Paracrine Effects of Mesenchymal Stem Cells on Dental Tissues – in vitro and in vivo studies

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Dedication:

This thesis is dedicated to my mothers and my wife

“I know the price of success: dedication, hard work, and an unremitting devotion to the things you want to see happen.”

Frank Lloyd Wright

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SCIENTIFIC ENVIRONMENT

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2. Animal facilities of the Vivarium

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ABBREVIATIONS

ALP	Alkaline phosphatase
Ang	Angiopoietin
BDNF	Brain-derived neurotrophic factor
BFGF	Basic fibroblast growth factor
BMSC	Bone marrow stromal cells
BMPs	Bone morphogenic proteins
Cbfa1/Runx2	Core-binding factor alpha 1/Runt-related transcription factor 2
Col 1	Collagen Type I
COX-2	Cyclooxygenase enzymes 2
CM	Conditioned medium
DSPP	Dentin sialophosphoprotein
DPC	Dental pulp cells
DPSC	Dental pulp stem cells
DMP-1	Dentin matrix protein–1
EGF	Epidermal growth factor
ECM	Extracellular matrix
ECR	External cervical resorption
ESRR	External surface root resorption
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDF-5	Growth differentiation factor-5
GDNF	Glial cell line-derived neurotrophic factor
hDPC	Human dental pulp cells
HGF	Hepatocyte growth factor
IFN-γ	Interferon-Gamma
IGF1	Insulin-like growth factor 1
IL	Interleukin
MSC	Mesenchymal stem cells

BMSC-CM	Bone marrow stromal cell-conditioned medium
MTT	Methylthiazole tetrazolium
NGF	Nerve growth factor
OC	Osteocalcin
OM	Osteo/odontogenic medium
OSTF1	Osteoclast stimulating factor1
PDL	Periodontal ligament
PGE_2	Prostaglandin E_2
PGP 9.5	Protein Gene Product 9.5
PDGF-BB	Platelet-derived growth factor-BB
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor-alpha
SD	Serum deprivation
VEGF	Vascular endothelial growth factor

ABSTRACT

In regenerative medicine or dentistry, it has been reported that stem cells induce the regenerative potential of injured tissues. In the present thesis, pulp and periapical tissues as well as pulpal cells were exposed to bioactive soluble molecules secreted by bone marrow mesenchymal stem cells (BMSC) cultured *in vitro* to determine the paracrine effects of MSC on tissue healing and regeneration.

In **Study I**, the proliferation and osteo/odontogenic differentiation of human dental pulp cells (hDPC) exposed *in vitro* to the exogenous recombinant growth differentiation factor-5 (GDF-5) and to a cocktail of soluble growth factors released by bone marrow stem cells in a conditioned culture medium (CM) were evaluated. Cell proliferation was examined by MTT, and osteo/odontogenic differentiation was assessed by Real-Time Quantitative Reverse Transcription PCR, alkaline phosphatase (ALP) staining, osteocalcin (OC) immunoreactivity and Alizarin Red Staining. It was found that CM collected from cultures of BMSC has higher osteo/odontogenic inductive effect on hDPC than GDF-5.

Study II was designed to evaluate the influence of CM on the healing responses of the dental pulp and periodontium of rat molars, following immediate replantation. CM had no effect on vascular endothelial growth factor (VEGF) mRNA and ALP mRNA in the dental pulp after 3 days, whereas an up-regulation of ALP mRNA was found in the tooth socket of the replanted teeth. Observations after 90 days showed that CM reduced the occurrence of external cervical and surface resorption and prevented extensive dentin production in replanted teeth.

Following the disclosure in **Study II** that CM had a protective effect on the pulp tissue following replantation, **Study III** was undertaken in order to investigate the underlying effect of CM on the release of inflammatory cytokines from hDPC *in vitro*, and on the gene expression of inflammatory cytokines following tooth replantation. *In vitro,* CM significantly stimulated production of prostaglandin E_2 (PGE_2), the inflammatory cytokines interleukin IL-10, -6 and -8, and chemokine RANTES, in hDPC compared with the control cells. Three days after tooth replantation, significantly lower mRNA levels of IL-1β, and-6, and TNF-α were associated with CM than with untreated replanted teeth.

These studies showed that BMSC-CM stimulates early differentiation and matrix mineralization, and the expression of inflammatory mediators in hDPC *in vitro*. BMSC-CM seems to attenuate the initial inflammatory reaction in pulp tissue, and enhance pulpal and periodontal healing following replantation of rat molars.

LIST OF PUPLICATIONS

The thesis is based on the following studies, referred to in the text by their Roman numerals:

I. **Niyaz Al-Sharabi,** Ying Xue, Masahito Fujio, Minoru Ueda, Cecilie Gjerde, Kamal Mustafa, Inge Fristad. **Bone marrow stromal cell paracrine factors direct osteo/odontogenic differentiation of dental pulp cells**. *Tissue Eng Part A. 2014 Nov; 20 (21-22):3063-72.*

II. **Niyaz Al-Sharabi**, Ying Xue, Minoru Ueda, Kamal Mustafa, Inge Fristad**. Influence of bone marrow stromal cells secreted molecules on pulpal and periodontal healing in replanted immature rat molars**. Dental Traumatology. 2015:n/a-n/a.

III. **Niyaz Al-Sharabi,** Manal Mustafa, Ying Xue, Minoru Ueda, Kamal Mustafa, Inge Fristad. **Conditioned media from cultured human bone marrow stromal cells attenuate the inflammatory reactions in dental pulp tissue.** Dental Traumatology - submitted.

The author also contributed to an original **Study** published recently but not included in the thesis:

Masahito Fujio, Zhe Xing, **Niyaz Al-Sharabi**, Ying Xue, Akihito Yamamoto, Hideharu Hibi, Minoru Ueda, Inge Fristad, Kamal Mustafa. **Conditioned media from hypoxic-cultured human dental pulp cells promotes distraction osteogenesis healing through blood vessel regeneration or enrichment**. J Tissue Eng Regen Med. 2015.

1. INTRODUCTION

Most tissue organs have limited regenerative capacity after injury. In this context, stem cells are of fundamental importance, primarily because of their inherent ability to differentiate into a variety of phenotypes to replace injured cells and structures [1]. Stem cell therapy, intended to enhance wound healing or tissue regeneration, is based on the concept that with appropriate induction, mesenchymal stem cells, usually bone marrow stromal cells, can differentiate into cell lineages appropriate to the injured tissue [2-4]. During *in vitro* culture to expand the quantity of stem cells, bioactive soluble molecules, such as growth factors, cytokines and microvesicles, collectively referred to as secretome, are secreted by the cells into the culture medium [5]. Previously discarded as a waste product after culture of the cells, this conditioned culture medium (CM) may be a readily accessible source of growth factors and cytokines, with potential clinical application, for example to enhance healing and moderate inflammation. Although the underlying mechanisms have not been fully clarified, recent research indicates that the action of the bioactive soluble molecules secreted by the stem cells may be as important to tissue repair as the differentiation of the stem cells themselves [6]. In wound healing, for example, CM, because of its secretome content, might have potential as a cell-free therapeutic agent.

In hard tissues such as tooth or bone, incomplete regeneration of tissues after trauma may result in functional impairment and compromised appearance. Dental trauma may cause injury to vascular, neural and hard tissues. Recovery may be compromised by pulpal necrosis and infection, arrested root formation and root resorption [7, 8]. In replantation of avulsed teeth, application of biological molecules or growth factors in the form of secretome might reduce the risk of root resorption, a common cause of failure.

Human dental pulp cells are multipotent, with high growth potential. There is increasing awareness of their potential as a readily accessible source of stem cells for use in other organs [9]. The mechanisms underlying the response of human dental pulp cells to trauma are not fully understood. In a broader context, further understanding of pulp cell biology and the role of secretome in pulpal healing may lead not only to

improved methods for regeneration of dental tissues but also to advances in clinical applications, in regenerative medicine.

The studies on which the present thesis is based were designed to investigate the potential of BMSC-CM to stimulate angio- and neurogenesis and connective tissue formation, namely bone, dentin and periodontal tissues, *in vitro* and in an animal tooth replantation model.

1.1. STEM CELLS

During the early stage of embryonic cell division, a pool of cells becomes specialized [10]. These specialized cells give rise to three embryonic germ layers; 1) the ectoderm, which forms the outer body layer and is also a precursor for the brain, the spinal cord and the nerves, 2) the mesoderm, which gives support to tissue cells (e.g. connective tissue) and 3) endoderm, which becomes the internal tissue lining (e.g. the lining of respiratory organs) [10]. Not all embryonic cells have the potential to progress to fully functional stem cells [10]. In adulthood, some residual cells persist, as reservoirs for tissue renewal and healing after injury [11].

There is also a subpopulation of quiescent resident cells, referred to as adult stem cells, somatic stem cells, or organ-specific adult stem cells [12], located in special microenvironments called stem cell niches, where they are regulated and maintained [13]. Although the bone marrow is regarded as the primary reservoir for stem cells, their presence has also been reported in a variety of tissues such as adipose tissue [14], dermis [15] and dental tissues [16]. In the studies on which this thesis is based, the stem cells investigated were harvested from human adult bone marrow (Lonza) and human adult dental pulp tissue.

1.1.1. Bone marrow stromal cells (BMSC)

Generally, the bone marrow microenvironment comprises specific cells in close proximity within a connective tissue network [17].

Within the bone marrow, there are two heterogeneous populations of stem cells: hematopoietic stem cells and multipotent mesenchymal stromal cells (MSC) [2]. MSC are undifferentiated (immature) adult progenitor cells, with the potential to

differentiate into several mesodermal tissues and cell lineages, including bone (osteoblasts), cartilage (chondrocytes) and fat (adipocytes) [2-4]. MSC may also be able to transdifferentiate into non-mesodermal lineages such as hepatocyte-like cells (endodermal lineages) [18] and neuronal and neuroglial cells (ectodermal lineages) [19].

Although stem cells constitute only 0.001 to 0.01% of the total cell population of bone marrow, MSC can easily be isolated and expanded *in vitro*. According to the criteria established by the International Society for Cellular Therapy (ISCT) [20], isolated MSC should attach to the plastic dish after culture. This property allows separation of MSC from the hematopoietic stem cells. MSC should also express the cell surface antigens CD105, CD73 and CD90 and be negative for CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR. A further criterion is that *in vitro,* MSC should retain the potential to differentiate into mesenchymal tissue-specific cells, such as osteoblasts, adipocytes, and chondroblasts [20].

To overcome the challenge of MSC heterogeneity, cell separation techniques have been proposed to isolate particular cell types. However, to date no specific cell surface marker candidate has been identified.

1.1.2. Dental pulp stem cells (DPSC)

The ability of dental pulp tissue to heal or regenerate after injury is dependent on the survival of existing cells, or their renewal by multipotent mesenchymal progenitor cells of the dental pulp tissue [16, 21, 22]. Under normal conditions, stem cells are quiescent (temporarily non-proliferative). Activity, triggered only in response to insults, is in the form of differentiation into odontoblast-like cells with secretory activity. Dental pulp cells (DPC) have been harvested from permanent and primary teeth and their potential application in dental pulp therapy has been investigated [23].

Fibroblastic cells isolated from the pulp tissues of deciduous and supernumerary teeth produce dentin-like nodules *in vitro* [24]. Dental pulp stem cells (DPSC) comprise a subpopulation of MSC in the dental pulp tissue, defined as fibroblast-like cells with standard stem cell properties, including clonogenic, self-renewal and multipotential differentiation properties [16, 23]. On the basis of their mesenchymal

and ectodermal origins, DPSC are regarded as a heterogeneous population of MSC [25].

As with other MSC, DPSC can be distinguished from non-mesenchymal stem cells by their pattern of cell surface marker expression. Although there is no specific marker(s) for this subpopulation, their expression pattern should be similar to that of BMSC [16]. When maintained in specific induction medium, these unique cells can also differentiate or transdifferentiate into different cell lineages, including neuronal, bone and endothelial cells [25] and have recently been used for dental and other tissue engineering purposes [9].

1.1.3. Applications of MSC and challenges

In vitro investigations confirm that MSC have the potential to differentiate not only into diverse mesodermal lineages (e.g., osteogenic, chondrogenic and adipogenic) but also beyond conventional mesodermal lineages, including hepatocyte-like cells [18], neuronal and neuroglial cells [19] and endothelial cells [26].

The homing capacity of stem cells has been demonstrated in a number of studies. In a mouse model, intravenously administered mesenchymal cells derived from bone marrow migrated into different irradiated tissues (bone, cartilage, and lung) [27]. In a canine model, transplantation of CD31-subfraction cells from bone marrow (BMSC), adipose tissue (ADSC) and DPSC into an empty root canal indicated the potential to regenerate dental pulp tissue [28]. MSC have also been investigated for their therapeutic effects in clinical conditions such as osteogenesis imperfecta [29], graft versus host disease (GVHD) after bone marrow transplantation [30], liver diseases [31] and burn-induced skin defects [32].

Despite promising potential, a major disadvantage of stem cell–based therapy is that the cells are generally unavailable in the quantities required for direct clinical application. This issue has largely been overcome by the development of *in vitro* cell expansion strategies. Several methods are available, including varying the serum content of the culture medium, modifying culture surfaces, and/or the addition of growth factors and cytokines. During these expansion procedures, the stem cells may lose their capacity to self-renew (stemness) [33].

Post-transplantation, stem cells are vulnerable to microenvironmental stress. They are generally found in low quantities, with poor engraftment properties and survival rates [34, 35]. This is generally attributed to microenvironmental factors such as hypoxia (low oxygen supply), ischemia (poor blood supply) and inflammatory cells and cytokines [36-38]. Not only is stem cell survival threatened, but the potential to differentiate into target tissues may also be compromised, with an adverse effect on the healing outcome (Figure 1).

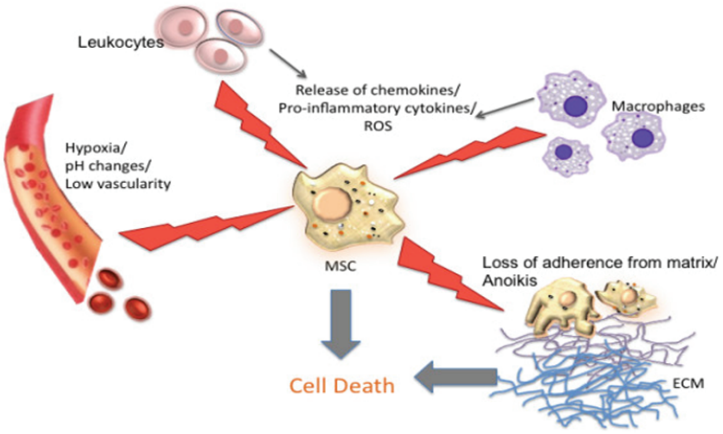

Figure 1 Effect of microenvironmental stress on MSC at the graft site. Adapted from Melanie Rodrigues et al. *Stem Cell Res Ther.* (2010), presented with permission from Biomed Central [36].

1.2. BIOACTIVE SOLUBLE MOLECULES

Bioactive molecules, secreted by inflammatory, progenitor and endothelial cells and fibroblasts [39] may be classified as inflammatory cytokines (pro- and anti-inflammatory cytokines), growth factors, vasculogenic and neurogenic factors [40].

The action of the biological molecules is generally determined by their proximity to the target area. Cellular changes can be triggered in numerous ways: 1) autocrine; (2) paracrine; (3) endocrine; (4) juxtacrine; (5) extracellular matrix mediated and (6) intracrine action [41]. In an autocrine response, the bioactive molecules act on the cell

itself. In a paracrine response, the adjacent cells are affected. However, when the distance is short-range, the terms “juxtacrine and intracrine” are sometimes used. Endocrine responses are those triggered in more distant cells.

There is considerable scientific evidence to support the beneficial effects of a number of growth factors in stimulating the healing process, for example members of the TGFβ superfamily [42-44], vascular endothelial factor (VEGF) and fibroblast growth factor (FGF) [45-47].

1.2.1. Growth differentiation factor-5 (GDF-5)

Exogenous growth factors, singly or in combination with numerous others, are involved in many aspects of tissue healing, including neuro- and angiogenesis and repair of bone-cartilage and tooth structure.

Growth differentiation factors are bone morphogenic proteins (BMPs), which are subsets of the signalling peptides of the transforming growth factor (TGF) β superfamily [48]. BMPs are pleiotropic morphogens involved in such biological responses as cell proliferation, differentiation and apoptosis [48] and exert their biological effects through specific receptors called TGFβ receptors (Type I and Type II). Smad 1, 5 and 8 are considered to be important intracellular transduction pathways of BMP receptors [49].

BMPs and their receptors are expressed in the developmental stages of many tissues such as bone, tooth, heart, neural tissue and cartilage [48, 50]. Currently, they are widely applied in hard tissue engineering, to stimulate differentiation of mesenchymal stem cells [51]. GDF-5, also known as BMP-14 or cartilage-derived morphogenetic protein-1 (CDMP1), has various biological effects on the development of connective tissues, including bone, joints, tendons and ligaments [52]. GDF-5 also has an angiogenic effect [53] and is considered to be a neurotrophic factor in the development of the nervous system [49]. Deficiency of this signalling peptide has been associated with delayed healing of bone fractures [54].

Exposure of MSC to GDF-5 *in vitro* results in formation of cartilage and further ossification, such as condensation, increased glycosaminoglycan deposition and collagen type II transcripts, indicating chondrogenic differentiation of BMSC [55]. The results of a number of studies of DPSC and dental tissues indicate that GDF-5 is

involved in neuro- and angiogenesis as well as in cell differentiation and mineralization of extracellular matrix protein [42, 43].

1.2.2. MSC-secretome

In regenerative medicine and/or tissue engineering applications, engrafted stem cells are vulnerable to ischemia and this may lead to poor survival of grafted cells and limited paracrine secretion and function [37]. Recent research shows that the paracrine effects of MSC secretome are an important factor in repair and healing after stem cell therapy [6, 56, 57]. Cell secretome comprises a group of soluble and vesicular trophic and immunomodulatory factors, such as extracellular proteins, growth factors, cytokines, chemokines and other molecules, which may influence the surrounding microenvironments and directly or indirectly modulate the biological phenotypes of different resident cells [5, 58].

There is considerable evidence that the paracrine effects of secreted soluble biomolecules are important in neuro- and angiogenesis. BMSC has been associated with detectable neuroprotective factors in ischemic areas, with reduction of apoptosis and enhancement of endogenous cellular proliferation [59]. A co-culturing system of MSC and dorsal root ganglion explants revealed that the majority of the secreted soluble molecules comprised extracellular matrix molecules, adhesion molecules and neurotrophic mediators, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), bFGF and ciliary neurotrophic factor (CNTF) [60]. Expression of angiogenic cytokines such as VEGF, angiopoietin 1 and 2 (Ang-1 and -2), bFGF, HGF, platelet-derived growth factors and insulin-like growth factor 1 (IGF1) have also been associated with the secretome of BMSC, indicating a pivotal role in angiogenesis [61].

MSCs have low inherent immunogenicity and modulate immunological responses [62]. BMSC have been shown to secrete interleukin-6, and 8 (IL-6 and IL-8), prostaglandin E_2 (PGE_2), and VEGF into culture medium and modulate inflammation through an effect on dendritic cells, natural killer cells, and naïve and effector T cells, to a more tolerant/anti-inflammatory phenotype [63]. Secretion of IL-6 and PGE_2 by BMSC was associated with reduced local inflammation in animals with arthritis [64]. IL-6 derived from BMSC is assumed to be a crucial factor for *in vitro* inhibition of

monocyte differentiation and skewing monocytes towards an anti-inflammatory producing cell type (IL-10) or type II activated macrophages [65].

