot tubes (50.000 cells/ tube). Single-cell suspensions

were incubated for 30 minutes at 4°C protected from light with saturating levels of monoclonal anti-human antibodies (Table 2.2.5). For indirect marker detection, cells labelled with CD90-biotin were additionally incubated with V450-Streptavidin secondary antibody for 20 minutes at 4°C in the dark. FcR Blocking Reagent was employed to reduce unspecific antibody binding. Background fluorescence was excluded using unlabeled cells and cells incubated with isotype-matched antibodies (Table 2.2.5).

Antibody	Conjugate	Isotype	Company
CD14	V450	mouse IgG2b *κ*	BD Biosciences
CD29	APC	mouse IgG1 *κ*	BD Biosciences
CD44	PerCP-Cy5.5	mouse IgG2b *κ*	BD Biosciences
CD45	V500	mouse IgG1 *κ*	BD Biosciences
CD73	PE	mouse IgG1 *κ*	BD Biosciences
CD90-biotin	Streptavidin-V450	mouse IgG1 *κ*	BD Biosciences
CD105	Alexa Fluor 488	mouse IgG1	AbD Serotec
TLR2	FITC	mouse IgG2a *κ*	eBioscience
TLR4	Alexa Fluor 488	mouse IgG2a *κ*	eBioscience

Table 2.2.5 Antibodies for analytical fluorescence-activated cell sorting.

After washing cells were transferred into FACS tubes and were analysed using a flow cytometer. Dead cells were excluded using a dead cell staining kit. After acquisition of light scattering and fluorescence data for each sample, the resulting information could be analyzed utilizing computer specific software that was associated with the flow cytometer. A minimum of 10.000 events were recorded per sample.

Material description	Type	Company
BSA	lyophilized powder	Sigma-Aldrich
EDTA	solution	Sigma-Aldrich
FcR Blocking Reagent	human	Miltenyi Biotec

Material description	Type	Company
LIVE/DEAD Fixable Dead Cell Stain Kit		Invitrogen
FACS tubes	5 mL polystyrene, round-bottom	BD Biosciences
Vortex mixer	Vortex Genie 2	Scientific Industries
pipetboy	acu	IBS Integra Biosciences
flow cytometer	BD FACS LSR II	BD Biosciences
data analysis software	FACSDiva 6	BD Biosciences

2.3 LPS treatment

After cell characterization both DFPCs and BMSCs were assayed for their ability to sense and respond to *P.gingivalis* LPS. Concretely, cells were treated with several doses of LPS in several time periods. Cells responses were analyzed in terms of cell viability, TLR2 and TLR4 expression, *in vitro* wound healing capacity and IL-6 secretion.

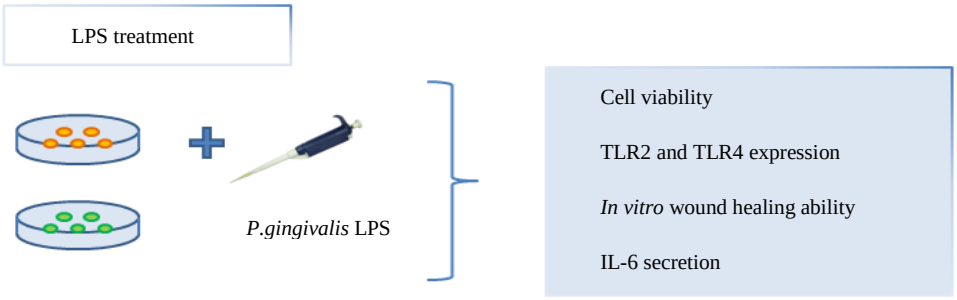


Figure 2.3 Overview of *in vitro* cell characterization after LPS treatment.

2.3.1 LPS treatment and cytotoxicity assay

Ultrapure LPS from *P.gingivalis* was obtained commercially and were used at final concentrations of 0, 1, 10 and 50 μg/ mL in MSCGM. To determine the cytotoxic effects of LPS, MTT assays were performed as described above (Section 2.2.2).

Material description	Type	Company
P.gingivalis LPS	ultrapure	InvivoGen
MSCGM	culture medium	Lonza

2.3.2 RNA extraction and complementary DNA synthesis

Total RNA was extracted from cells using an RNA purification kit at room temperature according to the manufacturer's instructions. Samples of 1 x 10^7 cells were lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate-containing buffer. This buffer leads to immediate inactivation of RNases, ensuring purification of intact RNA. Then ethanol was added to provide appropriate binding conditions and the sample was applied to a spin column. Total RNA were bound to column membrane and contaminants were efficiently washed away. RNA was then eluted in 20 - 30 µL RNase-free water. All bind, wash, and elution steps were performed by centrifugation at 22 – 25°C in a standard microcentrifuge set at 300 g. Genomic DNA contamination was eliminated using an on-column DNA digestion kit. The total RNA concentration and purity was determined by a spectrophotometer. Complementary DNA was synthesized from 2 µg of total RNA using oligo(dT)15 primer, dNTPs (10 mM), rRNAsin ribonuclease inhibitor, DTT (0.1 M), 5 x first-strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$) and 200 U/ µL SuperScript III Reverse Transcriptase in a 20 µL reaction volume. Annealing was performed by a thermocycler for 5 minutes at 65°C with rapid cooling at 4°C. Then reverse transcription was carried out for 60 minutes at 55°C, followed by 15 minutes at 70°C, with a final cool down to 4°C.

Material description	Type	Company
oligo(dT)15 primer	solution	Promega
ribonuclease inhibitor	rRNAsin	Promega
10mM dNTPs	solution	Invitrogen
reverse Transcriptase	SuperScript III	Invitrogen
RNA purification kit	RNeasy mini kit	Qiagen
on-column DNA digestion kit	RNase-free DNase set	Qiagen
centrifuge	Heraeus Primo R	Thermo Scientific
spectrophotometer	NanoDrop ND-1000	Thermo Scientific
thermocycler	MJ Mini	Bio-Rad Lab
vortex mixer	Galaxy mini	VWR

2.3.3 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed with a real-time PCR system using TaqMan Gene Expression Assays according to the instructions of the manufacturer. Reaction mixtures included specific primers for TLR2 and TLR4. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization of each sample (housekeeping gene). Relative gene expression of TLRs was determined based on the threshold cycle (C_T) values. Only C_T values less than 35.5 were included. Results were normalized according to the formula: $\Delta C_T = C_{T\ \text{target gene}} - C_{T\ \text{GAPDH}}$. ΔC_T values of samples were averaged and relative gene expression of LPS - treated cells(s) and calibrator(c) sample (i.e. untreated cells) were calculated following the delta/delta calculation method ($2^{-(\Delta\Delta Ct\,(s) - \Delta\Delta Ct\,(c))}$). Relative gene expression of the calibrator sample is always one. Standart error (SE) of normalized target gene expression relative to GAPDH was calculated from the initial SEs of target gene and GAPDH. Each sample was tested in quadruplicate. Calculations were performed with spreadsheet software.