1.2.2.1. Preparation of MSC secretome

Over the past decade, many attempts have been made to modulate cell behaviour towards improved cell survival, avoidance of immune rejection, and cell secretion. Cell preconditioning strategies comprise pre-treating or stimulating the cells during *ex vivo* or *in vitro* expansion before transplantation [66]. Among the strategies available today are physiological preconditioning [hypoxic conditions [61] and serum deprivation [67]], genetic manipulation [68], molecular or pharmacological treatment [69] and thermal preconditioning [37]. In the studies on which this thesis is based, serum deprivation was used to modulate the secretion of bioactive soluble molecules.

The process of *in vitro* culture of cells in either serum-free, reduced serum, and/or serum-protein free medium is collectively referred to as serum deprivation [70] and has recently been proposed as a tool to evaluate several cellular mechanisms, including cell secretion. It was shown that MSC cultured under serum deprivation conditions could survive for up to 1 year [67]. Moreover, serum deprivation induced the expression of angiogenic markers in MSC (e.g. insulin growth factor, angiopoietins and VEGF-A). By an autocrine mechanism, MSC change shape to form typical microtubules, like endothelial cells [67]. Compared with MSC cultured in complete medium, those cultured under conditions of serum deprivation were reported to be more resistant to oxidative damage (less DNA damage) and were stimulated to change their secretion of paracrine factors [71]. Moreover, MSC underwent epigenetic modifications during serum deprivation, which in turn increased secretion of pro-survival cytokines, including IGF1 and leptin [71].

Serum deprivation has also been shown to induce secretion of inflammatory healing mediators, including IL-6, IL-8 and chemokine (C-X-C motif) ligand 1 (CXCL1) from MSC [72]. Recently, the process has been evaluated as a physiological preconditioning model for ischemia, leading to secretion of a variety of bioactive soluble factors from MSC, including TGF-β1, VEGF-A, IGF1 and HGF, which in turn regulate a number of cellular activities, including growth, survival and angiogenesis [67, 73].

1.3. USE OF STEM CELL-DERIVED CONDITIONED MEDIUM (CM) IN REGENERATIVE MEDICINE

While stem cell transplantation is effective, especially in the treatment and regeneration of nonhealing tissues, recent *in vitro* and *in vivo* studies indicate that stem cell-CM can significantly enhance wound healing (section 1.3.1. and 1.3.2). CM is a supernatant of the cell culture and was previously regarded as waste, containing cell debris and artefacts. It is now recognized that CM contains a variety of bioactive soluble molecules and microvesicles secreted by the cells being cultured. A variety of biological functions has been attributed to CM containing factors secreted from MSC, such as immunoregulation, anti-apoptotic and anti-fibrotic properties [74, 75], cell homing or chemotactic effects [76], stimulation of angio/neurogenesis [77, 78] and connective tissue healing [73].

Cells can be cultured under different conditions, such as normal standard conditions, hypoxic (2% O_2) or serum deprivation conditions, and the duration can be regulated [79, 80]. The conditions under which the cells are cultured influence the composition of the culture medium.

The mechanisms by which CM promotes wound healing, independently of the stem cells, are not fully understood. The present thesis addresses the role of BMSC-CM as a stimulatory factor in angio/neurogenesis and connective tissue formation (e.g. bone and periodontal tissues).

1.3.1. Role in angio- and neurogenesis

The primary goal of angiogenic therapy is to enhance the growth of blood vessels in injured tissues, optimizing nutrient support for wound healing. Compared with CM collected from endothelial cell culture, BMSC-CM is reported to reduce apoptosis and to improve cell survival in hypoxic endothelial cells [74]. BMSC-CM has been shown to contain antiapoptotic and angiogenic factors, such as IL-6, VEGF, and monocyte chemoattractant protein. The observed effect on cell survival in hypoxic endothelial cells was attributed to activation of the PI3K-Akt signalling pathway [74]. In a similar study, BMSC-CM induced substantially longer vascular sprouts than a control (unconditioned) medium [67]. MSC grown under hypoxic conditions secreted greater

amounts of proangiogenic cytokines (e.g. stromal-derived factor-α, chemokine (C-X-C motif) ligand 1, RANTES, monocyte chemoattractant protein and macrophage colony-stimulating factor). However, both hypoxic and normoxic CM stimulated chemotaxis and adhesion of endothelial cells and promoted survival of cardiomyocytes [76]. BMSC-CM also contains a wide range of proteins with angiogenic effects, such as VEGF, monocyte chemoattractant protein and macrophage inflammatory protein-1α and b [77]. BMSC-CM stimulated complex capillary networks in canine endothelial cells (CVEC), and induced cellular migration and survival via decreasing apoptotic pathway of CVEC (caspase-3) [77].

Because of the importance of both angiogenesis and neurogenesis in healing and function, the role of BMSC-CM in these processes has been investigated in a number of studies. Collectively, these studies demonstrate that BMSC-CM has a profound neurogenic effect, promotes the growth and proliferation of nerve cells and increases the expression of neuronal markers [78, 81, 82].

1.3.2. Role in bone and periodontal tissue healing

The role of BMSC-CM in bone and periodontal healing has been investigated in several studies [73]. In one study, calvarial bone defects were treated with human BMSC-CM [80]. *In vitro,* after 48-h of incubation, the BMSC-CM was positive for IGF-1 and VEGF. BMSC-CM was able to induce migration of rat MSC. Moreover, BMSC-CM induced expression of the specific osteogenic markers osteocalcin (OC) and core-binding factor alpha 1/Runt-related transcription factor 2 (Runx2) in mouse MSC [80]. In the rat calvarial bone defect model, human BMSC-CM/agarose composite gel was implanted into the bone defects for 4 or 8 weeks. New bone generation was revealed in the defects implanted with the BMSC-CM /agarose composite gel. Regenerated bone induced by BMSC-CM was of higher quality than that achieved by the other groups. Human BMSC-CM also increased the migration and mobilization of injected labelled rat MSC into the implanted region [80].

Another study revealed that *in vitro,* MSC secrete a broad range of cytokines including IGF1, VEGF, TGF-β1, and HGF [73]. This study also showed that BMSC-CM (30% FBS) substantially enhanced the proliferation and migration of dog MSC and periodontal ligament (PDL) cells compared with serum-free DMEM. *In vivo,* intrabony

defects were filled with scaffolds of absorbable atelo-collagen sponge and loaded with BMSC-CM or PBS. After 4 weeks, BMSC-CM had induced new bone and cementum, with minimal inflammatory cell infiltration [73].

A more recent study in rabbits investigated the effect of human MSC-CM in accelerating bone formation after 2, 4 and 8 weeks [83]. *In vitro,* MSC-CM induced significant migration of rat MSC compared with serum-free DMEM. This study also showed that MSC-CM contains a group of secreted bioactive molecules, including IGF-1, VEGF, TGFb1, but not FGF-2, PDGF-BB, or BMP-2 [83]. This study also showed that after 2, 4 and 8 weeks, MSC-CM grafted onto a beta-tricalcium phosphate scaffold in the sinus cavity induced more bone formation in the entire sinus floor than a PBS/beta-tricalcium phosphate scaffold [83].

The potential of serum-free human BMSC-CM and MSC to accelerate bone healing has been compared in a distraction osteogenesis mouse model [56]. After 4 days, a high speed-distraction gap of 3.2 mm was filled with either MSC or BMSC-CM. After 11 days, both MSC and their CM had achieved a similar rate of new bone callus formation [56].

Another study has evaluated bone formation around titanium implants by immobilization (stabilization) of rat BMSC-CM [84]. Compared with the controls, immobilized rat BMSC-CM on the surface of the titanium implant positively modulated attachment of rat BMSC after 24 h *in vitro*, with an up-regulated gene expression of OC after 14 days. Moreover, extracellular matrix, signal transduction, protein synthesis and growth factors were detected in the implant culture. In regard to osseointegration around the titanium implant *in vivo*, rat BMSC-CM promoted significant bone formation after 7 and 14 days [84].

1.4. EFFECT OF BIOACTIVE SOLUBLE MOLECULES IN DENTAL TISSUES

In traumatized dental tissues, bioactive soluble molecules should stimulate neuro-angiogenesis within the root canal and the surrounding tissues, promote migration, proliferation and differentiation of endogenous stem cells and induce release of extracellular matrix proteins [85]. However, traumatic injuries to the dental hard

tissues are often complicated by vascular and neural damage, including pulpal necrosis and infection, arrested root formation and root resorption [7, 8]. Function or esthetics may be compromised [86]. In clinical dentistry, complete tooth displacement is usually treated by replantation. In successful cases, the structural integrity of the alveolar ridge is maintained and the vitality of pulp and periodontal tissues is preserved. Healing is achieved either by repair or regeneration, with formation of new tissue similar to the original [87]. A number of factors influence the healing outcome, including the severity of the impact, the age of the patient, the viability of periodontal ligament cells and the time elapsing between the trauma incident and tooth replantation or transplantation (immediate or delayed).

The capacity of bioactive molecules to induce cell differentiation and vascularisation has been investigated both *in vitro* and *in vivo*. The results of *in vitro* investigations of enzyme-dissociated and/or explant pulp cells from permanent and/or deciduous teeth indicate that dental pulp stem and progenitor cells could regenerate dentin-like structures [88]. DPC are sensitive to their surrounding local microenvironments and stimuli [89]; after culture in specific induction microenvironments, DPSC display morphological changes and form mineralization nodules [16]. During odontogenic differentiation, these cells can express specific odontoblasts and dentin-forming markers such as dentin sialoprotein (DSP) and dentin matrix protein (DMP-1). Together with expression of ALP and collagen type I (Col 1), the formation of mineralized nodules confirms their ability to form a dentin-like structure.

Dental pulp tissue healing is tightly regulated by metabolic changes and the microenvironments surrounding the quiescent stem cells or progenitor cells. Growth factors and cytokines have been investigated for their potential to stimulate cell differentiation and vascularization. VEGF and FGF-2 were shown to be likely candidates for the induction of angiogenesis during pulp repair [90]: VEGF induced neovascularization, FGF2 had a profound effect on proliferation of DPSC, and cells stimulated by FGF2 and TGF β1 showed odontoblast-like differentiation through increased gene/protein expression (DSP and DMP) and increased ALP.

The use of bioactive soluble molecules for promoting PDL and cementum regeneration following tooth trauma has also been investigated. [91]. BMP-7 did not

promote healing after complete removal of the PDL and cementum, but did so in teeth with partially intact cementum and PDL [91]. Another study evaluated the effect of NGF and/or epidermal growth factor (EGF) on the regeneration of periodontal tissues, alveolar bone and pulpal tissues in an auto-transplantation rat model [92]. Application of collagen, NGF and/or EGF was followed by extensive root resorption and minimal cementum coverage, but not ankylosis. In pulpal healing, collagen and EGF improved vascularization of the pulp [92].

Bioactive soluble molecules are less effective in inflamed pulp tissue [93], prompting experiments in which the pulp is extirpated and the root canal is filled with exogenous bioactive soluble molecules. In one such study, bFGF, VEGF, NGF, PDGF, and BMP7 were delivered individually or together into endodontically cleaned teeth before transplantation into the mouse dorsum [94]. New dentin-pulp like tissues were established with new blood vessels [94]. In another study, coating the root of extracted teeth with enamel matrix derivative (EMDOGAIN) resulted in normal PDL healing and reduced both replacement and inflammatory root resorption [95]. A study which evaluated the ability of both SDF1 and BMP7 to regenerate connective tissues (bone and PDL) used both ectopic and orthotopic models for tooth regeneration in an anatomically shaped artificial tooth. The results showed that SDF1 and BMP7 enhanced the regenerative capacity of the PDL and stimulated new bone formation [96].

1.5. RATIONALE

MSC-based therapy has shown promising results in wound tissue healing, through cell-cell and cell-matrix interactions [1]. Despite the disadvantage of an invasive collection procedure, therapeutic applications of bone marrow stem cells have been successful [16, 97]. It is now recognized that bioactive soluble molecules secreted by MSC into the culture medium during expansion *in vitro,* may induce significant biological effects, independently of cell transplantation. CM is easily prepared, can be freeze-dried, is relatively inexpensive and does not present the immune rejection problems which might complicate stem cell therapy [85]. Thus CM shows promise as an alternative approach to stem cell therapy and warrants further investigation. In recent experiments using dental healing models, CM has shown significant positive effects [73]. An important potential field of application would be in managing dental trauma, reducing the risk of root resorption after replantation of avulsed teeth. In the studies on which this thesis is based, CM is investigated with special reference to its effects on dental pulp cells *in vitro* and, in an *in vivo* animal model, its effects on post-traumatic tissue healing in teeth and supporting structures.

2. AIM

The overall aim of this thesis was to study the paracrine effects of mesenchymal stem cells (MSC) on pulpal cells and dental tissues. Utilizing *in vitro* and *in vivo* experimental models, the paracrine effects of MSC were investigated by exposing human dental pulp cells (hDPC) and periapical tissues to conditioned medium collected from cultures of human bone marrow cells (BMSC-CM).

Specific Aims

- To investigate the osteo/odontogenic potential of human dental pulp cells (hDPC) in response to BMSC-CM and recombinant growth differentiation factor-5 (GDF-5) **(Study I).**

- To investigate the effect *in vivo* of BMSC-CM on the healing process of pulp and supporting structures following replantation of rat molars (**Study II**).

- To determine the effect of BMSC-CM on the production of inflammatory mediators from hDPC *in vitro*, and on the mRNA expression of inflammatory mediators in rat pulpal tissues after tooth replantation **(Study III)**.

3. MATERIAL AND METHODS

The different experimental models, evaluation and methods of assay are summarized in Table 1. Further details are provided in each individual **Study** (I-III).

Table 1 *A brief outline of the whole* ***Study***

Experimental Models	**Evaluations**	**Methods**
***In vitro* culture of hDPC, stimulated by rhGDF-5 and/or BMSC-CM. (Study I)**	Cell Isolation Stem cell Characterization Cell Morphology Cell Proliferation Osteo/odontogenesis	Enzymatic digestion Flow cytometry Crystal Violet MTT Real-Time-qRT-PCR Western Blot Alizarin Red Staining ALP Staining Immunocytochemistry
Bilateral rat tooth replantation model. (Study II)	Blood and nerve supply Root resorption and dentin formation	Real-Time-qRT-PCR Immunohistochemistry Histology
Release of inflammatory mediators from hDPC *in vitro*, and gene expression in the pulp tissue of replanted molars *in vivo* (Study III)	Protein secretion Cyclooxygenase enzymes 2 (COX-2) protein expression PGE_2 production Gene expression of inflammatory mediators	Cytokine assay Western Blot Prostaglandin E2 assay Real-Time-qRT-PCR

3.1. CELL CULTURE

3.1.1. Primary dental pulp cell isolation (Studies I and III)

Dental pulp cells were isolated from the third molar teeth of healthy adult patients during routine operative procedures at the Dental Clinic at the University of Bergen, Norway by the enzyme dissociation method described previously [16, 98]. Briefly, dental pulp tissue was isolated by creating a groove (0.5 - 1 mm) with a sterile high-speed drill under copious irrigation with sterile saline solution along the cemento-enamel junction, followed by splitting with a chisel to reveal the pulp chamber. Harvested tissue was sectioned into small pieces and immersed in an enzymatic digestive solution of collagenase type 1 (4 mg⁄ml) and dispase (2 mg⁄ml) for 1 h at 37 °C. The digested tissue was centrifuged at 1400 rpm for 10 min, and thereafter filtered through a 70 µm strainer.

Single-cell suspensions were cultured and expanded with DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, before incubation at 37 °C in 5 % CO_2 atmosphere. The culture medium was changed twice weekly. When the cells reached ~75 % confluence, they were either subcultured or stored in liquid nitrogen.

The protocol was approved by the ethics committee at the University of Bergen, Norway (225.05, 3.2008.1750, 2009/610 and 2013/1248). All participants were informed verbally about the project and signed an informed consent form before the procedure.

3.1.2. Primary dental pulp cell characterization (Studies I and III)

To evaluate the mesenchymal stem cell markers on isolated human dental pulp cells, flow-cytometric analysis was carried out using Mouse Anti-Human antibodies against cell surface molecules (CD90-FITC, CD105-APC, STRO-1 PerCP-Cy5.5, and CD24-PE (R&D System). Cells at passage 1 were stained by incubation with conjugated antibodies in the dark for 1 hour, washed thoroughly with PBS and then centrifuged at 250-300x g for 5 min at 4°C. The supernatant was removed, and the cell pellets were

re-suspended in 10% paraformaldehyde and stored at 4°C until analysis. Flow-cytometric analysis was conducted by FACS flow cytometry (BD FACS Aria SORP). A total of 100,000 events were used for each sample.

3.1.3. Collection of BMSC-CM (Studies I-III)

In **studies I-III**, primary human bone marrow stromal stem cells (MSC) at passage 3 were seeded at an initial density of 2×10^6 into T-75 cm^2 culture flasks and then allowed to reach 80-90% confluence. The cells were then thoroughly washed three times with PBS and re-fed with serum free-DMEM for 48 hr. In **Study III**, DMEM serum-free medium was prepared under the same conditions and defined as a control. The cell supernatant was then collected and centrifuged at 3000 xg for 3 min at 4 ^oC and re-centrifuged at 1500 ×g for 5 min at 4 ^oC. The final supernatant was collected in small tubes and then stored at -80 and/or 4 ^oC for further experiments.

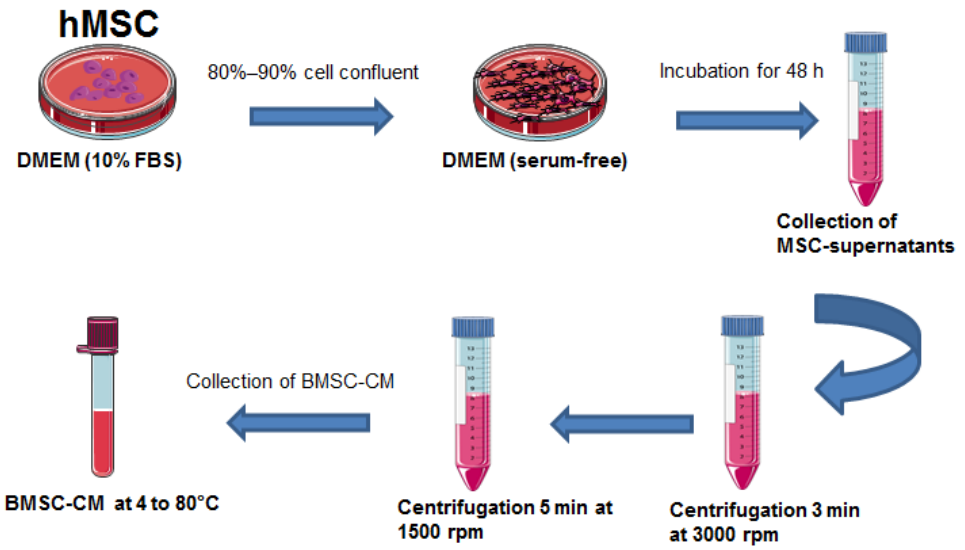

Figure 2 The BMSC-CM collection procedure. Figure made using Servier Medical Art.