Material description	Type	Company
Real-Time PCR System	StepOnePlus	Applied Biosystems
TaqMan Gene Expression Assay	TLR2, Hs01014511_m1	Applied Biosystems
TaqMan Gene Expression Assay	TLR4, Hs00152939_m1	Applied Biosystems
TaqMan Gene Expression Assay	GAPDH, Hs99999905_m1	Applied Biosystems
TaqMan Universal Master Mix	No AmpErase UNG	Applied Biosystems
spreadsheet software	Microsoft Excel	Microsoft Windows

2.3.4 TLR expression by FACS

To determine the expression of TLR2 and TLR4 on DFPCs and BMSCs, cells were treated with 50 μg/ mL *P. gingivalis* LPS for 24 hours and FACS analysis were performed as described above (Section 2.2.5). Antibodies used in the assay are shown in Table 2.3.4.

Antibody	Conjugate	Isotype	Company
TLR2	FITC	mouse IgG2a κ	eBioscience
TLR4	Alexa Fluor 488	mouse IgG2a κ	eBioscience

Table 2.3.4 Antibodies for analytical fluorescence-activated cell sorting.

2.3.5 *In vitro* wound healing assay

An established *in vitro* wound healing model was used for the study of cell wound healing rates. DFPCs and BMSCs were cultured in 24-well plates until they reached 90% confluence. Afterwards, a disposable plastic pipette tip (200 μL) was used to prepare a scratch across the monolayer of cells. Intact cells were gently washed twice with PBS to remove debris created by wounding and culture medium was added for the remainder of incubation. The extent of repopulation of the wound area was assessed for up to 24 hours by live imaging, processed by ELYRA PS.1 LSM-780. The latter is a confocal video-microscopy station, equipped with a motorized stage incubator for automated sample positioning and accurate control of air temperature and carbon dioxide concentrations, set at 37°C and 5% CO2, respectively. The use of a controlled large incubator, enclosing the entire stage and objectives, allowed the stable long-term video imaging of live cells. The sequential images were captured by a CCD video camera every 3 minutes, thus allowing the real-time observation of the *in vitro* healing process. During the assay, cells from the edges of the wounded area migrated toward the wound. The average wound dimensions were measured using graphic editing software. In order to determine the migratory activity of LPS-treated cells, rates of wound healing were calculated at several time points; normalized to untreated controls and finally expressed as percentages of cell migratory activity.

Material description	Type	Company
microscope	ELYRA PS.1 LSM-780	Carl Zeiss
CCD video camera	AxioCam MR	Carl Zeiss
graphics editing software	ZEN2011 software	Carl Zeiss
graphics editing software	AxioVision Rel 4.5 SP1	Carl Zeiss
computer	Extensa 3002 WLMi	Acer

2.3.6 Detection of IL-6 by enzyme linked immunosorbent assay (ELISA)

For quantitative detection of human IL-6 in cell cultures, supernatants collected from LPS-treated as well as untreated DFPC and BMSC cultures were analyzed by a commercially available sandwich ELISA kit. According to the instructions of the manufacturer, 96-well immunoplates were coated overnight at 4°C with 100 µL of coating buffer containing anti-human IL-6 capture antibody (diluted 1:100 in PBS). After removing the coating buffer plates were blocked with 300 µL of blocking solution at room temperature for 1 hour. Then blocking solution (containing PBS and 4% HSA) was poured off and 100 µL of culture supernatants was placed in wells at room temperature for 1 hour. After washing 5 times with wash solution (containing PBS and 0.1% Tween20), 100 µL of biotinylated detector antibody (anti-human IL-6, diluted 1:100 in reagent diluent) was placed in each well at room temperature for 1 hour. Serial dilutions of human recombinant IL-6 standard, whose concentrations spanned an effective assay range (8 - 1000 pg/ mL), were included in each well plate to obtain a standard curve. Wells containing culture medium only served as blank controls for nonspecific antibody binding. After washing, 100 µL of streptavidin diluted 1:8000 in reagent diluent was placed in each well for 30 minutes and incubated in the dark at room temperature. After washing, 100 µL of TMB substrate was added into each well, followed by incubation at room temperature for 20 minutes. The development of the colour reaction was controlled and as it was sufficiently proceeded 50 µL stop solution (2N hydrochloric acid) was added. Absorbance values were measured at 450 nm by a microplate reader. The quantity of IL-6 was calculated as the change in absorbance values which were proportional to the amount of protein captured in wells. For the standard curve, the absorbance values for different known concentrations of IL-6 standard were plotted and a best-fit line drawn through the points. The unknown concentrations of samples tested at the same time could be determined by reference to the standard curve. To estimate the relative amount of IL-6 assayed culture supernatants the absorbance values of the samples were compared to the absorbance values of standard IL-6.

Material description	Type	Company
ELISA kit	human interleukin-6	ImmunoTools
Multiwell cell culture plates	Nunc Maxisorp, 96 wells	Thermo Scientific
Tween20	viscous liquid	Sigma-Aldrich
Streptavidin-HRP	solution, 1.25 mg/ mL	Invitrogen
TMB substrate/ stop solution	solution	Sigma-Aldrich

2.4 Statistical analysis

The experiments were performed in at least three independent repeats for each cell population. All results are presented as means ± standard error (SE). Statistical analyses were performed utilizing the SigmaStat 3.5 software package. Differences were considered statistically significant at $p < 0.05$.

Material description	Type	Company
statistical software	SigmaStat 3.5	Systat Software
computer	VGN-NS21M	Sony

3. Results

3.1 Cell characterization

3.1.1 Isolation and culture of DFPCs

In the present study, human DFPCs were obtained from freshly extracted dental follicle tissues (n = 6). Cells were isolated by their ability to adhere to a plastic substratum. Non-adherent cells were discarded 2 - 3 days after isolation. Only a low number (n = 3 - 5) of attached single cells were able to grow and form primary cultures. DFPCs were characterized by a flattened, spindle-shaped, fibroblast-like morphology with multiple processes (Fig 3.1.1). DFPCs were cultivated in stem cell growth medium under standard culture conditions and reached 80% confluency in 3 weeks. Parallely, human BMSCs, isolated from bone marrow aspirates by plastic adherence, showed similar morphological features and were cultured under the same conditions as DFPCs. In the subsequent experiments DFPCs properties were analyzed and compared to BMSCs, a population of non-dental origin multipotent cells.