3.1.4. Experimental design (Studies I and III)

In **Studies I** and **III**, human dental pulp cells at passage 4-6 were cultured and treated with different media as illustrated in Figure 3.

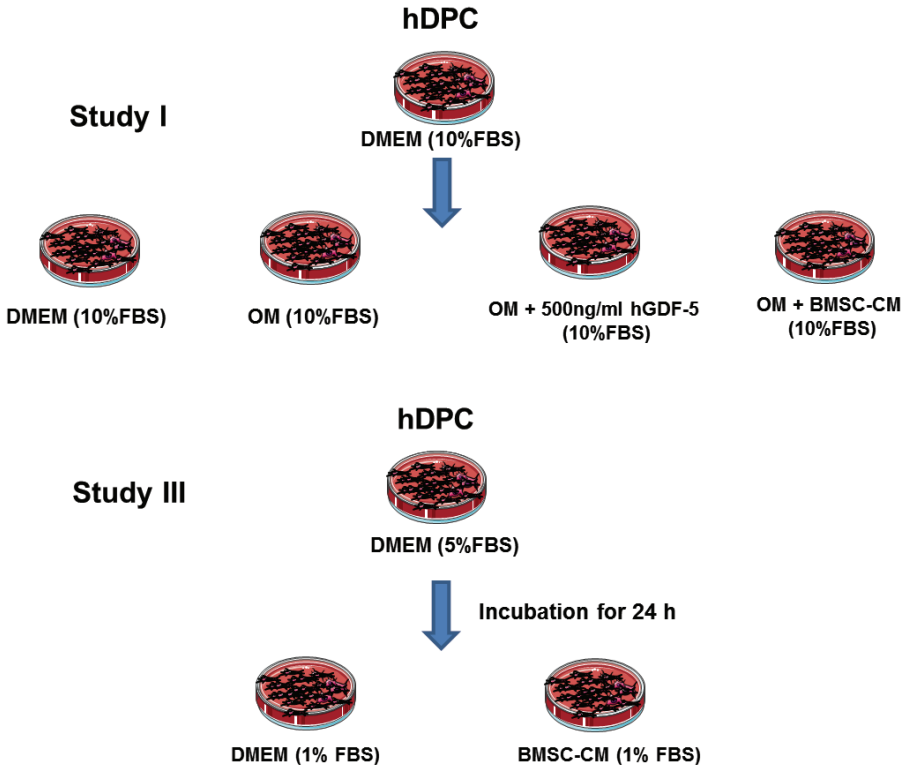

Figure 3 Outline of **Studies I** and **III**. In **Study I**, hDPC were cultured under complete growth media, trypsinized, counted and then cultured in different media: (1) DMEM (10% FBS), (2) OM (10% FBS), (3) 500ng/ml rhGDF-5 plus OM or (4) BMSC-CM plus OM. In **Study III**, hDPC were cultured with DMEM (5% FBS) for 24 hr. The cells were then cultured with either DMEM (1% FBS) or BMSC-CM (1% FBS). Figure made using Servier Medical Art.

3.1.4.1. Cell Morphology and proliferation assay (Study I)

To investigate the effect of different treatment media on the cell morphology, 2 × 10^4 hDPC were incubated in a 24-well plate for 48 and 120 hr. The cell morphology was then disclosed by crystal violet staining. Cell proliferation under different culture conditions was analysed by colorimetric assay for the quantification of MTT mitochondrial reaction of the viable cells. Briefly, hDPC were seeded at 1 x10^4/well in 96-well plates for 3, 24, 48, and 120 hr. After incubation, the cells were further incubated for 4 h with methylthiazol tetrazolium assay (MTT; Sigma Chemicals). Then, a solution of DMSO containing 6.25% (v/v) 0.1 M NaOH was added to the wells and incubated by shaking for 20 min at room temperature. The end product was quantified by microplate spectrophotometry at a wavelength of 570.

3.1.4.2. Western Blot (Studies I and III)

3.1.4.2.1. Protein Extraction

In **Study I**, hDPC, initial density 1 x 10^5 per cm^2, were seeded onto a six-well plate for 5 days. In **Study III**, hDPC, initial density 7 X 10^5 cells were seeded in 80 mm petri dishes for 24 hr. At the end of the incubation period, the cells were lysed using lysis buffer (RIPA buffer plus protease and phosphatase inhibitors; ThermoScientific) and centrifuged at 14,000 rpm at 4^{o}C for 20 min. Total protein concentration was measured by Pierce-BCA Protein Assay Kit (ThermoScientific).

3.1.4.2.2. Western Blot procedures

Twenty micrograms of total protein extracts were subjected to a 10% Mini-PROTEANR TGX™ Precast Gel for electrophoresis and transferred onto PVDF transfer membranes. The membranes were blocked in 5% non-fat dry milk, incubated overnight at 4°C with primary antibody, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. Immunoreative bands were visualized by use of enhanced chemiluminescence with Imun-Star™ WesternC™ Chemiluminescence Kit. Finally, the Gel Doc™ EZ System was used for photography, For **Study I**, protein expression was quantified using a densitometer normalized with the internal controls.

3.1.4.3. ALP and mineralization assay *(Study I)*

To examine the effect of different treatment modalities on the osteo/odontoblast differentiation of hDPC, cytochemical staining for ALP enzyme was carried out after 5 days. After washing and fixation with neutral buffered formalin (10%) for 1 min, ALP staining buffer (SIGMAFAST_BCIPR/NBT tablet; Sigma-Aldrich) was added for 10 min. For the mineralization assay, Alizarin R Staining was used to detect calcium nodule formation after 7 and 14 days. After washing and fixation with 70% ethanol for 1h at –20oC, Alizarin R Staining (40mM) was added and incubated with shaking at room temperature for 1 hr. Finally, the positive and negative stains were recorded using an inverted microscope equipped with a digital camera.

3.1.4.4. Immunocytochemistry analysis (Study I)

To examine the effect of growth factors on protein expression by hDPC, an immunocytochemistry assay was carried out after 5 days. After incubation and fixation with 4% paraformaldehyde, methanol with 0.3% hydrogen peroxide was added. The cell-plate was incubated overnight with blocking serum (5%, goat serum and/or donkey serum) at 4°C in a humidity chamber. Primary antibodies against OC, TUBβ3, DMP1 and DSP were added overnight at 4°C. Finally, the hDPC were washed three times in PBS and incubated firstly with goat secondary antibody and then with Avidin/Biotinylated enzyme Complex for 1 h at room temperature. Immunoreactivity was finally disclosed by incubation with substrate solution and examined with an inverted microscope equipped with a digital camera.

3.1.4.5. Prostaglandin E_2 production (Study III)

To evaluate the effect of BMSC-CM on PGE_2 production in hDPC, the quantity of PGE_2 was determined after 24 hr in the supernatant, using a commercially available ELISA kit (Prostaglandin E_2 ELISA Kit, Monoclonal, Neogen Corporation, Lansing, Michigan, USA). PGE_2 in both CM and control medium was also quantified. After incubation, the supernatants of all media were added to the monoclonal PGE_2 antibody pre-coated plate before the diluted enzyme conjugate was added, and then incubated at room temperature for 1 hr. The plate was washed by buffer and substrate solution was

then added for 30 mins. Finally, 50 ul of hydrochloric acid (1N) was added to stop the enzyme reaction. The PGE_2 content of all samples was quantified by microplate spectrophotometry (BMG LABTECH, GmbH) at a wavelength of 450 nm.

3.1.4.6. Multiplex Cytokine assay (Study III)

To determine whether BMSC-CM exerted an inflammatory response on hDPC, the concentrations of IL-10, -4, -6, and IL-8, and chemotactic cytokine RANTES were assessed after 24 hr, according to the manufacturer's protocol [99]. The concentration of selected cytokines in both CM and control medium was also measured. Briefly, the capture antibody-coupled beads were incubated with antigen standards and sample supernatants for 30 min, shaking in the dark at room temperature. The plate was then washed thoroughly with buffer to remove unbound materials. Next, the plate was incubated with biotinylated detection antibodies for 30 min by shaking in the dark at room temperature, and then washed thoroughly to remove the unbound biotinylated antibodies. Thereafter, 50 µl of reporter streptavidin-phycoerythrinconjugate (SA-PE) was added and incubated for 10 min in the dark at room temperature. Finally, after removing excess SA-PE, the beads were resuspended in 125 µl and the fluorescence was read and measured by Bio-Plex® MAGPIX™ Multiplex Reader. The observed concentrations were used to calculate the differences among the groups.

3.2. ANIMAL EXPERIMENTS (Studies II and III)

3.2.1. Replantation model using rat maxillary first molars

In Sprague-Dawley rats three weeks of age, immature maxillary first molars were extracted and replanted as previously described [100] (Figure 4A). Anaesthesia with Hypnorm-Dormicum (1 mL fentanyl/fluansion and 1 ml midazolam diluted in 2 ml sterile water) was administrated subcutaneously. The maxillary left first molar was extracted with a straight excavator, left with attached gingival tissue on the mesial side for 2 mins, and then gently replanted to its original position without further treatment. The same extraction procedure was carried out on the right side, but BMSC-CM (25 µl) was injected into the socket before replantation in the original position. The

replantation protocol and evaluation methods are summarized in Figure 4A and B, and Table 2. In **Study II**, un-operated left and right maxillary first molars were used as a reference group (Table 3).

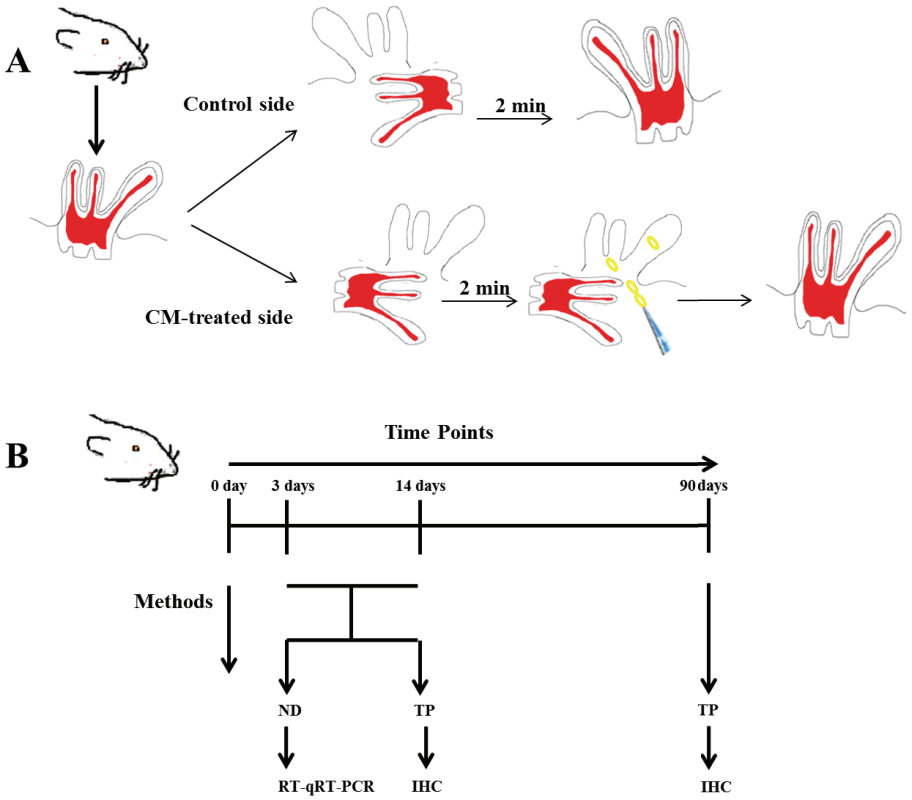

Figure 4 (A) Illustration of the replantation model (**B**) The study design includes three different time points (3, 14 and 90 days). After 3 and 14 days, the animals were euthanized by neck dislocation (ND) for Real-Time-qRT-PCR analysis (RT-qRT-PCR), and transcardiac perfusion (TP) for immunohistochemistry (IHC). After 90 days, all animals were euthanized with TP for IHC.

Table 2 *Replanted maxillary first molars: experimental group treated with CM and untreated control group (**Studies II** and **III**).*

Methods	Endpoint (days)	No. of Rats	Study II	Study III
IHC	**3**	5	x	__
	14	5	x	__
	90	5	x	__
RT-qRT-PCR				
Teeth	**3**	5	x	x
	14	5	x	x
Sockets	**3**	5	x	__
	14	5	x	__

Table 3 *Overview of the different methods used for the reference group (**Study II**).*

Methods	No. of Rats	Study II
IHC	**3**	x
RT-qRT-PCR		
Teeth	**3**	x
Sockets	**3**	x

3.2.2. Immunohistochemistry (IHC) (Study II)

Demineralized specimens intended for cryosectioning were immediately frozen in O.C.T. tissue-tech (Sakura Finetek, Tokyo, Japan). Cryosectioning was performed with a Leica CM 3050S (Leica Microsystems, Wetzlar, Germany) at −24°C. Immunohistochemical staining (IHC) for Laminin- and PGP 9.5-immunoreactivity was conducted as described elsewhere [101] and evaluated according to the different parameters shown in Table 4. An overview of different antibodies is presented in Table 5.

Table 4 *Immunohistochemical parameters evaluated*

Pulp healing pattern	Expression of Laminin- and PGP 9.5-immunoreactivity
Dentin formation	Dentin thickness from the furcal surface of the pulpal floor towards the internal pulp floor
Root resorption	Presence of ESRR and ECR

ESRR: External surface root resorption; ECR: External cervical resorption.

Table 5 *Antibodies* ***(Studies I-III)***

Full name	Abbreviation	Species	Manufacturer	Study
Beta-actin	β-Actin	Human	Santa Cruz	I
Osteocalcin	OC	Human	Santa Cruz	I
Dentin sialophosphoprotein	DSPP	Human	Santa Cruz	I
β3 tubulin	TUBB3	Human	Santa Cruz	I
Dentin matrix protein 1	DMP1	Human	Santa Cruz	I
Laminin	Laminin	Rabbit	StressGen Biotechnologies	II
Protein Gene Product 9.5	PGP 9.5	Rabbit	StressGen Biotechnologies	II
Cyclooxygenase 2	COX-2	Rabbit	Santa Cruz	III
Chemokine (C-C motif) ligand 5	RANTES	Human	Bio-Rad	III
Interleukin-10	IL-10	Human	Bio-Rad	III
Interleukin-6	IL-6	Human	Bio-Rad	III
Interleukin-4	IL-4	Human	Bio-Rad	III
Interleukin-8	IL-8	Human	Bio-Rad	III
Prostaglandin E_2	PGE_2	Human	Neogen Corporation	III

3.3. REAL-TIME-qRT-PCR (Studies I - III)

Details of Real-Time-qRT-PCR used in studies **I-III** are described elsewhere [102]. In **Study I**, total RNA was isolated using an isolation kit (Maxwell® 16 Total RNA Purification Kit, Promega), according to the manufacturer's protocol. In studies **II** and **III**, total RNA was isolated from tooth samples using the TRIZOL method (Invitrogen, Carlsbad, CA, USA). RNA purity and quantification were determined by spectrophotometry (ThermoScientific NanoDrop Technologies, Wilmington, DE).

The reverse transcription reaction was conducted using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), using 1000 ng total RNA dissolved in 40 µl nuclease-free water mixed with reverse transcriptase (RT) buffer, random primers, dNTPs and MultiScribe RT. The cDNA corresponding to 1 µg of mRNA was used as a template in each PCR reaction of primers. The real time PCR was performed under standard enzyme and cycling conditions on a StepOnePlus real-time PCR system using TaqMan® gene expression assays. Amplification was performed in 96-well thermal cycler plates for 30 cycles, with a final 10-min extension at 72 °C. For all studies, the relative expression of each gene was analysed using the comparative CT method ($2^{-\Delta\Delta Ct}$) and normalized to GAPDH, serving as an internal control. An overview is presented in Table 5.

Table 5 *Overview of gene expression assays*

Code	Full name	Abbreviation	Species	Study
Hs99999905-m1	Glyceraldehyde 3-phosphate dehydrogenase	*GAPDH*	Human	I
Hs00231692-m1	Runt-related transcription factor 2	*RUNX2*	Human	I
Hs00164099-m1	collagen type I	*COL1A2*	Human	I
Hs01029144-m1	Alkaline phosphatase	*ALPL*	Human	I
Hs01587814-g1	Osteocalcin	*OC (BGLAP)*	Human	I
Hs00171962-m1	Dentin sialophosphoprotein	*DSPP*	Human	I
Hs01009391-g1	Dentin matrix protein 1	*DMP1*	Human	I
Hs00964963-g1	β3 tubulin	*TUBB3*	Human	I
Hs01931883-s1	Glial cell-derived neurotrophic factor	*GDNF*	Human	I
Hs00375822-m1	Angiopoietin 1	*Ang-1*	Human	I
Hs00900055-m1	Vascular endothelial growth factor	*VEGF*	Human	I
Rn01749022-g1	Glyceraldehyde 3-phosphate dehydrogenase	*GAPDH*	Rat	II-III
Rn01512298-m1	Runt-related transcription factor 2	*RUNX 2*	Rat	II
Rn01511601-m1	Vascular endothelial growth factor A	*VEGFa*	Rat	II
Rn00686607-m1	Osteoclast stimulating factor 1	*OSTF1*	Rat	II
Rn00580432-m1	Interleukin 1β	IL-1β	Rat	III
Rn99999010-m1	Interleukin 4	IL-4	Rat	III
Rn99999011-m1	Interleukin 6	IL-6	Rat	III
Rn00563409-m1	Interleukin 10	IL-10	Rat	III
Rn00567841-m1	Interleukin 8	IL-8 (CXCR2)	Rat	III
Rn00562055-m1	Tumor necrosis factor alpha	TNF alpha	Rat	III

3.4. STATISTICAL ANALYSIS (Studies I-III)

Quantitative results were expressed as mean ± SD. In **Study I**, one-way analysis of variance was followed by a multiple-comparison Tukey test between the groups, control cell, OM, GDF-5 and CM-treated cells using IBM SPSS Statistics 19 (IBM). In **Study II**, one-way analysis of variance was followed by a multiple-comparison Tukey test between control and CM-treated replanted teeth and reference teeth. In **Study III**, Student's t-test was used for comparison between CM and control medium, between CM-treated cells and control cells and between CM-treated and control replanted teeth using IBM SPSS Statistics 22 (IBM). Differences between the means were considered statistically significant at $p < 0.05$.

4. RESULTS

4.1. DENTAL PULP CELLS: ISOLATION AND CHARACTERIZATION (Studies I and III)

Human DPC from young healthy donors (n=3) were successfully isolated, cultured and propagated after enzymatic digestion (Figure 5A). After 7 days, formation of clusters of cells was observed (Figure 5AI) and after 21 days, cells reached 70 to 80% confluence (Figure 5AII). Cells at passage 1 positively expressed the stem cell surface markers CD90 (45.4%), CD105 (5.8%), STRO-1 (6.1%) and CD24 (95.9%) (Figure 5).

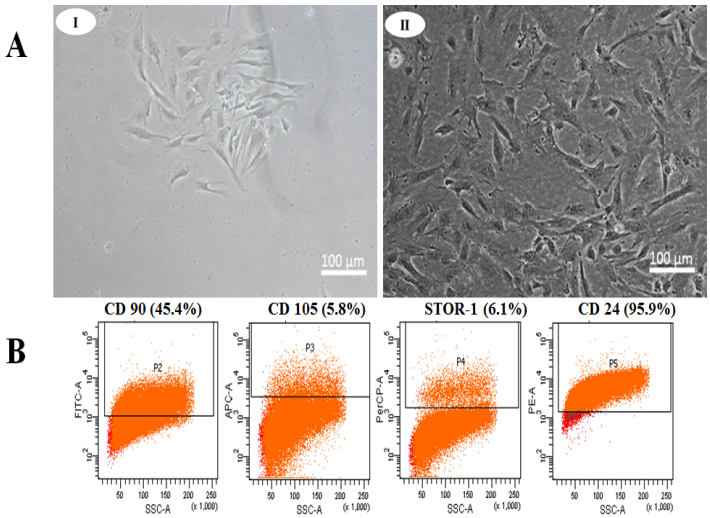

Figure 5 Human DPC after isolation (passage 1). (**AI**) after 7 days and (**AII**) after 21 days). (**B**) Flowcytometric analysis revealed that hDPC at passage 1 positively expressed selected mesenchymal stem cell markers. Modified from Niyaz Al-Sharabi et al. J Tissue Eng (2014), presented with permission of Mary Ann Liebert, Inc.

4.2. OSTEO/ODONTOGENIC DIFFERENTIATION OF ISOLATED hDPC UNDER DIFFERENT CULTURE CONDITIONS *IN VITRO* (Study I)

4.2.1. Cell proliferation under different cell culture conditions

CM significantly inhibited proliferation of hDPC after 24 and 48 hr compared with the OM, and after 120 hr compared with control cells, OM and GDF-5 treated cells (Figure 6).

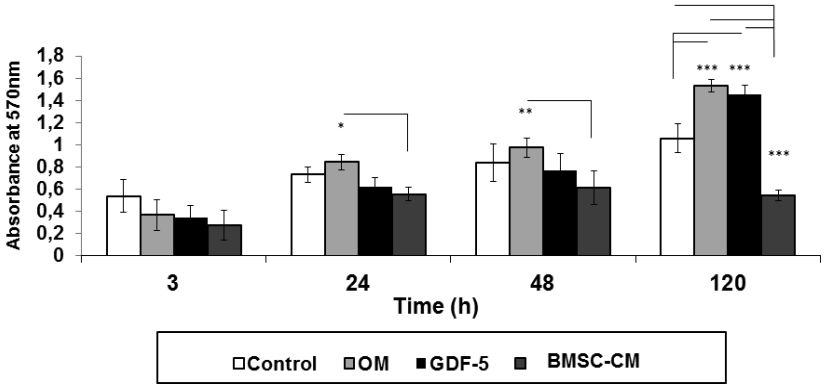

Figure 6 MTT assay reveals that CM has an inhibitory effect on cell proliferation compared with the other groups. The data are presented as mean ± standard deviation. (* $p<0.05$, ** $p<0.01$, and *** $p<0.000$). Adapted and modified from Niyaz Al-Sharabi et al. J Tissue Eng (2014), present with permission from Mary Ann Liebert, Inc.

4.2.2. mRNA level of neuro/angiogenic and osteo/odontogenic differentiation genes

The mRNA expression of TUBβ3 was significantly down-regulated in the cells treated with CM, compared with the other treatment groups after 2 days, and compared with cells treated with OM and GDF-5 after 5 days. Expression of GDNF was up-regulated in hDPC treated with CM, compared with the other groups after 2 days, and compared with the cells treated with OM and GDF-5 after 5 days. Compared with the other groups, the cells treated with GDF-5 exhibited high expression of Ang1

after 2 days: expression declined after 5 and 9 days, but was still higher than for cells treated with OM and CM after 9 days. mRNA expression of VEGF was lower in all treatment groups than in the control. There was no difference between the control cells and those treated with CM at any time point. Significant differences in the mRNA levels of VEGF were found between the cells treated with GDF-5 and control cells after 2 and 9 days. CM significantly down-regulated the mRNA expression of RUNX-2 compared with cells treated with OM and GDF-5 after 2 days. COL-1 and ALP expression were down-regulated in hDPC treated by CM compared with the controls, and cells treated with OM and GDF-5 at all time points. In contrast, compared with OM and GDF-5 treatment, CM treatment of hDPC significantly increased mRNA expression of the late differentiation marker (OC) after 2 and 5 days, and compared with all groups after 9 days. Figure 7 shows different mRNA levels of markers related to neuro/angiogenic and osteo/odontogenic differentiation on hDPC after 2 days.

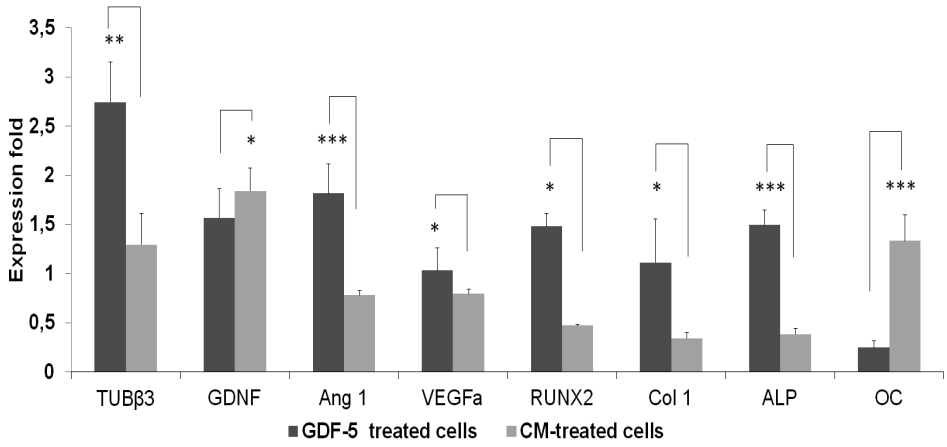

Figure 7 Real-time-qRT-PCR data in GDF-5 and CM-treated cells after 2 days. GAPDH was used for data normalization. The data are presented as mean ± standard deviation (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

4.2.3. Protein expression of TUBβ3 and OC

Immunocytochemical analysis revealed that hDPC after 5 days of culture in CM had the highest expression of TUBβ3 and OC of all the groups (Figure 8).

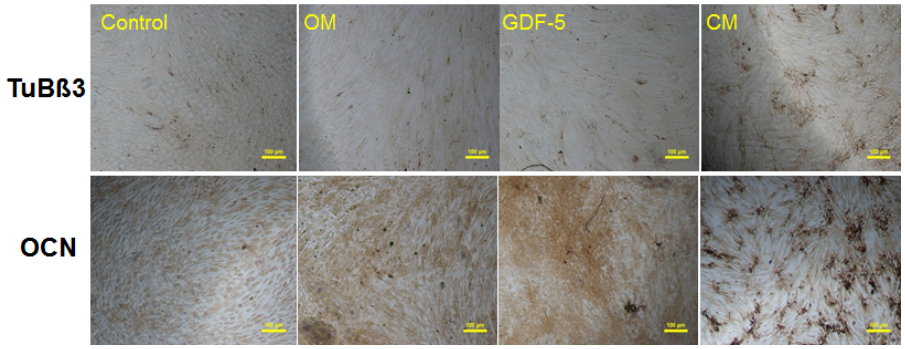

Figure 8 Immunocytochemistry for TUBβ3 and OC in hDPC treated under different culture conditions after 5 days. Modified from Niyaz Al-Sharabi et al. J Tissue Eng (2014), presented with permission from Mary Ann Liebert, Inc.

4.2.4. ALP staining and calcium nodule formation

The hDPC cultured in CM showed weak staining for ALP after 5 days, and highest calcium nodule formation of all the groups after 14 days (Figure 9). The GDF-5 group exhibited massive mineralized nodule formation compared with the OM group.

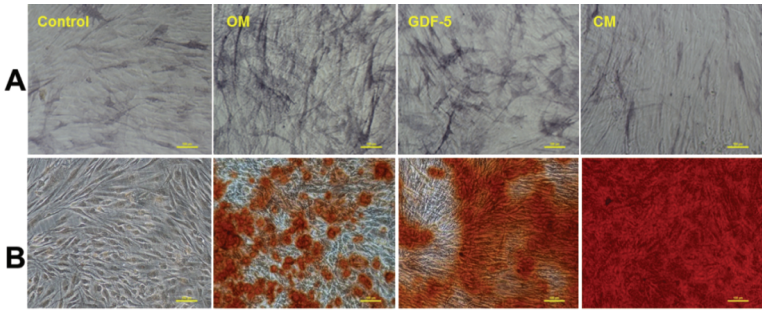

Figure 9 ALP and Alizarin Red Staining. (**A**) High positive staining for ALP in the OM group after 5 days compared with the other groups, the lowest in the CM group. (**B**) At 14 days the CM group had the highest red nodule staining of all groups. Adapted from Niyaz Al-Sharabi et al. J Tissue Eng (2014), presented with permission from Mary Ann Liebert, Inc.

4.3. EFFECTS OF CM ON PULPAL AND PERIODONTAL HEALING FOLLOWING TOOTH REPLANTATION *IN VIVO* (Study II)

4.3.1. Expression of VEGF and ALP mRNA in tooth pulp and socket samples

Real Time qRT-PCR data revealed expression of VEGF and ALP in the tooth samples of replanted teeth and reference teeth after 3 and 14 days. In the tooth samples, the expression level of VEGF and ALP was significantly higher in all replanted teeth than in the reference teeth. However, as shown in Figure 10, at no time point were there any differences in VEGF or ALP levels between the replanted teeth in the control and the CM groups. In the socket tissues, there was no difference between the groups in the level of expression of VEGF. After 3 days, statistically higher levels of ALP were observed for the replanted teeth of the control and CM groups compared with the reference teeth, but there were no differences after 14 days. Compared with the control group, ALP expression in the CM group was statistically higher after 3 days, with no differences after 14 days (Figure 10).

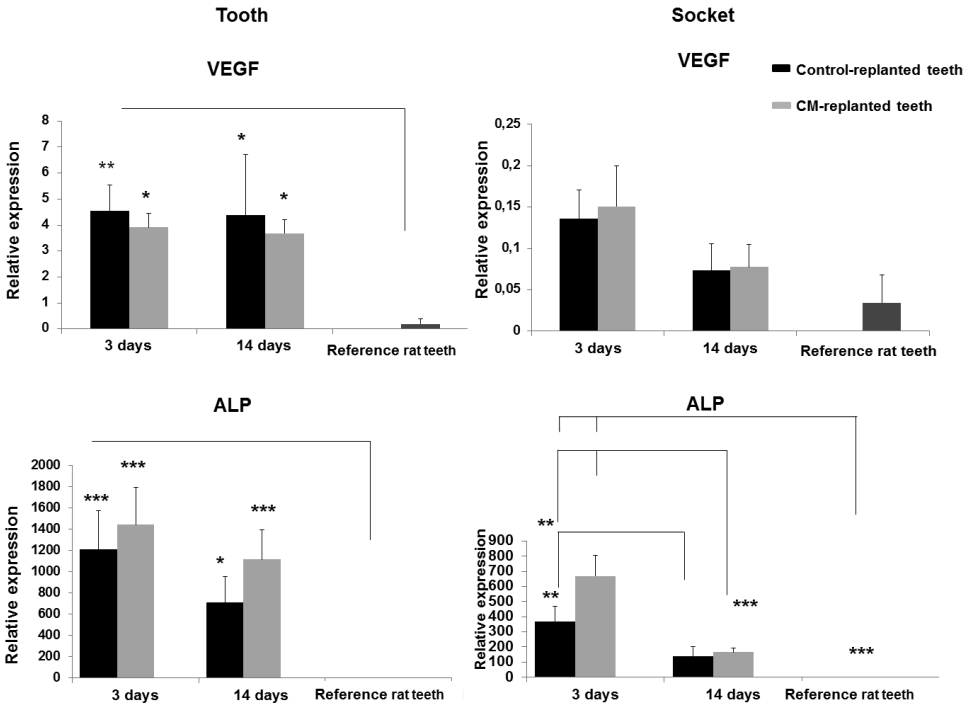

Figure 10 mRNA levels of VEGF and ALP in replanted teeth and reference group**.** GAPDH was used as a reference gene for normalization. The data are presented as mean ± standard deviation (*p < 0.05, **p < 0.01 and ***p < 0.001).

4.3.2. Histological findings

4.3.2.1. Expression of Laminin- and PGP 9.5-immunoreactivity in the pulp and supporting tissues

Histological examination demonstrated laminin-immunoreactivity in the pulp and supporting tissues of the replanted teeth. No morphological differences were observed among the groups of replanted teeth at any time point. The presence of PGP 9.5-immunoreactive nerve fibres was faint or undetectable in the tissue of the replanted teeth after 3 days, but normal expression was found after 14 and 90 days (Figure 11).

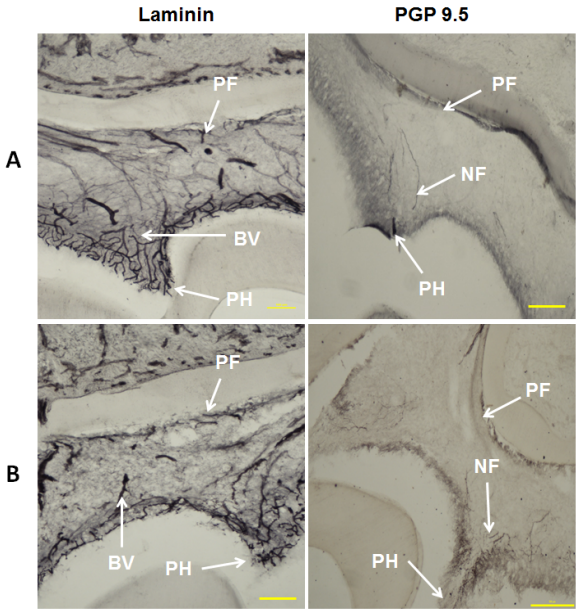

Figure 11 Expression of Laminin- and PGP 9.5-immunoreactivity in a replanted control tooth (A), and one from the BMSC-CM group (B) after 14 days. BV; blood vessels; NF, nerve fibers; PH, pulpal horn and PF; pulpal floor (magnification, 10×; scale bar, 50 μm).

4.3.2.2. Root resorption

After 3 days, no signs of root resorption were detected, whereas after 14 days ECR and ESRR were observed in 5 teeth: 4 from the control group and one from the CM group (Figure 12). After 90 days, all control replanted teeth (100%) showed signs of either ECR or ESRR, compared with only one specimen from the CM group (20%).

Figure 12 External surface root resorption in the apical third of both experimental groups at day 14, indicated by white asterisks (magnification, 10×; scale bar, 50 μm).

4.3.2.3. Periodontal ligament healing

At all observation time points, replanted teeth in both the control and CM groups were characterized by a distinct PDL reattachment. Destruction of the PDL was seen only in resorption areas after 14 and 90 days.

4.3.2.4. Root development

After 3 days, the replanted teeth were characterized by an open apex which continued to narrow at day 14. After 90 days, complete root development was observed in all replanted teeth.

4.3.2.5. Measurement of dentin thickness

There were no differences in dentin thickness between the replanted teeth after 3 days. After 14 days, there was an increase compared with 3 days. After 90 days, the dentin thickness of the replanted teeth in the control group was significantly greater than for the CM group (Figure 13) which in turn was significantly greater than in the reference rat teeth at this time point.

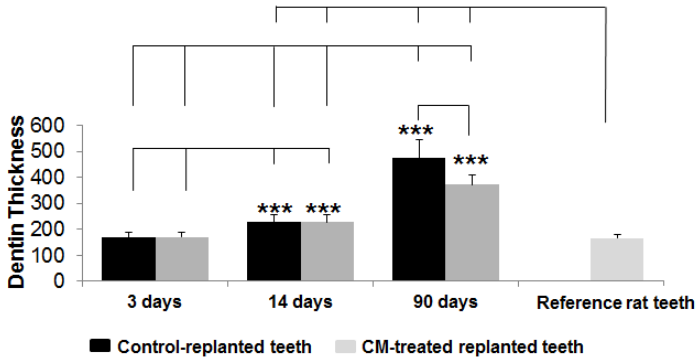

Figure 13 Dentin thickness measurements at day 3, 14 and 90 in all replanted teeth and reference teeth. The data are presented as mean ± standard deviation (***p < 0.001).

4.3.2.6. Bone-like tissue formation (BLT)

As shown in Figure 14, after 90 days, one replanted tooth specimen from the control group (20%) showed mineralized tissue formation in the central part of the pulp chamber. No such change was observed in the replanted teeth of the CM group.

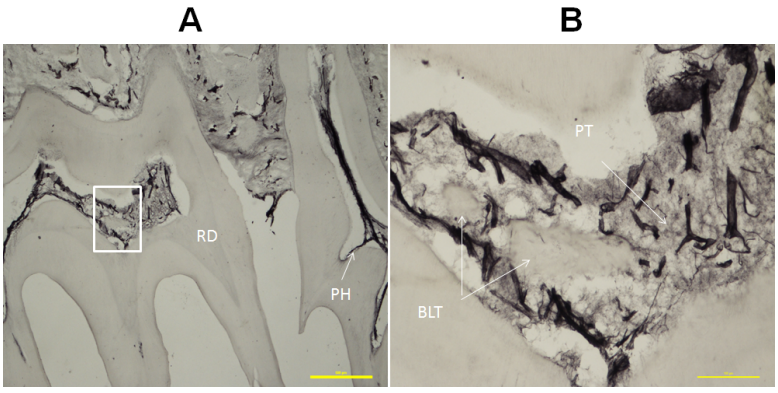

Figure 14 (**A**) In a replanted tooth of the control group pulp calcification (framed) in the central part of the pulp chamber, is surrounded by extensive new dentin formation (magnification, 10x; scale bar, 50 µm. (B) Higher magnification of (A) showing bone-like tissue formation, surrounded by soft tissue. RD: reparative dentin, PH: pulpal horn, BLT: bone- like tissue PT: pulp tissue (magnification, 20×; scale bar, 100 µm).

4.4. RELEASE AND GENE EXPRESSION OF INFLAMMATORY MEDIATORS *IN VITRO* AND *IN VIVO* (Study III)

4.4.1. Concentration of inflammatory mediators *in vitro*

IL-10, -6, and -8, and RANTES were detected in both control medium and CM, but in differing concentrations: the levels of IL-6 and -8, and RANTES were significantly higher in the CM than in the control medium (Figure 15).

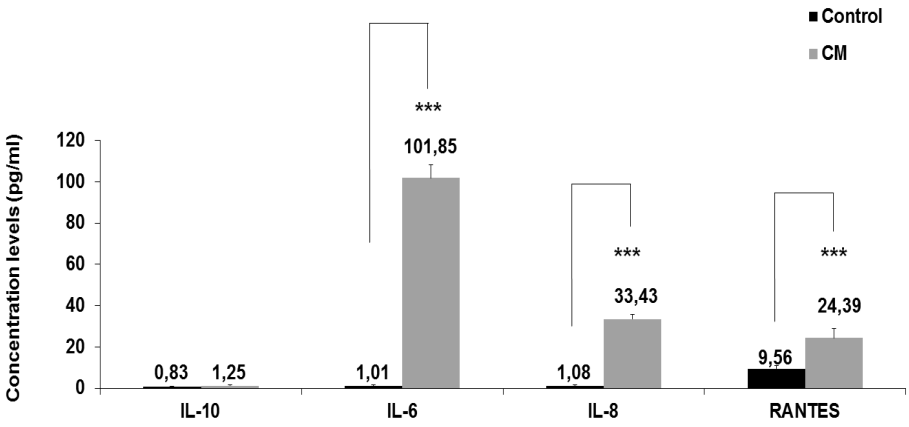

Figure 15 Concentration of inflammatory mediators in CM compared with control. The data are presented as mean ± standard deviation (*p < 0.05 and ***p < 0.001).