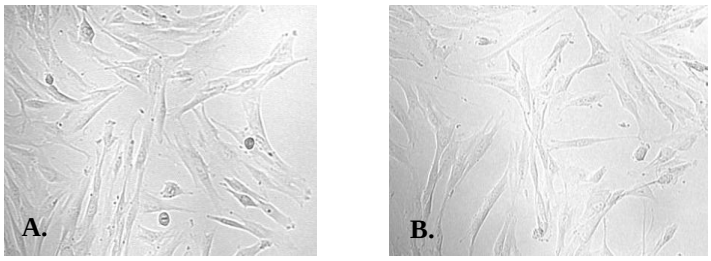


Figure 3.1.1 Microscope images showing typical (A) DFPCs and (B) BMSCs morphology, 100x.

3.1.2 Clonogenic and proliferative properties of DFPCs

First passage DFPCs and BMSCs were evaluated for their ability to form colonies. Single-cell suspensions of both cell populations were seeded at low densities. After 12 days in culture the total number of colonies was determined microscopically. The colony forming efficacy (CFE) of cells derived from dental follicle tissues was 20.4 ± 2.8%, while the incidence of BMSCs CFE (5.3 ± 1.5%) was significantly lower (p < 0.05) (Fig. 3.1.2a).

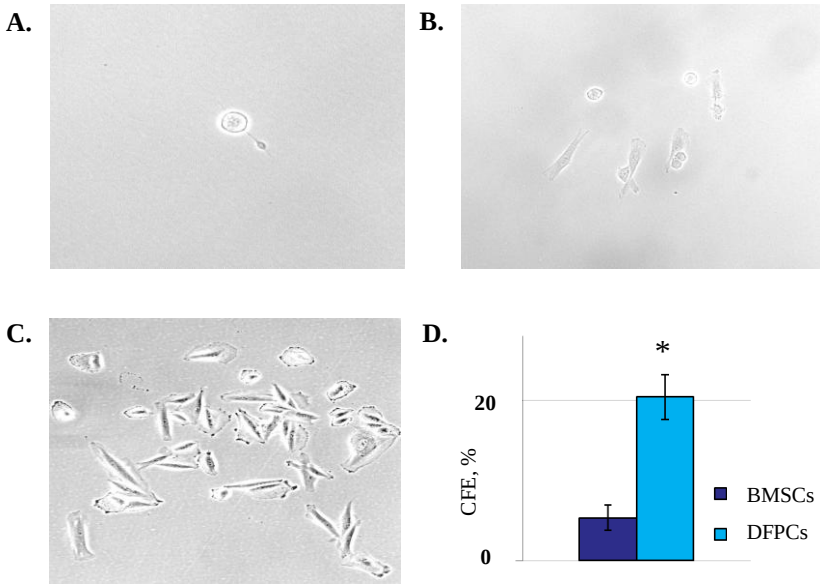


Figure 3.1.2a Representative light microscope images of a DFPCs colony after (A) 1 day, (B) 4 days and (C) 7 days of cultivation, 100x magnification. (D) Colony forming efficiency of DFPCs and BMSCs; DFPCs $n = 5$, BMSCs $n = 4$, values represent means ± SE, $*p < 0.05$ (Student's *t*-test).

Cell proliferation of both DFPCs and BMSCs was assessed by continuous 4-day MTT assays. Results were expressed as percentages of cell viability and corresponding growth curves were plotted. Statistical analysis revealed no significant differences between the proliferation rates of both cell populations (Fig. 3.1.2b).

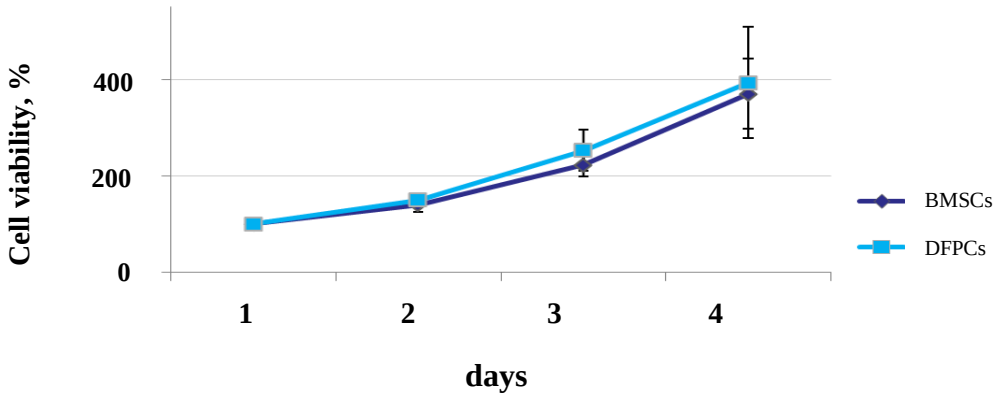
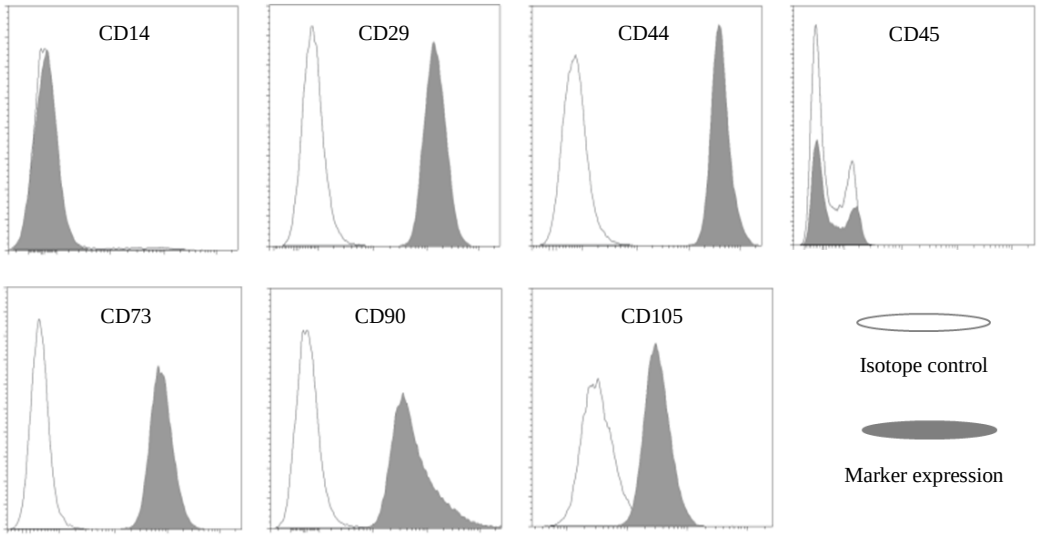


Figure 3.1.2b Cell proliferation rates of DFPCs and BMSCs assessed by MTT dye reduction assay; DFPCs $n = 5$, BMSCs $n = 4$, values represent means ± SE.