4.4.2. CM and inflammatory mediators secreted by hDPC *in vitro*

After culture of hDPC, the levels of IL-10, -6, and -8, and RANTES were statistically higher in CM than in the control medium (Figure 16).

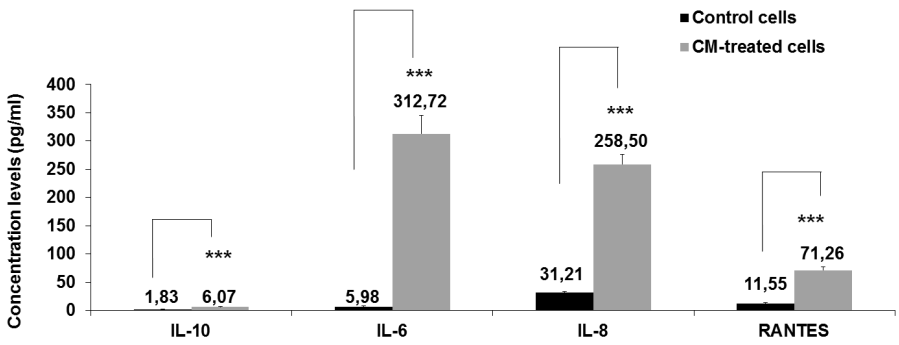

Figure 16 Level of inflammatory mediators in cells from the CM and control groups. The data are presented as mean ± standard deviation (***p < 0.001).

4.4.3. PGE_2 production and protein expression of COX-2 *in vitro*

Production of PGE_2 was significantly higher in CM than in the control medium and in cells cultured with CM compared with control cells (Figure 17 A). Western blot analysis revealed higher expression of COX-2 in hDPC treated by CM than in controls (Figure 17 B).

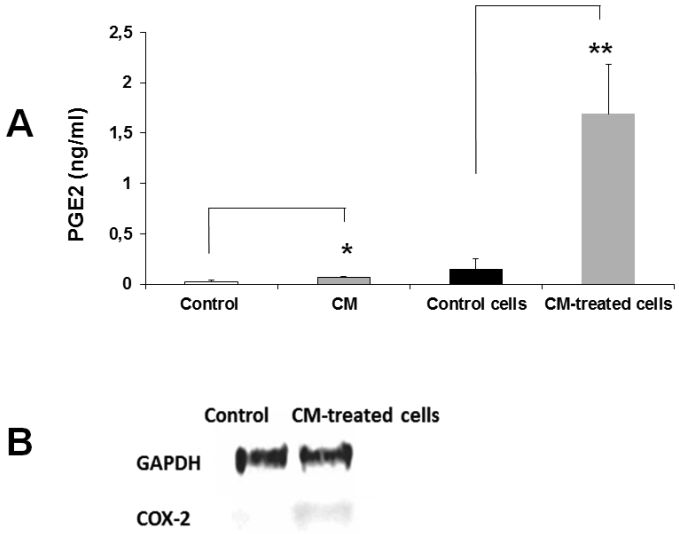

Figure 17 (**A**) Concentrations of PGE_2 in CM compared with control, and in cells from the CM and control groups. (**B**) Western blot data showing expression of COX-2. GAPDH was used as a reference control in the western blot analysis. The data are presented as mean ± standard deviation (*p < 0.05 and **p < 0.01).

4.4.4. Expression of IL1β, -10, -8 , and -6, and TNF-α in replanted rat molars

Three days after replantation of the rat molars, mRNA expression of the pro-inflammatory cytokines IL-1β, and -6, and TNF-α was lower in the CM teeth than in the control teeth; there was no difference between groups in regard to the mRNA level of IL-10 and -8. At 14 days, there was no intergroup difference in the mRNA levels of IL1β, -10, -8 and -6, and TNF-α (Figure 18).

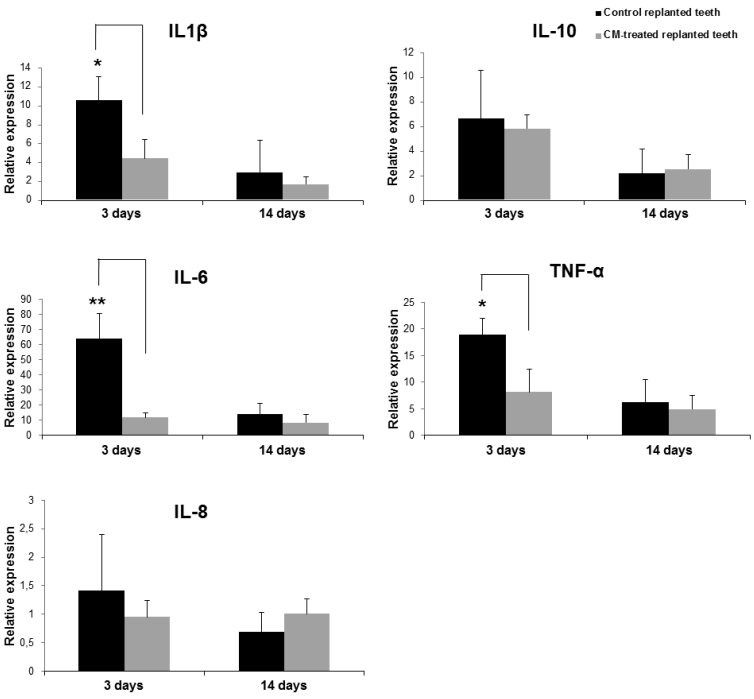

Figure 18 mRNA levels of IL-1β, -10, -8 and -6, and TNF-α in replanted teeth treated with CM and replanted control teeth. GAPDH was used for data normalization. The data are presented as mean ± standard deviation (*p < 0.05 and **p < 0.01).

5. DISCUSSION

Recent research has shown that stem cells can mediate healing responses without prior differentiation, a property attributed to bioactive soluble molecules which are secreted by the stem cells into the surrounding culture medium during expansion *in vitro* [103].

Conditioned culture medium was previously discarded as waste after culture and expansion of stem cells. However, it is now of interest as a readily accessible source of growth factors and cytokines with potential clinical application, to moderate inflammation and enhance healing.

The present thesis is based on *in vivo* and *in vitro* studies of the effects of signalling molecules, released from BMSC during culture, on dental pulp cells and dental tissues. The results show that BMSC-CM stimulates early differentiation and matrix mineralization and the expression of inflammatory mediators in hDPC *in vitro*. The *in vivo* experiments show that BMSC-CM seems to attenuate the initial inflammatory reaction in pulp tissue, and enhance pulpal and periodontal healing following replantation of rat molars.

5.1. DENTAL PULP CELL ISOLATION AND CHARACTERIZATION

In **Studies I** and **II**, hDPC from young adults were successfully isolated, cultured and propagated after enzymatic digestion. Of several methods for harvesting dental pulp cells, enzymatic digestion yields cells with high mineralization potential [104]. Stem/progenitor cells from young adults are preferred. Such cells are less likely to be adversely affected by environmental factors, and can be cultured and propagated to adequate numbers, circumventing any age-related loss in numbers or functionality, which might result in decreased regenerative capacity, including proliferation [105, 106]. The third molars are frequently extracted in early adulthood, at a stage when the structure of these teeth is still immature; hence the pulps harbour a large reservoir of undifferentiated cells necessary to complete tooth development.

To meet the criteria for MSC, stem cells derived from the pulp should express protein surface markers such as CD 90, STRO-1 and CD 105. In accordance with earlier studies

[107], the heterogeneous population of dental pulp cells in the present **Study** positively expressed these markers. STRO-1 antibody is regarded as a marker for purification of stromal cell precursors in bone marrow and dental pulp [108] and has been detected in dental pulp cells and odontoblasts at the stage of completion of crown and root formation [109].

CD 24 antibody is expressed by many cell types, including stem cells derived from the apical papilla (SCAP) [110, 111]. The DPC in the present **Study** exhibited relatively high expression of CD 24. As both SCAP and DPC are derived from neural crest cells [112], this might be attributable to contamination with SCAP during harvesting of the dental pulp tissue, or to the similar origin of the tissue types [112].

It should be noted, however, that inconsistencies in reports about expression of cell surface markers from different studies could be due to many variables such as passage number, composition of the culture medium and individual variations among donors.

5.2. *IN VITRO* DIFFERENTIATION OF DENTAL PULP CELLS

Study I showed that BMSC-CM inhibited hDPC proliferation and induced expression of markers such as TUBβ3 and OC, associated with neurogenic and osteogenic differentiation, respectively. After short-term culture, there was a high rate of mineralization.

There is a strong correlation between cell proliferation and differentiation: terminal differentiation is associated with a shift from a proliferative to a differentiating state [113]. The inhibitory effects of CM on cell proliferation found in **Study I** have also been reported previously [114]. However, contradictory results are also reported: BMSC-CM has been shown to increase proliferation of both PDL cells and MSC *in vitro* [73, 80]. Nor did GDF-5 exert any mitotic effects on hDPC at early time points, but increased cell proliferation after 120 hr. GDF-5 is reported to have no mitotic effects on mouse DPC and porcine cells derived from the dental papilla, but increases the proliferation of stem cells derived from the dental follicle and periodontal ligament stem cells [42, 43, 115]. These inconsistencies regarding the effects of both CM and GDF-5 on cell proliferation might be attributable to the different target cells, cell

culture design or synergistic effects between BMSC-CM and the chemicals in the osteogenic medium [116]. Further study is warranted in order to gain a more detailed understanding of the mitotic effects of GDF-5 and BMSC-CM and the underlying cell signalling pathways.

Col 1, ALP and calcium deposition are often used to identify the early and late stages of differentiation and maturation of cells. ALP expression is greatest when the cell begins to differentiate, followed by a decrease and subsequent calcium deposition [117]. Down-regulation of the core-binding factor alpha 1/Runt-related transcription factor 2 (Cbfa1/RUNX2) gene is an important indicator of fully differentiated odontoblasts [118]. In accordance with these findings, **Study I** revealed that the inhibition of cell proliferation by BMSC-CM was associated with a low level of transcription factor Cbfa1/RUNX2 and ALP staining. Up-regulated gene and protein expression of bone-matrix protein (OC), together with accelerated mineralization were also observed. This is in accordance with previous observations [42, 56, 119].

In addition to the cytokines and growth factors demonstrated in CM, MSC secrete PGE_2. The production of PGE_2 by MSC transplanted to stimulate tissue formation confirms the dual effect of PGE_2 on tissue healing and regeneration [120]. In **Study III**, CM enhanced the expression of PGE_2 and its enzyme (COX-2) by hDPC. A similar response is reported after stimulation of hDPC with bone inductive materials such as Mineral Trioxide Aggregate [121]. In **Study III,** CM induced secretion of IL-6 from hDPC after short-term culture *in vitro* (24 hr). IL-6 has an important immunoregulatory function [65, 122] and affects the angiogenic and bone remodelling processes. IL-6 expression also decreases when MSC undergo cell differentiation into different lineages [123]. Exogenous IL-6 and IL-6 produced by stem cells were found to induce osteoblastic differentiation of periodontal ligament cells as well as adipose stem cells *in vitro* [124, 125]. The ability of MSC-CM to induce mineralization in mouse BMSC *in vitro* was inhibited by antibodies against IL-6, indicating a role of IL-6 signaling in prompting osteogenesis of mouse BMSC [56]. Although the concentrations of cytokines after cell differentiation were not evaluated in this project, it is assumed that the higher paracrine and autocrine levels of IL-6 and PGE_2 found in

Study III might have an effect on initiation of osteo/odontogenic differentiation of hDPC [56, 126].

DPSC are of neural crest origin and might have a therapeutic effect when transplanted to the damaged central nervous system [127]. In **Study I** the hDPC cultured in BMSC-CM expressed TUBβ3 mRNA and protein, indicating neurogenic properties of the cells. Previous research has also shown the induction of neuronal differentiation of DPSC, as evidenced by up-regulation of neurogenic mRNA markers and increased protein expression of TUBβ3 [128].

Revascularization is crucial to healing and repair of the dental pulp. Under specific inductive conditions, DPC, can transdifferentiate into vessel-forming cells with expression of specific cell tissue markers [129]. In **Study I**, BMSC-CM did not induce substantial up-regulation of VEGF and Ang1 mRNA compared with the treated control cells. However, after 2 days, expression of VEGF mRNA was higher in cells cultured with BMSC-CM than in those cultured with GDF-5 and the latter exhibited higher expression of Ang-1 mRNA. The addition of osteogenic supplements to the MSC culture media is reported to decrease VEGF expression and secretion [130], suggesting that in **Study I** the osteogenic supplements together with the BMSC-CM might have reduced mRNA expression of VEGF and Ang1. These results indicate the need for further investigation of potential negative interactions between constituents of OM (e.g., dexamethasone) and proangiogenic cytokine expression and secretion.

While distinct osteo/odontogenic differentiation of hDPC was demonstrated in **Study I**, trans-differentiation of hDPC into vessel and/or neuronal phenotypic cells was more difficult to assess. Therefore, further investigation is warranted into the influence of the current treatments, especially BMSC-CM, on the potential of hDPC to transdifferentiate into phenotypical and functional neuronal- and /or endothelial-like cells.

5.3. BMSC-CM MODULATES INFLAMMATION AND HEALING OF DENTAL TISSUES

It has been proposed that the paracrine effects of MSC secretome can accelerate wound healing, especially when administered to the site of injury [131]. The results of the *in vitro* studies on which this thesis is based show that BMSC-CM modulates osteo/odontogenic differentiation. Based on these observations, and the fact that BMSC-CM is known to contain bioactive soluble molecules with cyto-protective and inflammatory properties [75], a rat tooth replantation model was used in order to evaluate the effects of BMSC-CM on the healing of dental tissues. As inflammation is an integral part of the healing process, the influence of BMSC-CM on the inflammatory response to dental trauma was also investigated.

5.3.1. Root resorption and periodontal healing

In clinical dentistry, despite attempts at immediate replantation of traumatically avulsed teeth, the healing outcomes are unpredictable [132]. Root resorption may result in reversible or irreversible damage to cementum, dentin and bone [133]. Delayed replantation, with drying and damage to the PDL cells (cementoblast necrosis) is regarded as a major contributory factor to clinical failure [134]. Injury to or loss of the protective layers (per-cementum and pre-dentin) may trigger an inflammatory reaction and root resorption [8]. PDL damage can be limited by immediate replantation. Hence in **Study II**, the teeth were replanted within two minutes of extraction. Evaluation after 90 days revealed that BMSC-CM reduced the number of cases of external root resorption in replanted teeth. These findings are in accordance with those of a previous study of PDL cells, showing that CM derived from cultured gingival fibroblasts had a significant effect on the viability of PDL cells [135].

Root resorption is attributed to clastic cells on the root surface following an inflammatory response to trauma or bacterial irritants [8]. Inhibiting osteoclast activity might reduce the negative effects of the inflammation. In **Study II**, the finding that

BMSC-CM reduced the number of teeth with resorption after replantation is in accordance with evidence from previous studies. In **Study II**, factors secreted by MSC induced up-regulated production of lipid mediator PGE_2 from hDPC *in vitro*. It has been suggested that the *in vitro* stimulatory effect of PGE_2 on RANKL and OPG expression in a cementoblast cell line, as well as the production of OPG by cementoblasts, can in turn protect the root surface from resorption [136]. Thus BMSC-CM may enhance healing not only by modulating the inflammatory state surrounding the tooth, but also through reduction in osteoclastic activity [73, 137].

5.3.2. Root development

During tooth formation, the pulp has an inherent capacity for continued root development. After trauma, immature permanent teeth exhibit an inherent healing capacity. The healing pattern has been categorized into normal, partial closure and arrested root development [138, 139]. These developmental patterns are influenced by the viability of the PDL, pulp revascularization and the survival of Hertwig's epithelial root sheath [138, 139]. In **Study II** the replanted teeth exhibited normal or complete root development at 90 days. This might be attributable to the developmental stage of the teeth at replantation and the short interval between extraction and replantation, limiting the severity of the trauma and the inflammatory response. Hertwig's epithelial root sheath was preserved and root formation continued [140]. Surviving mesenchymal stem cells, residing in the apical papilla, are thus important for continued root formation [141].

5.3.3. Pulpal healing

The dental pulp is enclosed in dentin. If the dental hard tissues are unaffected by tooth displacement and replantation, damage is limited primarily to disruption of pulpal nerve and blood supply and injury to the PDL.

5.3.3.1. Nerve and blood supply

In the intact pulp, nerve fibres are located along the blood vessels, close to the odontoblast layer and predentin as well as in the inner part of dentin [142]. Following

tooth replantation, these nerve fibres play an important role in pulp tissue healing [143, 144]. In **Study II**, pulpal healing following replantation was rapid. Expression of the neuronal marker PGP 9.5 was faint or weak 3 days after replantation, but had resumed close to normal levels at day 14, indicating a high potential for recovery or regeneration of odontoblasts in young teeth. Expression of PGP 9.5 has been proposed as an indicator of differentiation of odontoblasts or odontoblast-like cells. [145].

An important aspect of the inflammatory response is the increase in blood supply. This facilitates migration of inflammatory cells which are important for elimination of microbial elements and recovery of pulpal function [146]. As the pulp lacks a collateral blood supply, necrosis may develop after injury. The necrotic pulp is more susceptible to invasion of microorganisms and subsequently to development of apical pathology. MSC and their secreted factors may enhance cell migration/differentiation and reduce inflammation and clastic activity [73, 80, 137]. Immature teeth with an open apex have high revascularisation potential [147]. In **Study II** the higher expression of VEGF mRNA and Laminin-immunoreactivity observed after tooth replantation indicates vascularization of the replanted teeth.

5.3.3.2. Hard tissue formation and inflammatory reaction

The *in vivo* **Study** showed that compared with untreated replanted teeth, CM treatment resulted in attenuated gene expression of inflammatory mediators (IL-1β, and -6, and TNF-α) and a corresponding formation of mineralized tissue along the pulp-dentin interface.

In vitro, expression of early markers of inflammation is associated with early expression of odontogenic differentiation and mineralization markers in hDPC [121, 148, 149]. Although pulpal inflammation is regarded as a negative factor in pulpal disease, the initial inflammatory reaction in the pulp tissues might in fact be a prerequisite for healing [121]. However, resolution of inflammation is then required to eliminate further tissue damage caused by pro-inflammatory mediators. Inflammation is thus a balance between pro-inflammatory mediators (e.g., IL-1β and TNF-α) and anti-inflammatory mediators (e.g., IL-4 and IL-10) that are important for recovery of

tissue homeostasis. Although **Study III** disclosed no difference in the mRNA levels of IL-10 between control and CM treated replanted teeth, IL-10 expression in the latter was associated with low mRNA levels of IL-1β, and -6, and TNF-α, 3 days after replantation, indicating a possible immunoregulatory effect of the MSC secretome [6].

In **Study III,** CM stimulated production of IL-10 by hDPC, compared with the control medium (DMEM). Two *in vitro* studies support this disclosure: firstly, human dental pulp showed higher production of IL-10 in healthy than in inflamed dental pulp cells and this up-regulation was associated with suppressed proliferation of activated T cells [150] and secondly, secretion of IL-10 from mesenchymal cells was shown to inhibit cardiac fibroblast proliferation and collagen expression [75]. Thus IL-10 released from hDPC stimulated by culture in BMSC-CM may serve a protective function for the pulp tissue.

Study III revealed high concentrations of RANTES and IL-8 in BMSC-CM, increasing further after treatment of hDPC with BMSC-CM. It is unclear whether this effect is associated with osteo-/odontogenic-like phenotype differentiation of hDPC *in vitro*, as we did not evaluate their production after cell differentiation. However, a previous study has shown that the ability of MSC-CM to induce osteogenic differentiation of mouse BMSC was not affected by antibodies against RANTES or SDF-1 *in vitro* [56]. In contrast, the *in vivo* part of **Study III** disclosed no differences in mRNA levels of IL-8 among the replanted groups. These results might be attributable to the effect of immediate replantation on chemokine expression, or IL-8 might have a minor impact in the current model. It is therefore necessary to document earlier and later time points to achieve more comprehensive mapping of the cytokine and chemokine profiles and the cellular and tissue responses of MSC-CM over time.

Pulp calcification and inflammation after tooth replantation are closely dependent mechanisms [151]. At least three forms of tissue obliteration of the pulp tissue space have been reported after replantation; dentin-like, bone like or both, and fibrotic tissue with irregular calcification [152, 153]. This form of hard tissue is assumed to be produced either by physiological stimulation or abnormal regulatory mechanisms of pre-existing odontoblast and/or un-/differentiated pulpal stem cells [154]. In

accordance with the correlation between inflammation and the rate of hard tissue formation following tooth injury, increased mineralized tissue formation in the tooth pulp of replanted control teeth might be a consequence of the high mRNA levels of IL-1β, and -6, and TNF-α found three days after replantation. CM might therefore reduce the initial inflammatory reactions in the dental pulp, thus reducing mineralized tissue formation along the pulp-dentin interface.

Although in **Study I** high rates of mineralization were observed after stimulating hDPC with CM *in vitro*, this effect was not replicated in **Study II**. This discrepancy could be attributable to the impact of a single dose versus continuous stimulation, the time points studied, the amount of CM or to the presence of osteo/odontogenic supplements added to the culture medium in **Study I**. However, the long term effect of CM as a single dose *in vivo*, as observed in **Studies II** and **III,** was not anticipated. Although repeated application of CM has shown positive effects on bone healing, possibly mediated by increased vascularization [155], the present model precluded repeated application.

There is a correlation between a high rate of hard tissue formation in the pulp space and inflammation and resorption on the root surface [144, 156]. However, in the present studies the rate of inflammation on the root surface was not evaluated. The reduction in the mineralization rate along the pulp-dentin interface, as well as the marked reduction in the number of teeth undergoing external root resorption after CM treatment, might thus be related to a lower level of inflammation in the root and surrounding tissues.

6. CONCLUDING REMARKS

Traumatic injuries to hard tissues such as bone or teeth trigger many physiological responses such as inflammation, vascularization, neurogenesis and differentiation of endogenous stem/progenitor cells. The studies on which this thesis is based addressed the role of paracrine secretions of stem cells in tissue repair and healing. The *in vitro* studies analysed the effect of bone marrow mesenchymal stem cells conditioned medium on human dental pulp cells. *In vivo,* dental trauma and replantation of avulsed molar teeth were simulated in a rat model, in order to investigate the influence on root resorption of conditioned medium containing secretome from cultured human bone marrow stem cells.

The results of the studies suggest the following conclusions:

• Compared to exogenous recombinant GDF-5 and odontogenic medium, CM has a pronounced stimulatory effect on differentiation of hDPC, evidenced by expression of osteocalcin and a corresponding rapid formation of mineralized nodules.

• In a rat model simulating avulsion and replantation of molar teeth, BMSC-CM reduces the number of teeth undergoing external root resorption and the rate of hard tissue formation along the pulp-dentin interface.

• BMSC-CM attenuates the initial inflammatory reaction in the pulp tissue following replantation of rat molars, as evidenced by decreased gene expression of IL-1β, IL-6, and TNF-α.

• BMSC-CM contains a group of inflammatory healing mediators, including IL-6 and PGE_2. Production of IL-6 and PGE_2 by hDPC is significantly up-regulated when hDPC are stimulated by BMSC-CM *in vitro*.

• BMSC-CM contains only insignificant amounts of anti-inflammatory IL-10, but production increases significantly when hDPC are stimulated with BMSC-CM *in vitro*.

7. FUTURE PERSPECTIVES

• Cells cultured in CM contain a group of microvesicles with bi-lipid membranes [157]: detailed investigation of the properties of these components might disclose other stem cell paracrine/endocrine factors which may favourably influence dental tissue repair and regeneration.

• The results of these studies show that stem cell secretome in CM can induce early and rapid osteo/odontogenic differentiation *in vitro*. However, further investigation is warranted to determine the relative effects of CM on migration of DPSC, and their differentiation into different lineages.

• The results also highlight the contribution of stem cell secretome to tissue healing by inducing production of relevant inflammatory healing mediators (e.g., IL-10, IL-6 and PGE_2) *in vitro*, and reducing the initial inflammatory reactions in pulp tissue *in vivo*. It would therefore be of interest to investigate whether CM might have an immunomodulatory influence on pulp-dentin regeneration and repair of pathological conditions of the pulp.

• While the present project addressed the effect of CM on pulpal cells and the preservation of pulp vitality after tooth replantation, the effect of CM on pulp regeneration also warrants investigation.

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Influence of bone marrow stromal cell secreted molecules on pulpal and periodontal healing in replanted immature rat molars

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Abstract – *Aim*: To investigate the effect of paracrine factors secreted from human bone marrow stromal cell (BMSC-CM) on pulpal and periodontal healing following immediate replantation of maxillary rat first molars. *Material and Methods*: Fifty maxillary rat first molars were elevated and replanted after 2 min. The left teeth were replanted without treatment, whereas BMSC-CM was injected into the right socket prior to replantation. Twelve un-operated teeth served as reference teeth. The expression of vascular endothelial growth factor A, alkaline phosphatase, Runt-related transcription factor 2 and osteoclast stimulating factor 1 was studied by real-time reverse transcription polymerase chain reaction at day 3 and 14. The dentin thickness together with Laminin- and PGP 9.5-immunoreactivity were studied after 3, 14 and 90 days. *Results*: Real-Time qRT-PCR data showed up-regulated expression of ALP mRNA in the socket specimens of conditioned medium treated replanted teeth after 3 days. No morphological differences were found for the expression of Laminin and PGP 9.5 between control and conditioned medium treated replanted teeth. At day 14, external cervical and surface root resorption was found in one BMSC-CM and one control tooth. At 90 days, all control replanted teeth had external cervical and surface root resorptions, whereas only one sample was seen among the conditioned medium treated teeth. At day 90, more extensive dentine formation with narrowing of the pulpal space was observed in the control compared with conditioned medium treated teeth. *Conclusions*: The present findings showed that BMSC-CM treatment reduced the number of replanted teeth with external root resorption and resulted in a significant reduction in new dentin formation.

Traumatic dental injuries (TDI) are the most common facial injuries that affect the integrity of the natural dentition (1). After tooth avulsion, replantation may preserve the alveolar-ridge and orofacial development and growth. Despite attempts for immediate replantation of traumatically avulsed teeth, many factors may impair the healing process. Pulpal changes after rupture of blood vessels and nerve fibres, damaged odontoblast function and degree of attachment damage, may affect the healing outcomes after trauma (2, 3).

Several studies have focused on treatment of the root surface to control periodontal complications after replantation or transplantation. Despite attempts to control periodontal ligament (PDL) repair and tooth revascularization, uncertain healing outcome has been found (4–7). Bioactive soluble molecules, which are a group of signalling molecules able to control several endogenous activities, have shown promising results in some healing models. Bone morphogenic protein 7 (BMP-7) was shown to increase cementum formation, improve eruption and survival of transplanted teeth in immature mini-pigs (8). In addition, BMP-6 enhanced periodontal wound healing and cementogenesis in dogs (9). For enhanced pulpal revascularization, vascular endothelial factors (VEGF) has been suggested as a crucial angiogenic factor for traumatically avulsed teeth (10), whereas endothelial growth factor (EGF) improved revascularization of the pulp tissue after auto-transplantation in rat molars (11).

It has been shown that transplanted exogenous stem cells produce a variety of bioactive immunoregulatory, angiogenic and neurotrophic molecules that can be used for improving and accelerating wound healing (12). In particular, secreted factors derived from bone marrow mesenchymal stem cell (BMSC) or bone marrow mesenchymal stem cell conditioned medium

(BMSC-CM) has been shown to promote wound healing (13, 14). For dental tissues, BMSC-CM is able to induce osteo/odontogenic differentiation of dental pulp cells *in vitro* (15), and promote alveolar bone and cementum regeneration in infra-bony defects in rats (16). The existence of mesenchymal stem cells in pulpal and periodontal tissues may therefore participate and influence healing after tooth avulsion. Therefore, the aim of the presented study was to investigate the effect of human bone marrow stromal cell secreted molecules as a conditioned medium (BMSC-CM) to enhance periodontal and pulpal healing following replantation of rat molars.

Materials and methods

Animals and replantation procedures

The experimental protocol was approved by the Regional Committee for Animal Research Ethics, University of Bergen, under supervision of the Norwegian Experimental Animal Board.

Thirty-one female Sprague-Dawley rats, aged 3 weeks and approximately 100 g in weight were used (National Public Health Institute, Norway). The animals were fed standard pellet diets with water *ad libitum* and acclimatized for 7 days prior to the operation procedures. Anaesthesia was achieved with a subcutaneous injection of Hypnorm-Dormicum (1 ml fentanyl/fluansion diluted in 1 ml sterile water mixed with 1 ml midazolam diluted in 1 ml sterile water, 0.2 ml kg^{-1} body weight). Before the surgical procedure, the crown was cleaned with 0.3% chlorhexidine gel. The left and right first maxillary molars were selected in this study. The teeth were elevated with a sterile elevator placed at the distal aspect of the crown, according to an established protocol (17). The left tooth was first elevated for 2 min and replanted without any treatment (denoted as control replanted teeth). The contralateral right tooth was elevated in a similar manner, but received injection of BMSC-CM (lyophilized reagent diluted in 100 ml serum-free medium, 0.025 ml $tooth^{-1}$) in the socket before replantation (denoted as CM treated replanted teeth). No postoperative fixation was used. Postoperative pain killer (Temgesic 0.3 mg ml^{-1}, 0.05 ml 100 g^{-1} body weight) and antibiotic prophylaxis (benzylpenicillinprocaine 300 000 IE ml^{-1}), 0.020 ml 100 g^{-1} body weight, were given. The animals were kept on a soft diet after the operation to facilitate feeding and drinking. At the end of the observation periods (3, 14 and 90 days), all animals were anaesthetized subcutaneously with Hypnorm-Dormicum (approximately 0.4 ml rat^{-1}). Five rats at 3, 14 and 90 days were processed for immunohistological analysis. The rest of the rats at 3 and 14 days were euthanized with neck dislocation for gene analysis. Six reference teeth (three rats) were processed for the gene analysis, and six reference teeth (three rats) were processed for immunohistological analysis (Table 1).

Table 1. Replanted and reference rat upper first molars

Endpoint (days)	IHC	Gene analysis	Rats No.
3 days	5 rats	5 rats (10 teeth and 10 sockets)	10 rats (20 teeth)
14 days	5 rats	5 rats (10 teeth and 10 sockets)	10 rats (20 teeth)
90 days	5 rats		5 rats (10 teeth)
Reference teeth	3 rats	3 rats (6 teeth and 6 sockets)	6 rats (12 teeth)
Total rats	18 rats	15 rats	31 rats (62 teeth)

Tissue preparation

For gene analysis, the upper jaw of the rats was dissected out and separated into left and right parts. Teeth and socket specimens were prepared and immediately submerged in RNA later and stored at −80°C. For the histological procedures, animals at day 3, 14 and 90 underwent transcardiac perfusion by heparinized saline (0.9% NaCl/0.03% heparin), followed by 10% ethylene-diamine-tetra acetate (EDTA), pH 7.4, at room temperature (Table 1). Briefly, after transverse abdominal incision, a fine needle was inserted into the descending aorta and immobilized by needle forceps before infusion of perfusion solution. The maxillary jaws were then dissected out and immersed in demineralizing solution (10% EDTA) at 4°C for 4 weeks. Decalcified specimens were rinsed in PBS for 24 h, subsequently soaked in 30% sucrose in 0.1 M phosphate buffer, pH 7.4 for 24 h, embedded in O.C.T. tissue-tech (Sakura Finetek, Tokyo, Japan), and immediately kept at −80°C.

Gene analysis

Total RNA was isolated using TRIZOL method and the RNA purity and quantification were determined by spectrophotometry (ThermoScientific NanoDrop Technologies, Wilmington, DE, USA). The reverse transcription reaction was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), using 1000 ng total RNA dissolved in 40 µl nuclease-free water mixed with reverse transcriptase (RT) buffer, random primers, dNTPs and MultiScribe RT. The cDNA corresponding to 10 ng of mRNA was used as a template in each PCR reaction of primers (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was conducted by a StepOnePlus real-time PCR system using TaqMan® gene expression assays: glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Rn01749022-g1), Runt-related transcription factor 2 (RUNX 2, Rn01512298-m1), Vascular endothelial growth factors A (VEGFa, Rn01511601-m1) and Osteoclast stimulating factor 1 (OSTF1, Rn00686607-m1) (Applied Biosystems, Foster City, CA, USA). The data were analysed using the $2^{-\Delta\Delta CT}$ method and GAPDH served as house-keeping gene for normalization.

Immunohistochemical procedure (IHC) using Laminin and PGP 9.5

Cryosectioning was performed on a Leica CM 3050S (Leica Microsystems, Wetzlar, Germany) at −24°C into 25-µm sections. Sections were fixed with cold acetone

and treated with absolute methanol containing 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity. Sections were incubated with PBS containing 2.5% normal goat serum for 60 min at room temperature before incubation with rabbit polyclonal anti-Laminin (1:5000; StressGen Biotechnologies, Victoria, Canada) and rabbit polyclonal anti-PGP 9.5 (1:400; StressGen Biotechnologies, Victoria, BC, Canada) overnight. After several rinses in PBS, sections were incubated with biotinylated anti-rabbit IgG for 60 min, and finally with biotin/avidin solution (VECTASTAIN Elite ABC Kit, Vector Laboratories, Inc. Burlingame, CA, USA). Following washes with PBS, substrate solution-cell reactions (DAB Substrate Kit, Vector Laboratories, Inc., Burlingame, CA, USA) were added at room temperature. The expression of Laminin and PGP 9.5 was examined with an inverted microscope equipped with a digital camera (FV 500, Olympus, Tokyo, Japan).

Immunohistochemical evaluation

The immunostaining was evaluated according to the following parameters; pulp healing pattern including an analysis of angiogenic and neurogenic marker expression in the pulp chamber, and reparative or new dentin formation. Dentine formation was evaluated by calculating the dentine thickness from the furcal surface of the pulpal floor towards the internal pulp floor.

For analysis of root resorptions, several parameters were used; ESR, External surface resorption; IRR, Internal root resorption; EIRR, External inflammatory root resorption; ECR, External cervical resorption; Ankylosis (replacement resorption). All parameters were evaluated by a blinded observer.

Statistical analysis

The mRNA expression of the reference teeth was considered as the normal level of uninjured teeth. Quantitative data of dentin thickness after 3, 14 and 90 days and the mRNA expression levels were presented as mean ± standard deviation. For statistical analysis, one-way analysis of variance was followed by a multiple-comparison Tukey test, IBM® SPSS® Statistics 22.0 (IBM, USA). Statistical significance was determined at $P < 0.05$.

Results

Real-Time Quantitative Reverse Transcription PCR (Real-Time qRT-PCR)

The Real-Time qRT-PCR data revealed ALP, Cbfa1/Runx2 and OSTF1 (bone remodelling related genes), and VEGFa (angiogenic related gene) mRNA expression in all teeth and sockets samples after 3 and 14 days (Fig. 1).

Higher VEGFa mRNA expression was found in all tooth specimens of replanted teeth after 3 and 14 days compared with reference rat teeth. Although not statistically significant, the VEGFa mRNA level was slightly up-regulated in all socket specimens of replanted teeth after 3 and 14 days compared with reference rat teeth. No intergroup differences were found between the controls and CM treated tooth and socket specimens.

The ALP mRNA expression was significantly up-regulated in the tooth specimens of control and CM treated replanted teeth after 3 and 14 days compared with reference rat teeth. Compared to 3 days, the ALP mRNA expression was declined at 14 days. In the socket specimens, a significant up-regulation of ALP mRNA was detected in all replanted teeth after 3 days compared with reference rat teeth. A significant up-regulation of ALP mRNA expression in the socket specimens was also found for the CM treated replanted teeth compared with control replanted teeth after 3 days. Compared to 3 days, the ALP mRNA expression in the socket specimens was significantly down-regulated in the control and CM treated replanted after 14 days.

For the RUNX2 mRNA expression, up-regulation was found in the control replanted teeth compared with the reference rat teeth after 3 days. After 14 days, statistical down-regulation was found in the control replanted teeth compared with same group after 3 days. No intergroup time differences in the mRNA level of RUNX2 was found for the CM treated replanted teeth, although the expression after 14 days was declined. For the sockets, no differences were detected between replanted teeth and reference rat teeth at 3 and 14 days.

Increased mRNA expression of OSTF1 was found in all replanted teeth compared with the reference rat teeth, although not at a statistical level. On the other hand, the mRNA expression of OSTF1 in the sockets were down-regulated in all replanted compared to the reference rat teeth, and the expression of OSTF1 was statistically down-regulated in control replanted compared with reference rat teeth after 14 days.

Histological analysis

Reference rat teeth

The reference rat teeth showed normal distribution of Laminin and PGP 9.5 in pulp and periodontium (Fig. 2).

Three days after replantation

The pulp of control and CM treated replanted teeth demonstrated immunoreactivity to both Laminin and PGP 9.5 (Fig. 3). The PGP 9.5 staining was faint compared with reference rat teeth. The PDL was separated from the alveolar bone by organized connective tissue in all replanted teeth. No statistical differences were found between the experimental groups for the dentin thickness (Fig. 4).