3.1.3 Immunophenotypic characterization of DFPCs

In order to determine the phenotypic profile of DFPCs, fluorescence-activated cell sorting (FACS) was performed. A typical panel of stem cell markers was used to identify surface antigens of cultured DFPCs. According to results DFPCs exhibited a strongly positive expression of a variety of surface markers (CD29, CD44, CD73, CD90 and CD105). DFPCs failed to react with hematopoietic markers CD14 (monocyte and granulocyte marker) and CD45 (common leukocyte antigen). Analysis of surface epitopes by FACS is shown in Figure 3.1.3. The immunophenotypic analysis of BMSCs revealed similar results (data not shown) (Mark 2013).

A.



B.

CD14	CD29	CD44	CD45	CD73	CD90	CD105
0 ± 0%	99.8 ± 0.02%	99.7 ± 0.08%	0 ± 0.02%	98.7 ± 0.5%	82.4 ± 1.6%	82.9 ± 6.2%

Figure 3.1.3 (A) Immunophenotyping of human DFPCs by flow cytometry after staining for specific CD surface markers. DFPCs were positive for typical stem cell markers CD29, CD44, CD73, CD90 and CD105. No expression of haematopoietic markers CD14 and CD45 was detected. Bright areas indicate CD marker isotope controls. (B) Surface marker expression values are presented in percentages; $n = 3$, values represent means ± SE.

3.1.4 Differentiation potential of DFPCs

The multipotency of DFPCs was verified by *in vitro* functional differentiation assays. DFPCs were cultured in various induction media and their ability to differentiate towards multiple lineages was evaluated. Adipogenic induction was apparent by the accumulation of lipid-rich vacuoles within the cells, when cells were placed in adipogenic medium for 2 weeks. Differentiation became evident after 7 - 9 days after induction and most adipocytes were observed near the well center. Lipid droplet formation in adipocytes was verified by Oil Red O staining (Fig. 3.1.4a) and immunostaining (Fig. 3.1.4d). When placed in osteogenic differentiation medium for 2 weeks, cells formed mineralization nodules, as revealed by Alizarin red staining (Fig. 3.1.4b) and immunostaining (Fig. 3.1.4e). The calcium deposits were scattered throughout the cell moonolayer as single mineralized clusters.Finally, culturing cells into chondrogenesis induction medium for 3 weeks resulted in the development of chondrocyte pellets. Safranin O staining (Fig. 3.1.4c) revealed a homogeneous distribution of sulfated proteoglycans within the cell pellets matrix and was confirmed by positive immunostaining (Fig. 3.1.4f). Immunostaining negative controls, applied in the absence of primary antibody, did not stain (data not shown). BMSCs used in the study were also able for multiple mesodermal lineage differentiation (Mark 2013).

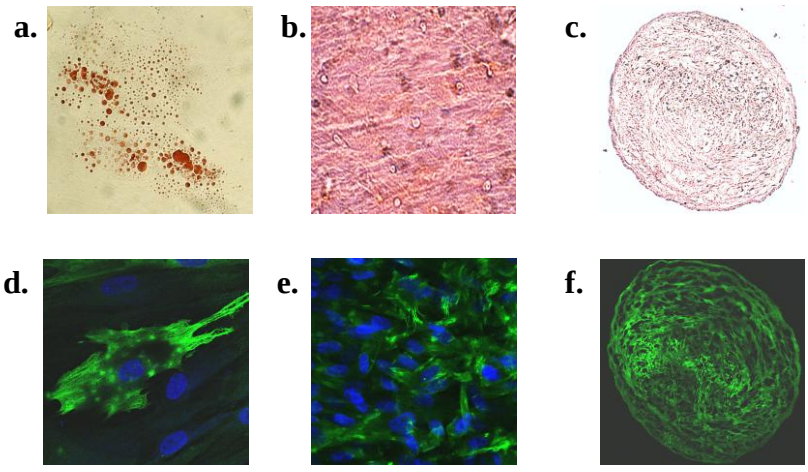


Figure 3.1.4 Multiple mesodermal lineage differentiation capacity of DFPCs *in vitro*. Adipogenesis was confirmed by Oil Red O staining (a) and immunostaining (d), 400x. Osteogenesis became evident after Alizarin Red staining (b) and immunostaining (e), 100x. Chondrogenesis was verified by Safranin O staining (c) and immunostaining (f), 10x; $n = 3$.

3.2 LPS treatment

3.2.1 LPS cytotoxic effects on DFPCs

To verify whether LPS evokes cytotoxicity effects on DFPCs and BMSCs, cell viability was examined by MTT assays. Interestingly, cell viability of both populations was not affected, even when cells were treated with a high LPS dosage (50 µg/ mL) or for a long period (72 hours) (Fig. 3.2.1).

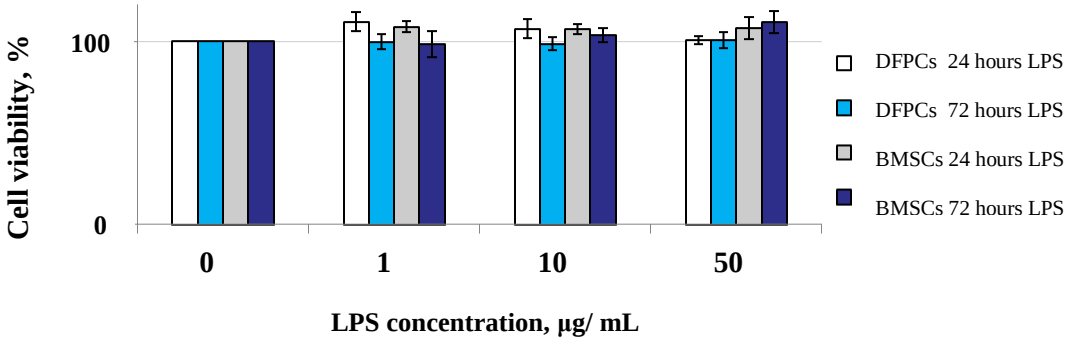


Figure 3.2.1 Cell viability rates of DFPCs and BMSCs assessed by MTT dye reduction assay; DFPCs $n = 7$, BMSCs $n = 6$.

3.2.2 TLR2 and TLR4 expression in DFPCs

The expression of TLR2 and TLR4 in both DFPCs and BMSCs was comparatively analyzed. The gene and protein expression levels of TLRs were determined by qRT-PCR and flow cytometry, respectively.

According to the results, both cell populations expressed low levels of TLR2 and TLR4 mRNA. Notably, the mRNA levels of TLR4 expression were significantly higher when compared to TLR2 gene expression ($p < 0.05$). The expression of TLR4 was higher in BMSCs compared to DFPCs ($p < 0.05$). Further, TLR2 and TLR4 gene expression was tested in cells stimulated for 24 hours with 50 µg/ mL *P. gingivalis* LPS. This high dose LPS treatment did not influence the expression level of TLR2 mRNA, whereas gene expression of TLR4 was significantly downregulated ($p < 0.05$, Fig. 3.2.2a).