Fourteen days after replantation

Increased immunoreactivity of Laminin was found in both control and CM treated replanted teeth, as compared with reference rat teeth (Fig. 5). Laminin- and PGP 9.5-immunoreactivity was densely distributed close to the odontoblast layer, with a pattern similar to reference rat teeth. The intensity of the Laminin- and

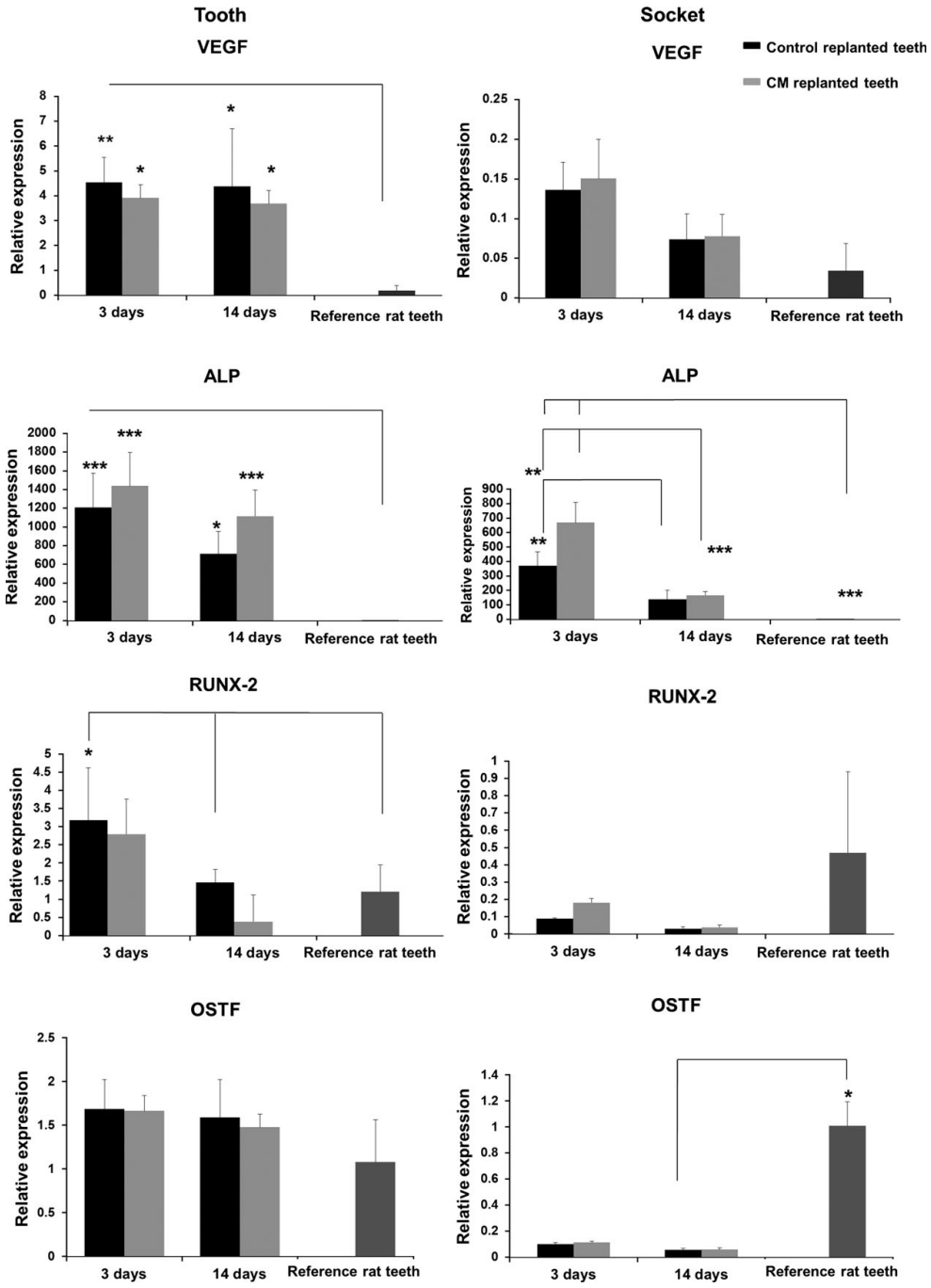

Fig. 1. Real-Time qRT-PCR analysis at day 3 and 14 in teeth and tooth socket specimens. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for data normalization. The data are presented as mean ± standard deviation (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

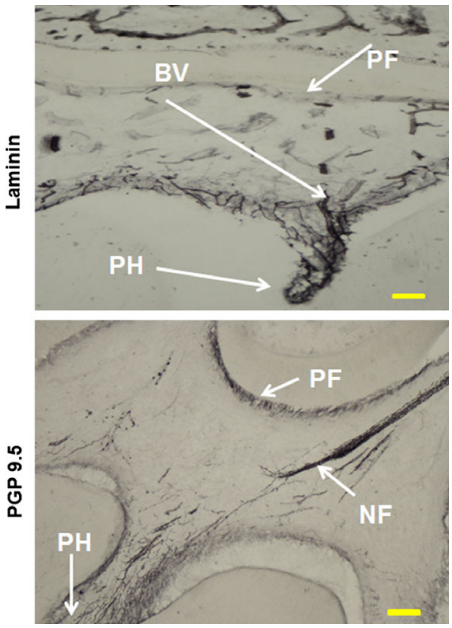

Fig. 2. Laminin- and PGP 9.5-immunoreactivity distribution, as demonstrated in reference rat teeth. BV, blood vessels; NF, nerve fibres; PH, pulp horn and PF, pulp floor (magnification, 10×; scale bar, 50 μm).

PGP 9.5-immunoreactivity was higher in the mesial root as compared with the distal root of the replanted teeth. For the dentin formation, statistically more dentin was formed in both replanted groups compared with day 3 groups and the reference rat teeth (Fig. 4, ***$P < 0{,}001$). However no intergroup difference was found between control and CM treated replanted teeth. Continued root development was observed in all replanted teeth comparable with the reference rat teeth. External cervical and surface root resorptions were noted in the mesial part of the mesial root of one specimen in each of the replanted groups (Fig. 6A).

Ninety days after replantation

Complete root development was shown with distinct PDL in the replanted teeth. Laminin and PGP 9.5-immunoreatctivity was observed in the pulp tissue, with a similar pattern as found for reference rat teeth. External cervical and surface root resorptions were noted in the replanted teeth (Fig. 6B). Resorptions were demonstrated in all control replanted teeth, whereas resorptions were only found in one CM treated replanted tooth. Extensive dentin formation was found at this time point, and was significantly higher in the control replanted teeth as compared with the CM treated replanted teeth (Fig. 4 and 7, ***$P < 0.001$). Dentin formation in both control and CM treated replanted teeth was significantly higher after 90 days as compared with 3 and 14 days, and to the reference rat teeth (Fig. 4, ***$P < 0.001$). Dentin formation was

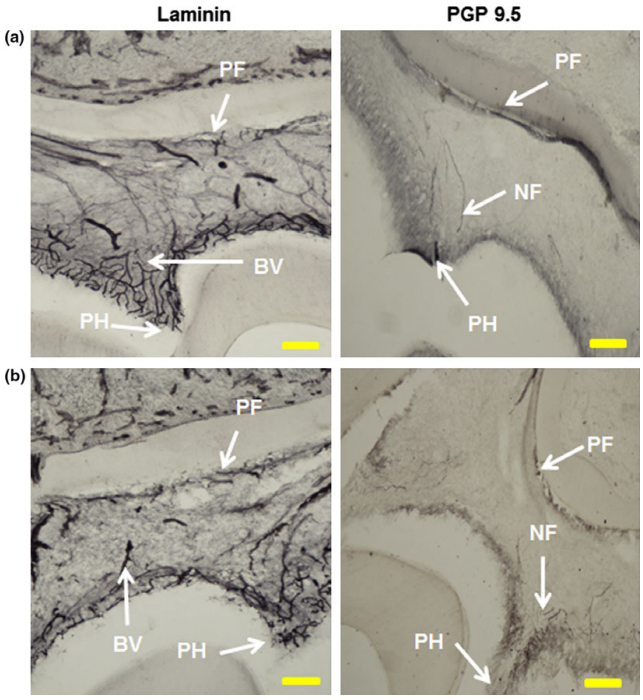

Fig. 3. Laminin- and PGP 9.5-immunoreactivity 3 days following tooth replantation. (a) control and (b) CM treated replanted teeth. Laminin and PGP 9.5-immunoreative distribution is demonstrated in both control and CM treated replanted teeth. BV, blood vessels; NF, nerve fibres; PH, pulp horn and PF, pulp floor (magnification, 10×; scale bar, 50 μm).

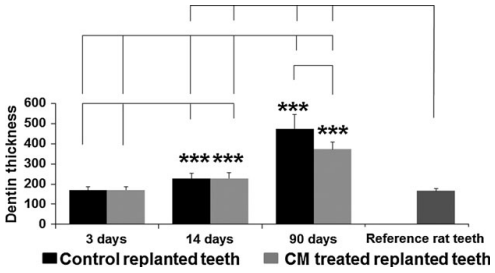

Fig. 4. Dentin thickness at day 3, 14 and 90. At day 3, no statistical differences were found among the experimental groups. At day 14, dentin thickness was significantly increased in all replanted teeth as compared to the same groups at day 3, and reference teeth at day 14. At day 90, CM treated replanted teeth exhibited less dentin thickness compared with the control replanted rat teeth. Both replanted groups had increased dentin thickness compared with the same groups at day 3 and 14. The data are presented as mean ± standard deviation (***$P < 0.001$).

characterized by narrowing of the pulp chamber and scattered calcified tissues was seen in one of the control replanted teeth (Fig. 7).

Discussion

The present study revealed that bone marrow stromal cell conditioned medium (BMSC-CM) reduced the number of teeth with external root resorption and prohibited excessive reactive dentin formation.

As angiogenesis is indicated as a critical step in pulp healing (18), this study used Laminin and VEGFa as markers for pulpal blood supply. Laminin is presents in the basement membrane of blood vessels and cell membranes (19). Laminin is a member of proteins and glycoproteins that has many biological functions including cell-cell interactions, and encourage neurite outgrowth after tissue damage (19). In the current study, gene and immunohistological data showed the expression of blood vessels markers even 3 days following replantation. At day 14, Laminin immunoreactivity appeared to be up-regulated in the pulp tissue of the replanted compared with reference rat teeth. This may reflect a role for Laminin in the tissue healing. The mesial root of the replanted teeth was characterized by intensive Laminin immunoreactivity compared to the distal root, indicating a more severe luxation trauma to the distal root during the procedure. These results might indicate better revascularization, possibly due to less sever luxation trauma and the fact that this root is bigger with a larger volume. Increased density of Laminin-positive blood vessels in the pulp after dentinal injury has been reported, reflecting localized increased blood flow (20).

Pulpal nerve fibres are shown to affect the pulp tissue homeostasis, blood flow and healing (21). In normal pulp tissue, nerve fibres are densely distributed in the odontoblast layer, predentin, and the inner part of dentin. In the root they are more centrally located along the blood vessels (22). During pulpal injury, nerve fibres react by sprouting of their terminal axons, indicating an important role in the healing process (23). PGP 9.5 has been used as neurochemical marker for intra-pulpal nerves during tooth development (24). In agreement with previous studies the odontoblasts also expressed PGP 9.5, possibly reflect-

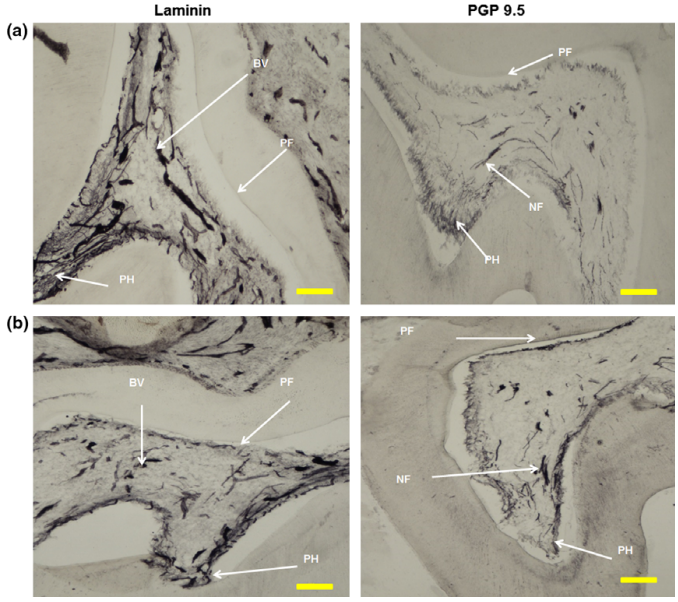

Fig. 5. Laminin- and PGP 9.5-immunoreactivity in the pulp chamber of replanted teeth at day 14. (a) Laminin- and PGP 9.5-immunoreative staining in a control replanted tooth. (b) Laminin- and PGP 9.5-immunoreative staining in a CM treated replanted tooth. BV, blood vessels; NF, nerve fibres; PH, pulp horn; PF, pulp floor (magnification, 10×; scale bar, 50 μm).

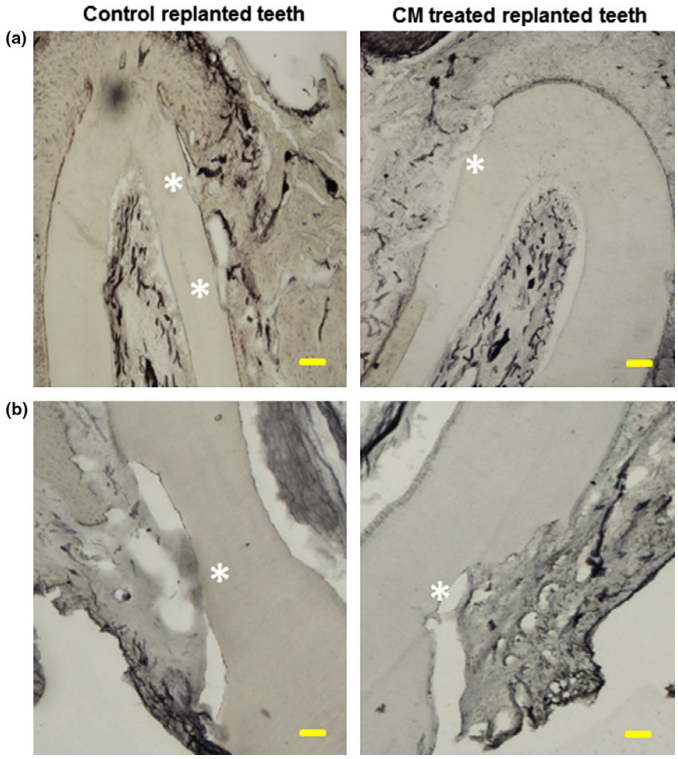

Fig. 6. (a) External surface root resorptions on both control and CM treated replanted teeth at day 14 indicated by white asterisks (magnification, 4×; scale bar, 500 um). (b) External cervical root resorptions in both experimental groups at day 90 indicated by white asterisks (magnification, 10×; scale bar, 50 μm).

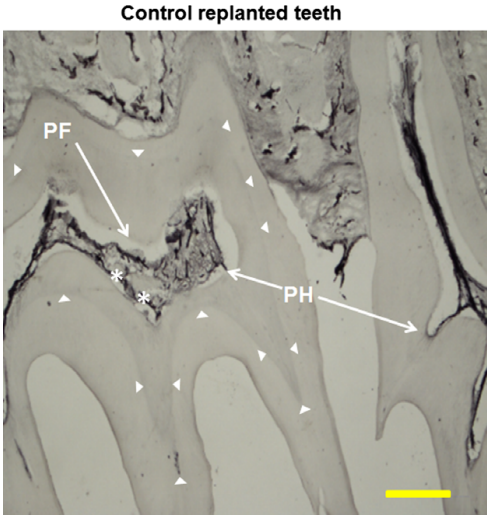

Fig. 7. Extensive dentin formation with narrowing of the pulp space seen in a control replanted tooth at day 90. Pulp calcifications (white asterisks) occupy the pulp space beneath an extensive amount of tertiary dentin. Arrowheads indicate the new dentin formation. PH, pulp horn; PF, pulp floor (magnification, 20×; scale bar, 100 μm).

ing their neural crest origin (24). It has previously been documented that the PGP 9.5-immunoreactivity in pulpal nerves and odontoblasts disappeared 1–3 days following replantation, and that some fragmented immunoreative structures in the coronal pulp remained (25). That study also found that PGP 9.5-immunoreactive regenerating axons returned after 5 days (25). In the present study the PGP 9.5-immunoreactivity was weak or disappeared at day 3, possibly reflecting the presence of the protein and not functional nerve fibres.

Post-traumatic external root resorption is virtually a consequence of damage and inflammation in the PDL and the root cementum, and is considered as a self-limiting condition that is followed by repair (26). Cervical root resorption is also an inflammatory mediated external resorption of the root, but is more progressive in nature (27). Periodontal healing is strongly affected by the extent of damage to the PDL cells (28), and is mainly dependent on time and extra-oral storage (17). Numerous attempts to preserve the PDL cells and reduce the clastic activity have been suggested to minimize root resorption. For example, topical alendronate (ALN) treatment of dog teeth was found to inhibit the osteoclast activity, reflected by a reduction in the incidence of root resorption (29). Enamel matrix derivative (EMDOGAIN) was found to reduce the number of teeth with inflammatory root

resorption after replantation of permanent incisors in Beagle dogs (26). In the present study, we found a reduction in external surface resorption after BMSC-CM treatment at 90 days. These findings are in line with results showing that BMSC-CM treatment induced proliferation of PDL cells *in vitro*, and improved regeneration of cementum in a dog one-wall intrabony defect model (16). In addition, the newly regenerated bone and cementum in the dog one-wall intrabony defect model was associated with minimal inflammatory cell infiltration compared with the other treatment groups (16). Also, BMSC-CM prevent the activity of osteoclast cells, which is a prerequisite for bone resorption (30). Taken together these results indicate that BMSC-CM can promote periodontal healing of replanted teeth.

Damage to the neurovascular supply can induce pulp-dentin complex responses and accelerate deposition of hard-tissue. The severity of the damage, reflecting the extent of pulpal inflammation, influences the deposition of dentin along the periphery of the pulp space (31). A previous study has suggested that rupture of the blood vessels may cause pulpal ischaemia and degeneration of primary odontoblasts, thus triggering the underlining mesenchymal stem cells to form new odontoblast-like cells (32). However, three or more healing events with respect to the dentin-pulp reaction after tooth trauma have been presented. This includes dentin-like, bone-like, mixed tissue and/or fibrotic tissue healing (33, 34). The present study found dentine-like tissue with increased thickness in control replanted teeth compared with BMSC-CM treated and reference teeth. Evidence of calcified tissue in the central portion of the pulp chamber was found in one of the control replanted teeth at day 90. Central deposition of hard tissue may be produced by odontoblast-like cells originating from migrated and differentiated stem cells residing in the perivascular area. Based on the present results we are not able to conclude whether the dentin deposition in the replanted teeth is a result of surviving primary or newly differentiated secondary odontoblasts. It may be speculated that a reduced inflammation, as found for infrabony defects treated with BMSC-CM, may be responsible for reduced dentine thickness found in the present study.

Conclusions

Based on the current findings, BMSC-CM treatment was able to reduce the number of teeth with severe root resorptions and prevent excessive new dentin formation in the pulp chamber that was observed in untreated replanted maxillary immature teeth.

Acknowledgements

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III

Conditioned medium from human bone marrow stromal cells attenuates initial inflammatory reactions in dental pulp tissue

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Abstract

Purpose: To evaluate the effect of paracrine factors secreted from bone marrow stromal cells as a conditioned medium (BMSC-CM), on the secretion of pro- and anti-inflammatory cytokines from dental pulp cells (hDPC) *in vitro*, and on the gene expression *in vivo* after replantation of rat molars.

Materials and Methods: hDPC were cultured in CM for 24 hr, and the concentration of interleukin (IL)-10, -4, -6, and -8, RANTES, and prostaglandin E_2 (PGE_2) in the media were measured by Multiplex assay and ELISA, respectively. Expression of cyclooxygenase-2 (COX-2) was also examined by Western blot analysis after 24 h. Left and right maxillary first rat molars (n=20) were elevated for 2 minutes and then replanted with or without CM injected into the tooth sockets. Levels of IL-1β, -10, -4, -6 and -8, and tumor necrosis factor-alpha (TNF-α) mRNA were evaluated by Real-Time qRT-PCR 3 and 14 days following tooth replantation.