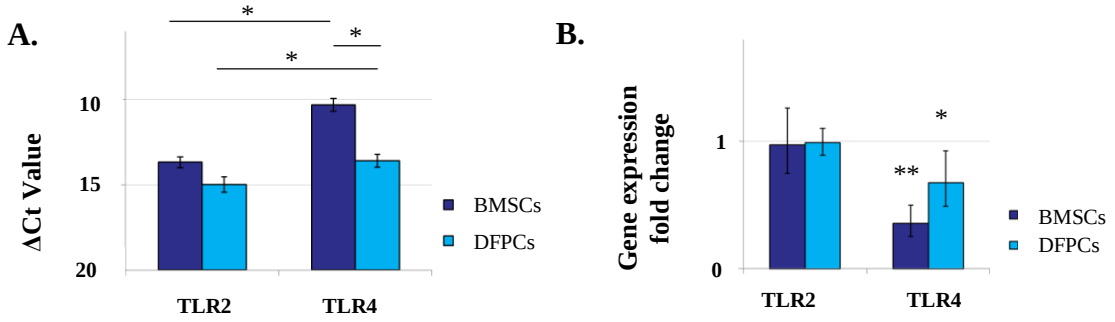


Figure 3.2.2a (A) Relative gene expression of TLR2 and TLR4 analysed by qRT-PCR. Histogram scale is inverted, so that higher bars represent higher levels of mRNA; DFPCs n = 4, BMSCs n = 3, values represent means ± SE, *p < 0.05 (Student's t-test). (B) Gene expression fold change of TLR2 and TLR4 after LPS treatment. Data were analyzed by the delta/delta calculation method; DFPCs n = 4, BMSCs n = 3, values represent means ± SE, *p < 0.05, **p < 0.01 (Student's t-test).

The expression of TLR2 and TLR4 was confirmed also at protein level for both DFPCs and BMSCs. According to FACS analysis, both cell populations expressed low levels of TLR2 and TLR4 (Fig. 3.2.2b). Protein levels of TLR4 were significantly higher than TLR2 on DFPCs (p < 0.001). The expression of TLR4, but not TLR2, was upregulated on LPS-treated BMSCs (p < 0.05). Finally, TLR2 and TLR4 protein expression on DFPCs was not significantly affected by LPS treatment.

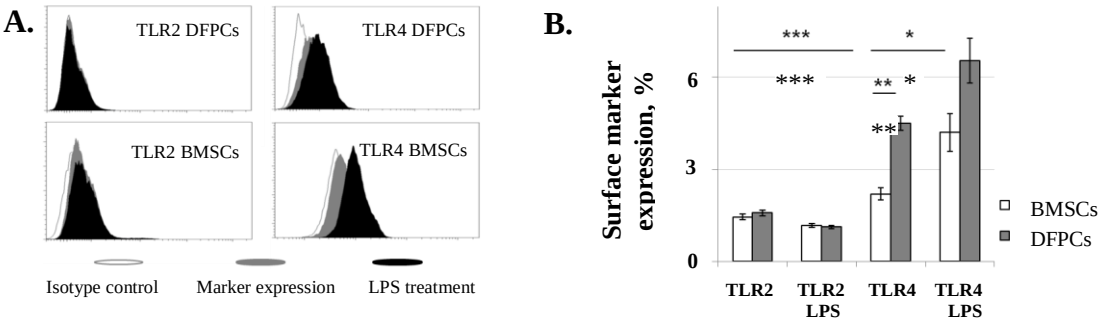


Figure 3.2.2b (A) Protein expression of TLR2 and TLR4 in DFPCs and BMSCs was evaluated by flow cytometry. Representative FACS histograms of TLR2 and TLR4 expression are shown. (B) TLR2 and TLR4 were expressed at low levels on both DFPCs and BMSCs. The expression of TLR2 was significantly lower than TLR4. The TLRs expression level of DFPCs was not significantly influenced by LPS treatment, while the expression of TLR4 on LPS-treated BMSCs was elevated; DFPCs n = 5, BMSCs n = 4, values represent the means ± SE, *p < 0.05, **p = 0.01, ***p < 0.001 (Student's t-test).

3.2.3 Migration potential of DFPCs

To test whether LPS can affect cell migration, DFPC as well as BMSC cultures were subjected to *in vitro* scratch assays. This assay allows the observing of the healing process *in vitro*. Data analysis indicated that cells migrated in a linear fashion. As shown in Figure 3.2.3, LPS-treated DFPCs had a 43.5% higher migratory capacity compared to untreated controls ($p < 0.05$), suggesting that LPS may promote DFPCs basal motility. Interestingly, LPS treatment had no significant impact ($p > 0.05$) on BMSCs migration rates.

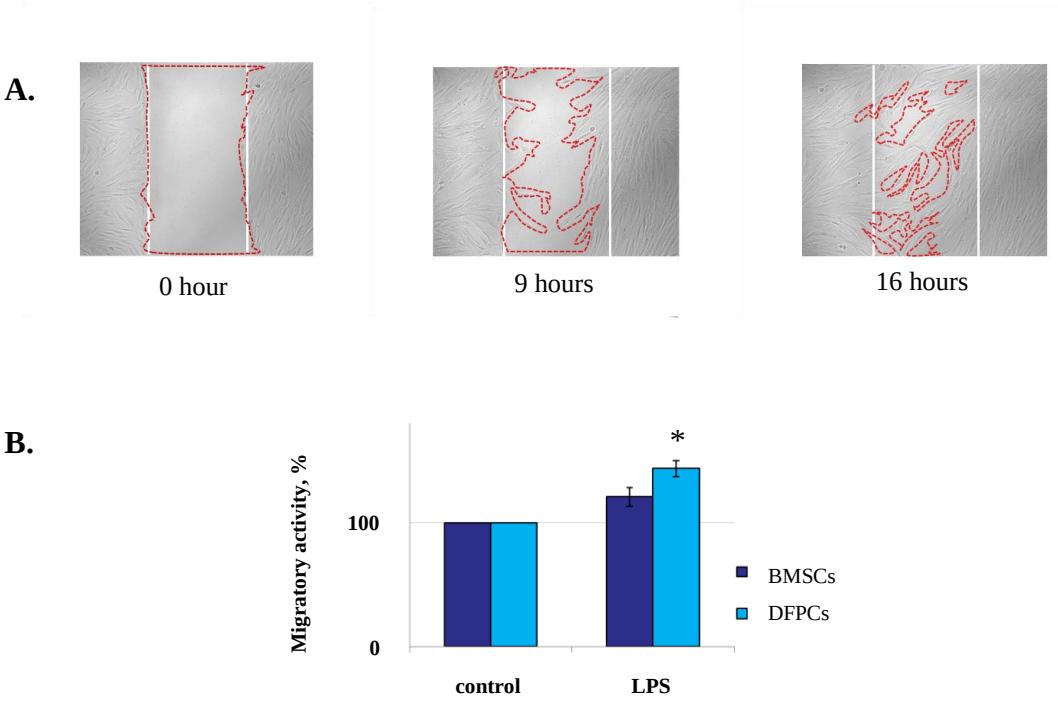


Figure 3.2.3 (A) Representative images of *in vitro* wound healing scratch assay. After scratching a confluent cell monolayer of cells, surrounding cells migrated into the scratched area (time after scratching is indicated). White lines represent wound edges at $t = 0$ hour. Dotted red lines represent wound dimensions during cell migration at three different time points, 10x. (B) Migratory activity rates after LPS treatment. Average wound dimensions were measured at several time points; data were normalized to untreated controls and results were expressed as percentages of migratory activity; DFPCs $n = 4$, BMSCs $n = 3$, values represent means ± SE, $*p < 0.05$ (Student's *t*-test).

3.2.4 IL-6 secretion by DFPCs

Next, LPS induced IL-6 secretion was analyzed by examining culture supernatants of both cell types. No IL-6 could be measured in the supernatants of DFPCs. In all tested samples the detected signal was never higher than that of medium control. On the contrary, BMSCs produced IL-6 (Fig. 3.2.4). Cytokine secretion by BMSCs was upregulated after 24 hours of LPS treatment in a dose-independent manner ($p > 0.05$). Treatments for 72 hours also led to elevated IL-6 secretion, which was also independent from the LPS dosage used ($p > 0.05$). These results suggest that BMSCs produced daily approx. 300 pg/ mL of IL-6. Interestingly, cytokine secretion seemed to reach a plateau of approx. 1600 pg/ mL, even by high LPS concentration. It is also important to mention that the elevated cytokine production by BMSCs was not due impaired cell viability, as this was not affected by any LPS treatment.

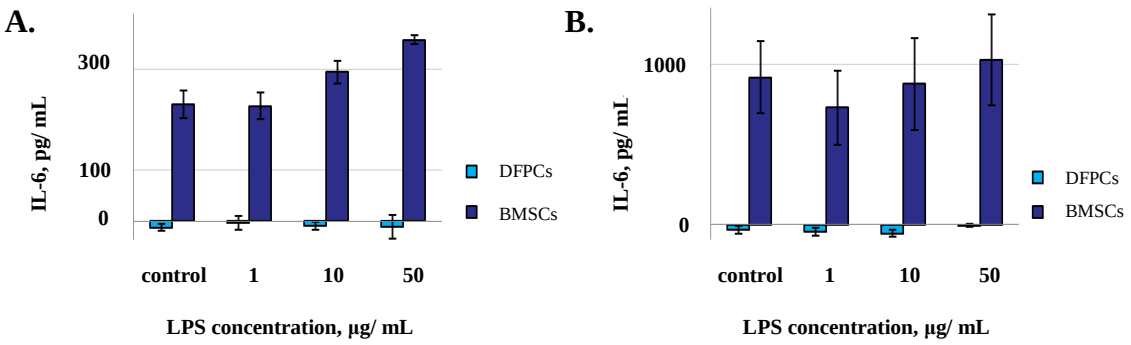


Figure 3.2.4 Histograms show IL-6 secretion by DFPCs and BMSCs, measured after 24 hours (A) and 72 hours (B) of LPS stimulation by ELISA. Limit of detection was 8 pg/ mL. Each sample was tested in triplicate; DFPCs $n = 3$, BMSCs $n = 5$, values represent means ± SE, $p > 0.05$ (Student's *t*-test).

4. Discussion

Dental follicle progenitor cells represent a population of multipotent cells derived from the loose ectomesenchyme-derived connective tissue surrounding the developing tooth germ prior to eruption (Ten Cate 1997). Here, human DFPCs were isolated from freshly extracted dental follicle tissues of wisdom teeth by applying previously developed methodology (Morsczeck 2005).

4.1 Cell characterization

Human DFPCs, as BMSCs, represent a heterogeneous population of cells with the capacity to adhere to plastic surfaces and form clonogenic, fibroblastic-like colonies (Morsczeck 2005; Honda 2010).

The high colony forming efficiency and proliferation of DFPCs has already been reported in studies comparing DFPCs to other dental-derived stem cells (Jo 2007; Tomic 2011; Schilardi 2012). These characteristics are attributed to the high telomerase activity in DFPCs (Guo 2013). Here, in comparison to BMSCs, DFPCs showed a higher colony-forming efficiency, but same proliferation rates. The origin of DFPCs could be an explanation for their superior clonogenic potential (Gronthos 2000; Jo 2007; Schilardi 2012), as DFPCs are obtained from developing tissues (dental follicle). On the contrary, source of BMSCs are matured tissues (bone marrow aspirates) at a later developmental state with a lower incidence of clonogenic cells (Gronthos 2003). Proliferation potential of DFPCs might be affected by the *in vitro* culture conditions, which also results in changes of cell/ culture morphology (De Sá Silva 2012). Thus, the fact, that the CFE rates of both cell populations may be influenced by the differential isolation protocols, should be taken into account.

The present study demonstrated the multilineage differentiation capacity of DFPCs *in vitro*. Interestingly, although osteogenic differentiation capacity of DFPCs is well documented *in vitro* (Morsczeck 2005; Honda 2010), the ability of DFPCs to differentiate along the adipogenic and chondrogenic pathway have been controversially discussed. These conflicting results have been attributed to the inherent variability of DFPCs populations (Lindroos 2008; Haddouti 2009; Honda 2011; Mori 2012). It is suggested that the heterogeneity of DFPCs could in fact be reflective of the cell

maturity stage along the adipogenic and chondrogenic differentiation pathways (Luan 2006; Guo 2013). Here, DFPCs were demonstrated to have the ability to differentiate towards the osteogenic, adipogenic and chondrogenic lineage, when cultured with special induction media, as already reported in the literature (Kemoun 2007; Jo 2007; Yagyuu 2010; Tomic 2011).

Further, both DFPCs and BMSCs were analyzed for expression of typical stem cell markers by flow cytometry. In accordance with reports from the literature (Nauta 2007; Huang 2009; Gaebel 2011), DFPCs and BMSCs were identified as CD29, CD44, CD73 and CD105 positive cells. Both cell populations expressed these cell markers with similar high intensity while no expression of CD14 (monocyte and granulocyte marker) and CD45 (common leukocyte antigen) was observed. Thus, together with the multilineage differentiation potential of DFPCs, the minimal criteria for defining DFPCs as multipotent mesenchymal stem cells were fulfilled (Dominici 2006).

4.2 Effects of *P.gingivalis* LPS on DFPCs *in vitro*

4.2.1 Effects on cell viability

In this study several concentrations of *P.gingivalis* LPS (0, 1, 10 and 50 μg/ mL) and three time points (0, 24 and 72 hours) were used in order to determine the effects on cell viability of both DFPCs and BMSCs. It is already demonstrated that the concentrations and compositions of subgingival microflora vary greatly depending on the local micro-environmental conditions (Socransky 2005). Thus, *P.gingivalis* LPS concentration used here was thought to be superoptimal to resemble the high LPS concentrations likely to be found in the subgingival plaque of periodontal pockets. Besides, this experimental model may allow us to support the clinical meaning of our *in vitro* study.

Interestingly, *P.gingivalis* LPS had no statistically significant influence on both cell populations - independent from LPS dose used and treatment duration. These results are in agreement with previous literature reports demonstrating the low cytotoxic activity of *P.gingivalis* LPS (Kadono 1999; Mo 2008; Wang 2010; Zhang 2010; Sipert 2013). This fact could be explained by the low endotoxic potencies of *P.gingivalis* LPS in comparison to lipopolysaccharide derived from enteric bacteria, like *Escherichia coli* (Horiba 1989; Dixon 2005; Muthukuru 2005; Belibasakis 2007; Kocgozlu 2009; Jain

2010; Jotwani 2010). Specifically, *P.gingivalis* LPS is characterized by the unique structure of lipid A (Millar 1986; Ogawa 2007), which is also supposed to be responsible for the weak immunogenic properties of *P.gingivalis* (Darveau 1998; Liu 2008a).

4.2.2 Effects on TLR2 and TLR4 expression

In order to unveil DFPCs responsiveness to LPS, analyses of TLR2 and TLR4 expression were performed. According to our results, DFPCs express both TLR2 and TLR4 in mRNA and protein levels. Tomic *et al.* also demonstrated the expression of TLR3 and TLR4 on human DFPCs (Tomic 2011). Parallely, the expression of TLR2 and TLR4 was confirmed in BMSCs. These results are in accordance to recent studies showing the expression of TLRs in MSCs (Liotta 2008; DelaRosa 2010). Further, Pevsner-Fischer *et al.* confirmed the expression of functional TLRs by analyzing the responses of MSCs to TLR agonists (Pevsner-Fischer 2007).

Here, the impact of LPS treatment on the expression of mRNA encoding for TLR2 and TLR4 was comparatively evaluated in DFPCs and BMSCs. The significant down-regulation of TLR4 mRNA in both cell populations might be part of an adaptive mechanism of cells being exposed to bacteria, as already proposed (Mo 2008). On protein level the expression of TLR4 was significantly elevated only on LPS-treated BMSCs, while the expression levels of TLR4 in DFPCs remained unchanged. Activated TLRs are supposed to deliver signals produced by injured tissues, thus contributing to host immune response and tissue repair processes (Wang 2002). Hwa Cho *et al.* already proposed the idea that activation of hMSCs through TLR ligands may alter their TLR expression pattern and stem cell fate (Hwa Cho 2006). The fact that LPS treatment did not influence significantly the expression of TLRs by DFPCs might signal the tolerant character of this cell population in the presence of toxins. Nevertheless, the underlying mechanism for this tolerizing phenomenon is not yet known.

Notably, LPS did not influence the TLR2 gene expression in both cell populations, whereas the expression of TLR4 was significantly altered. These results support the idea that LPS could be recognized through TLR4 (Darveau 2004), as TLR2 is supposed to be mainly responsible for lipoprotein and lipopeptide sensing (Jin 2008). On the

contrary, Bainbridge *et al.* reported that *P.gingivalis* LPS may activate both TLR2 and TLR4 in human embryonic kidney cells (Bainbridge 2002). However, the same study demonstrated the heterogeneity of *P.gingivalis* LPS used in the experiments. These controversial data might be explained by the differential experimental approaches and emphasize the complexicity of signaling cascades that activate TLRs.

4.2.3 Effects on cell migration potential

The innate regenerative potential of the periodontium has been extensively investigated and clearly appears to be dependent on wound management (Ivanovski 2006). Current research focuses on identifying biologic factors that favor migration and proliferation of stem cells, thus promoting healing and regeneration of the periodontium (Fuseler 2012; Felthaus 2013). A profound understanding of biological and clinical variables could be critical for the effective optimization and increased predictability of periodontal-regenerative therapies (Polimeni 2006).

In fact, migration to the appropriate site of injury is considered to be critical for the therapeutic efficacy of stem cells (DelaRosa 2010; Vertelov 2013). It is proposed that migratory activity of stem cells is strongly dependent on their local or systemic inflammatory context (Ponte 2007). Several studies have already described an enhancement of MSCs mobility after stimulation with TLR agonists. Waterman *et al.* suggest that MSC polarization could be an explanation to the effect of TLR stimulation and its downstream consequences on the migratory properties of stem cells (Waterman 2010). Another study on human BMSCs supports the notion that the stimulation of BMSCs with TLR agonists led to the activation of downstream signaling pathways, including NF-κB, Akt and MAPK (Tomchuck 2008). Park *et al*, also, demonstrated that LPS may promote the migration ability of murine odontoblast-like cells via TLR4 through the ERK and PI3/Akt signaling pathways (Park 2011).

In this context, we sought to analyze the effects of TLR stimulation on the migration rates of DFPCs by an *in vitro* wound healing model. Interestingly, LPS treatment enhanced significantly the wound healing efficiency of DFPCs compared to the untreated controls. These data suggest a positive impact of LPS on the mobility of DFPCs, which could play a pivotal role in tissue repair processes. On the contrary, LPS

treatment had no significant impact on the migration rates of BMSCs in this study. The differential migration rates of DFPCs and BMSCs could be explained by the proposed theory that responses to TLR ligands are cell-type specific (Lundberg 2007). The fact that DFPCs originate from developing tissues (dental follicle) may also provide an explanation for their enhanced migratory potential upon stimulation (Gronthos 2000).

4.2.4 Effects on IL-6 secretion

There are numerous signalling molecules involved in cell migration. Signaling pathways that control migration of MSCs, involve chemoattractant-receptor axes and intracellular signalling pathways. Moreover, extracellular matrix and biophysical factors play important role in guiding migration of MSCs (Li 2011). IL-6 is a multifunctional cytokine, involved in the initiation of host inflammatory processes against periodontal pathogens, leading to periodontal bone loss (Graves 2008). Indeed, high levels of IL-6 have been associated with the presence of chronic periodontitis in patients (Nibali 2011). Besides, the direct induction of IL-6 secretion after cell stimulation with *P.gingivalis* LPS is well documented (Steffen 2000; Imatani 2001; Feldman 2011; Herath 2011; Zhao 2012).

Here we focused on the possible role of IL-6 in the migration of LPS-treated DFPCs. Recent reports support the notion that secreted IL-6 may act in a paracrine fashion on MSCs, thus enhancing migratory potential and cell survival (Schmidt 2006; Rattigan 2010; Tsai 2012). Remarkably, the analysis of IL-6 secretion showed no cytokine sekretion by DFPCs, as previously reported (Morsczeck 2012). These results could imply that DFPCs do not participate in the initiation of inflammatory processes and retain a tolerant character under the influence of toxins. Further, it could be assumed that IL-6 was not responsible for the enhanced migratory activity of LPS-treated DFPCs. The exact mechanisms leading DFPCs migration remain to be elucidated. On the contrary, the secretion of IL-6 by BMSCs was elevated after LPS treatment. Raicevic *et al*. already demonstrated that MSCs responsiveness to TLR ligands may cause alterations of their cytokine secretion profile (Raicevic 2010). These data may support the idea that TLR signalling is involved in the production of IL-6 by MSCs, as part of their pro-inflammatory shift, at sites of inflammation (Frost 2005; Pevsner-Fischer 2007; DelaRosa 2010; Waterman 2010).

5. Conclusions

In conclusion, analysis of data presented here indicates that DFPCs represent a population of multipotent cells with unique properties. According to the results, DFPCs shared common properties with BMSCs, but also significant differences in their biological responses. Thus, DFPCs were highly clonogenic and proliferative, while expressing same stem cell surface markers, as BMSCs. Furthermore, it was demonstrated that both DFPCs and BMSCs were positive for TLR2 and TLR4 expression. Notably, LPS treatment provoked differential responses in both cell populations. In case of TLRs, their expression in DFPCs seemed to remain unchanged after LPS treatment. On the contrary, TLR4 expression in BMSCs was significantly upregulated. Further, LPS treatment resulted in elevation of DFPCs migration rates. Nevertheless, DFPCs did not produce IL-6 even under the influence of LPS. These results let us speculate that the enhanced migratory ability of DFPCs was not IL-6 driven. On the contrary, BMSCs were responsive to LPS by showing higher levels of IL-6 production. Moreover, the *in vitro* wound healing ability of BMSCs was not affected either by LPS stimulation or by the elevated levels of IL-6.

According to the above results, we suggest that DFPCs are able to response to bacterial LPS. However, DFPCs seem to retain a tolerant character under the influence of toxins. We speculate that DFPCs may not actively participate in the initiation of immune response of the host. The enhanced cell viability and wound healing rates of LPS-treated DFPCs may indicate the applicability of this cell population in cell-based regenerative approaches. A better understanding of DFPCs behavior in sites of infection is needed to support this tempting conception.

6. Summary

Periodontitis represents one of the major oral health burdens worldwide. It is a bacterially induced inflammatory disease, characterized by the destruction of tooth-supporting tissues. Particularly, certain bacteria strains, such as *Porphyromonas gingivalis*, are believed to be greatly involved in the initiation and progression of the disease. Thus, conventional periodontal treatments are based on root surface debridement and disinfection of diseased periodontal tissues. Nevertheless, these therapeutic approaches have proved to be insufficient to attain complete and predictable regeneration of the periodontium.

Currently, much progress has been made in understanding the cellular and molecular events involved in formation of the periodontium. The presence of multipotent cells in dental tissues has been demonstrated. These cell populations are proposed as suitable candidates for cell-based periodontal therapies. Specifically, dental follicle is considered as source of multipotent cells. Experimental studies have shown that dental follicle progenitor cells (DFPCs) may support repair and regeneration of the periodontium *in vivo*. Thus, DFPCs have been proposed as biological grafts for cell-based therapies. Nevertheless, it is speculated that delivery of DFPCs in bacterially colonized periodontal pockets may cause alterations in their functional and phenotypic profile.

Aim of this study was to investigate the ability of DFPCs to sense and respond to *P.gingivalis* lipopolysaccharide (LPS). The influence of LPS on DFPCs was evaluated in terms of cell viability, TLR2 and TLR4 expression, migratory capacity and IL-6 secretion. Additionally, DFPCs properties were compared to bone marrow stem cells (BMSCs), a well-studied class of adult stem cells.

Human DFPCs were isolated from dental follicle tissues of freshly extracted wisdom teeth. After testing their multipotent characteristics, DFPCs were treated with different doses of *P.gingivalis* LPS at several time periods. Toll-like receptors (TLR) 2 and 4 are believed to be essential for the recognition of *P.gingivalis* LPS. The analysis by qRT-PCR and flow cytometry indicated that DFPCs, similar to BMSCs, expressed low levels of both TLR2 and TLR4 at gene and protein level, respectively. Concretely, the expression of TLRs in DFPCs remained unchanged after LPS treatment, while TLR4

expression in LPS-treated BMSCs was significantly upregulated. According to *in vitro* scratch assays, LPS treatment resulted in elevation of DFPCs migration rates that is essential for wound healing processes. Furthermore, assays on the secretion of interleukin-6 (IL-6), a pro-inflammatory cytokine and potent stimulator of cell migration, have been undertaken. Interestingly, the levels of IL-6 secretion of DFPCs and BMSCs remained unchanged after LPS treatment. Finally, conduction of MTT assays demonstrated no influence of LPS on viability rates of both cell populations.

Taken together, these results suggest that DFPCs were able to sense and respond to *P. gingivalis* LPS. However, DFPCs seemed to retain a tolerant, non-inflammatory character under the influence of toxins. On the other hand, the enhanced wound healing rates of LPS-treated DFPCs may indicate the applicability of this cell population as biological graft in cell-based regenerative approaches. Further *in vivo* studies are needed to support this tempting conception. Conclusively, this study provides new insights into understanding the physiological role of dental-derived progenitor cells in sites of periodontal infection.

7. Thesis statements

1. Dental follicle progenitor cells (DFPCs) are able to sense and respond to *Porphyromonas gingivalis* lipopolysaccharide (LPS).
2. DFPCs responses to *P. gingivalis* LPS are differential in comparison to bone marrow stem cells (BMSCs).
3. DFPCs, similar to BMSCs, express low levels of both Toll-like receptors (TLR) 2 and TLR4, which are believed to be essential for the recognition of *P.gingivalis* LPS.
4. LPS treatment has no impact on TLR2 and TLR4 expression by DFPCs, while TLR4 expression in LPS-treated BMSCs is significantly upregulated.
5. According to *in vitro* wound healing assays, LPS treatment results in elevation of DFPCs migration rates.
6. LPS treatment has no impact on DFPCs secretion levels of interleukin-6 (IL-6), a pro-inflammatory cytokine and potent stimulator of cell migration.
7. DFPCs have the potential to promote periodontal wound healing under the influence of toxins.

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10. Appendix

A. Curriculum Vitae

B. Selbständigkeitserklärung