Results: The production of IL-8, -10 and -6, RANTES and PGE_2 by cells cultured in CM was significantly higher than production by cells cultured in standard medium (DMEM). At day 3 following replantation *in vivo*, the levels of IL-1β and -6, and TNF-α mRNA were significantly lower in the CM-treated replanted teeth compared with control teeth. Further, at day 3 the IL-6/IL-10 ratio was significantly lower in the CM-treated replanted teeth compared with control. At day 14 following replantation, no differences in the mRNA levels and ratios were detected between the pulp tissues of replanted and control teeth.

Conclusions: These findings indicated that CM promotes secretion of pro- and anti-inflammatory cytokines from hDPC *in vitro*, and attenuates the initial inflammatory response in the rat dental pulp *in vivo* following tooth replantation.

Introduction

Teeth and supporting tissues are well equipped with tissue specific stem cells/progenitor cells that are activated during tissue injury and inflammation [1]. The fate of stem cells in terms of migration, self-renewal and differentiation into specialised phenotypes, is governed by the surrounding local microenvironment and soluble molecules [2]. Exogenously transplanted mesenchymal stem cells (MSC) are shown to stimulate a wide range of biological activities in immune cells as well as endogenous resident stem cells/progenitor cells [3]. It has been shown that MSC are potent cells for tissue healing and repair, an effect exerted either by cell-cell contact or via the secretion of soluble factors including growth factors, cytokines and chemokines [4, 5]. Although bone marrow stromal/stem cells (BMSC) and dental stem cells differ in their osteo/odontogenic phenotype characteristics [6], bone marrow is widely used as a standard available source for adult stem cells. It has been shown that BMSC participate in regeneration of tooth-like structures when transplanted into the alveolar socket together with scaffold and dental bud cells in mice [7]. BMSC are also able to be re-programmed to give rise to odontoblasts and ameloblast-like cells in proximity to embryonic epithelium [8, 9].

Conditioned medium (CM) from mesenchymal stem cells (MSC-CM) contains growth factors and cytokines, and has been shown to mimic the regulatory effects of stem cells on immunocompetent cells [10, 11]. CM from stem cells derived from adipose tissue has been shown to enhance secretion of anti-inflammatory IL-10 from T-helper cells *in vitro* [12]. More recently, CM from bone marrow stromal cells (BMSC-CM) was found to induce formation of new bone and cementum in intrabony defects with minimal inflammatory cell infiltration [13].

Dental traumas are complex in nature and affect both hard and soft tissues. The healing outcome is thought to depend on a successful interplay and balance between progenitor cells

residing in different tissue compartments [14]. Recent data have suggested that dental pulp resident stem cells/progenitor cells are involved in production of inflammatory mediators that promote cell differentiation and pulpal healing [15]. Although replantation after tooth displacement can preserve the tooth and supporting tissues, minimizing pulpal inflammation is important after replantation [16].

Since growth factors and cytokines play a role in healing events following injury [17], we hypothesised that the growth factors and cytokines present in the BMSC-CM can induce production of cytokines with a protective function. Thus, the first aim of the present study was to examine the effect of BMSC-CM on the release of interleukin (IL)-10, -4, -6, and -8, RANTES, and PGE_2 from hDPC *in vitro*. Next, the *in vivo* effect of BMSC-CM on mRNA expression of IL-1β, -10, -4, -6, and -8, and TNF-α in pulp tissue of replanted teeth was evaluated.

Materials and methods

BMSC culture and collection of conditioned medium

Primary human bone marrow stromal cells (hBMSC) were purchased from European Service Center for Lonza Bioscience (Lonza, Verviers, Belgium). The BMSC were then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Cells at passage 3 were seeded into T-75 cm^2 culture flasks at an initial density of 2 x 10^6. When the cells reached 80-90% confluence they were washed with PBS three times, supplemented with fresh serum-free DMEM containing 1% penicillin-streptomycin and collected after 48 hr. The supernatants from different culture flasks were pooled, then centrifuged at 4°C at 3000 x g for 3 min followed by 5 min at 1500 x g and collected as a CM. DMEM serum free medium incubated under the same conditions served as a control. The media were stored at −80°C for further experiments.

Isolation of human dental pulp cells (hDPC) and cell culture

According to the protocol approved by the Regional Committees for Medical and Health Research Ethics (REC) at the University of Bergen, Norway (225.05, 3.2008.1750, 2009/610 and 2013/1248), human third molar teeth (n=3) from healthy adult patients aged 18–25 were extracted during routine operative procedures at the Dental Clinic at the University of Bergen, Norway. The dental pulp cells (hDPC) were enzymatically isolated and characterised by phenotype on the basis of positive expression for mesenchymal cell surface markers, such as CD90, CD105, STRO-1, and CD24 [18]. Cells from passages 4-6 were used for the *in vitro* study and the experiments were performed in triplicate. The isolated hDPC were cultured with DMEM containing 5% FBS and 1% penicillin-streptomycin (DMEM-5% FBS) at an initial density of 6 x 10^5 for 24 hr. Next, the cells were incubated with either DMEM-1% FBS (control cells) or BMSC-CM-1% FBS (CM-treated cells).

Multiplex assay of cytokine Levels

Supernatants from control or CM-treated hDPC, as well as samples of BMSC-CM or control medium, were tested for the presence of IL-10, -4, -6, and -8, and RANTES by use of Bio-Plex Pro Human Cytokine Group I 6-plex Assay (human multiplex bead-based immunoassay kit, Bio-Rad Company, USA) after a 24 h incubation. The multiplex cytokine assay allows simultaneous quantification, and was performed according to the manufacturer's instructions (Bio-Plex™ Cytokine Assay, Bio-Rad Company, USA) [19]. The level of released cytokines was measured in pg/ml, using a Bio-Plex® MAGPIX™ Multiplex Reader (Bio-Rad Company, USA).

PGE_2 determination by Enzyme Linked Immunosorbent Assay (ELISA)

The collected supernatants used for determination of cytokine level were also tested for the presence of PGE_2 using a commercial ELISA kit (Prostaglandin E_2 ELISA Kit, Monoclonal, Neogen Corporation, Lansing, Michigan, USA), according to manufacturer's instruction. The

PGE_2 content of the samples was quantified by microplate spectrophotometry (BMG LABTECH, GmbH, Germany) at a wavelength of 450nm, and the concentration was calculated in ng/ml according to standards.

Western blot analysis

To measure the protein expression of COX-2 in hDPC after BMSC-CM treatment, 7×10^5 cells were seeded in 80 mm petri dishes and cultured for 24 h. Cell total protein fractions were extracted using RIPA buffer according to the manufacturer's instruction (RIPA buffer plus protease and phosphatase inhibitors, ThermoScientific, USA). The concentration of total proteins was measured with the Pierce™ BCA Protein Assay Kit (ThermoScientific, USA). Twenty microgram of total protein extracts were separated by electrophoresis and transferred as previously described [18]. The membranes were blocked with 5% nonfat-milk in TBS containing 0.1% Tween 20 for overnight at 4^oC with rabbit anti-COX-2 antibody (1:1000 dilutions, Abcam®, Cambridge, U K). The membranes were then incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:2000 dilutions, Santa Cruz Biotechnology; Santa Cruz, CA, USA), in 5% nonfat-milk in TBS containing 0.1% Tween 20 for 1 h. Rabbit anti-GAPDH served as an internal control for the total expression of COX-2 (1:1000 dilutions, Santa Cruz Biologicals, Santa Cruz, CA, USA). Protein detection was done with an enhanced chemiluminescence (ECL) kit and the bands were visualised by the Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA).

Replantation model using rat maxillary first molars

To study the effect of BMSC-CM on the gene expression of IL-1β, -10, -4, -6 and -8, and TNF-α *in vivo*, a tooth replantation model using rat maxillary first molars was used. Thirteen female Sprague-Dawley rats, aged 3 weeks (approximately 100 g in weight), were included (National Public Health Institute, Norway). General anesthesia was achieved in each animal with 0.2 ml/kg of Hypnorm-Dormicum (1 mL fentanyl/fluansion and 1 ml midazolam diluted

in 2 ml sterile water). Before surgery, the tooth crown was cleaned with 0.3% chlorhexidine gel, and the left and right first maxillary molars (n=20) were elevated mesially for 2 mins, and then replanted as previously described (Figure 3A) [20]. The left first maxillary molars (n=10) were first elevated and replanted without treatment (control replanted teeth), whereas the right first maxillary molars (n=10) were replanted after injection of 25 ul of CM in the tooth socket (CM-treated replanted teeth). No postoperative fixation was used. Three and 14 days following replantation all animals were anaesthetized with Hypnorm-Dormicum and euthanized with neck dislocation. Next, the upper jaw of the rats was dissected out and the teeth were then extracted, submerged in RNAlater and stored at −80°C. The animal experiments were approved by the Regional Committee for Animal Research Ethics at the University of Bergen, under regulations of the Norwegian Experimental Animal Board.

Real-Time Quantitative Reverse Transcription PCR (Real-Time qRT-PCR)

To study the *in vivo* effects of BMSC-CM on gene expression of IL-1β, -10, -4, -6 and -8, and TNF-α in the rat tooth pulp 3 and 14 days following tooth replantation, total RNA was isolated using the TRIZOL (Invitrogen, Carlsbad, CA, USA). RNA purity and quantification were determined by spectrophotometry (ThermoScientific NanoDrop Technologies, Wilmington, DE, USA), and the reverse transcription reaction was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The cDNA corresponding to 1 μg of mRNA was used as a template in each PCR reaction of the different primers (Table 1), and the RT-PCR was performed on a StepOnePlus real-time PCR system using TaqMan® gene expression assays (Applied Biosystems, Foster City, CA, USA). Rat glyceraldehyde 3-phosphate dehydrogenase was used as a normalised reference gene, and the relative expression of each gene was analysed using the $2^{-\Delta\Delta CT}$ method.

Data analysis

Quantitative results were expressed as mean ± SD. For the *in vitro* experiments, a student t-test was used for comparison between CM and controls. A similar comparison was performed between CM-treated cells and control cells. For the *in vivo* experiments, the expression of each cytokine was calculated, and then the ratios of the pro-inflammatory cytokines IL-1β, -6 and TNF-α to the anti-inflammatory IL-10 were calculated. A student t-test was used for comparison to determine differences between CM-treated and control replanted teeth. The analysis was performed using IBM SPSS Statistics 22 (IBM® SPSS® Statistics 22.0, USA), and the differences between the means were considered statistical significant at $p < 0.05$.

Results

Secretion of inflammatory mediators *in vitro*

The levels of IL-6, and -8, and RANTES were significantly higher in CM than the control medium (Figure 1). When hDPC were cultured in CM, the levels of IL-10, -6 and -8 and RANTES were significantly higher in the supernatant compared with control cells (Figure 1). IL-4 was detected in low levels in CM and control medium, and the levels were not enhanced in the supernatant from the control and CM-treated cells (data not shown).

The effect of CM on the expression of COX-2 and PGE_2 in hDPC

The effect of CM on PGE_2 release and expression of COX-2 was further examined at the protein level. CM contains significant higher level of PGE_2 compared with control, and induced significantly higher release of PGE_2 from hDPC compared with control cells (Figure 2A). Since CM resulted in up-regulated production of PGE_2, COX-2 expression was assessed in hDPC. The protein expression of COX-2 was greater in CM-treated cells compared with control cells (Figure 2B).

Effect of CM on mRNA levels of IL-1β, -10, -6 and -8, and TNF-α in replanted rat teeth

Three and 14 days following tooth replantation *in vivo*, mRNA for IL-1β, -10, -6 and -8, and TNF-α was detected in all replanted teeth (Figure 3B). No mRNA expression of IL-4 was detected in the tooth samples at all time points (data not shown).

At day 3, a significantly lower level of IL-1β and -6, and TNF-α mRNA was detected in the CM-treated replanted teeth than in the control (Figure 3B). There was no statistical difference in the mRNA level of IL-10 and -8 between the groups. At day 14, no-intergroup differences were found for the mRNA levels of IL-1β, -10, -6 and -8, and TNF-α between the CM-treated and control replanted teeth (Figure 3B).

At day 3 following replantation, the IL-6/IL-10 ratio was significantly lower in CM-treated compared to control replanted teeth. The ratios of IL-1β/IL-10 and TNF-α/IL-10, however, did not differ between the groups (Figure 4). By day 14 following replantation, the ratios of pro-inflammatory cytokines IL-1β and -6, and TNF-α to anti- inflammatory cytokine IL-10 did not differ between the groups (Figure 4).

Discussion

The present study showed that BMSC-CM induced higher secretion of PGE_2, IL-10, -8, and -6, and RANTES in hDPC *in vitro*, and attenuated the gene expression of IL-1β, -6 and TNF-α in pulp tissues three days following tooth replantation *in vivo*.

IL-6 is a pleiotropic cytokine that acts as a major mediator in the host response following tissue injury, with an effect on bone osteoclasts and bone resorption through an autocrine/paracrine manner [21]. The result of the present study showed that a significant high level of IL-6 was detected in the CM both before and after incubation with hDPC *in vitro*. It has been reported that MSC secrete high levels of IL-6 [22], and the cytokine has previously been reported to be involved in the immunoregulatory effects exerted by MSC [23]. In the pulp tissue, the mRNA level of IL-6 has been found to be significantly up-regulated in teeth with irreversible pulpitis compared with healthy teeth [24]. It has been reported that continued

expression of IL-6 correlates with the degree of inflammation in the pulp following tissue injury and infection [25]. Three days following tooth replantation, IL-6 was significantly lower in the CM-treated than the control teeth, a finding indicating an effect of CM on the immediate inflammatory response. Notably, the mRNA level of IL-6 declined in pulp tissues of replanted teeth after 14 days in both groups, suggesting a normal resolution process. One has to take into consideration that the single application of BMSC-CM used in this study was not expected to have a long-term effect. Although repeated administration of CM has been shown to be beneficial for bone healing [26], continued application is technically difficult in the present model.

In an *in vivo* inflammatory arthritic model, MSC secretion of the immunoregulatory factor PGE_2 showed a concomitant IL-6 up-regulation [4]. The current study found higher levels of PGE_2 in the CM. Moreover, an enhanced level of both PGE_2 and the enzyme involved in PGE_2 biosynthesis (COX-2) was detected after culture of hDPC in CM. PGE_2 has been shown to induce osteoblast and cementoblast differentiation and mineralisation *in vitro* [27, 28], and higher production of COX-2 and PGE_2 by hDPC was found after stimulation with mineral trioxide aggregate (MTA) [15]. Low concentrations of exogenous PGE_2 is able to induce calcified nodule formation by hDPC *in vitro* [29]. It is therefore tempting to speculate that the presence of PGE_2 in CM, and the subsequent increase after cultivation of hDPC in CM *in vitro*, could be involved in the differentiation and mineralisation of endogenous dental pulp cells [18]. Although the expression of PGE_2 was not examined *in vivo*, the finding that PGE_2 is present in CM suggests a possible role of CM during pulp healing following tissue injury.

IL-10 is a key anti-inflammatory cytokine that inhibits production of pro-inflammatory cytokines including IL-1β, -6 and TNF-α [30], thereby having an immunoregulatory effect. Increased secretion of IL-10 has also been detected in rats with acute kidney injury treated with BMSC-CM, suggesting a significant role of IL-10 in survival and protection of the

kidney [31]. We found no difference in the level of IL-10 between the CM and control medium, but an elevated level was detected in the medium of CM-treated compared with the control hDPC, suggesting stimulatory effect/s of CM on hDPC for the production of the resolution molecules. This is in parallel with a previous finding showing that CM from adipose stem cells induces secretion of IL-10 from CD4+FoxP3+ cells *in vitro* [12]. It has been reported that the mRNA level of IL-10 is up-regulated in the pulp beneath deep caries compared with shallow caries [32], and also in teeth with irreversible pulpitis [33]. Following tooth replantation, we found no difference in the mRNA level of IL-10 between CM-treated and control replanted teeth. However, the expression of IL-10 in CM-treated replanted teeth was previously reported to be associated with a reduction in the mRNA level of IL-1β, -6 and TNF-α, suggesting an immunoregulatory effect of CM [34].

IL-4 is another anti-inflammatory cytokine and plays a role in formation of TH-2 cells, and in shifting the macrophage phenotypes into IL-10 producing cells [35]. IL-4 is also known to stimulate fibroblast proliferation and inhibit osteoclast activities [36]. The present *in vitro* experiments did not find an elevated level of IL-4 either before and/or after CM treatment. Moreover, mRNA level of IL-4 was not detected in the replanted teeth. The relevance of these findings is unclear and further molecular investigations to examine this undetected gene are needed.

IL-1β is considered a strong inflammatory mediator, important in acute host responses [37]. Three days following tooth replantation, IL-1β mRNA expression was significantly lower in CM-treated compared with control replanted teeth. It is well known that IL-1β activates osteoclasts, resulting in bone destruction and root resorption [38]. Continued release of IL-1β might thus impair tissue healing. As for IL-6, IL-1β mRNA declined after 14 days, indicating attenuation of inflammation over time.

In agreement with reports showing that chemokines are constitutively produced and secreted from stem cells [39], we found high level of RANTES and IL-8 in CM, with a significant increase when hDPC were cultured in CM. In dental pulp tissue both the mRNA level and protein expression of IL-8 have been found to increase in inflamed pulp [24, 40]. As for IL-1β, continued expression of IL-8 may contribute to root and bone destruction of injured teeth [25]. The present *in vivo* results found no differences in the mRNA level of IL-8 between the groups, suggesting a minor role for IL-8 in the current model.

TNF-α, a potent pro-inflammatory mediator, is able to induce secretion of other pro-inflammatory cytokines, including IL-6 [41]. TNF-α has been detected during orthodontic tooth movement, indicating a role for TNF-α in inflammation and root resorption [42]. TNF-α has been found in teeth with symptomatic irreversible pulpitis [43], and in the gingival crevicular fluid after tooth injury [25]. However, no difference in the mRNA level of TNF-α was found between teeth with healthy pulps and reversible pulpits, [44]. Down-regulated mRNA level of TNF-α in CM-treated replanted teeth after 3 days compared with controls might suggest that CM treatment attenuates the initial inflammatory reaction after tooth replantation.

Collectively, the lower level of IL-1β, -6 and TNF-α in the CM-treated replanted teeth supports an immunoregulatory action of the stem cells paracrine factors. However, cytokines always act in a network and some cytokines can block the effects of others. In particular, it has been shown that increased IL-6/IL-10 ratio during pulpitis could be used as an indicator for pulp disease [33]. Based on this, the ratio of each pro-inflammatory cytokine IL-1β, and -6 and TNF-α to the anti-inflammatory cytokine IL-10 was investigated. IL-6/IL-10 ratio was significantly lower in the CM-treated replanted teeth than the control replanted teeth. No difference was found in the IL-1β, TNF-α /IL-10 ratio. These findings suggest an impact of CM on the inflammatory/resolution process through the IL-6 and IL-10 network. Although

the pro/anti-inflammatory ratios were lowered after 14 days in all replanted teeth, the level of inflammatory reaction at early time points might affect the healing outcome negatively. In this context, it has been shown that pulp tissue mineralisation and inflammation are closely related mechanisms [45-47].

Conclusion

We conclude that CM promotes secretion of pro- and anti-inflammatory cytokines from hDPC *in vitro*. Moreover, CM attenuates the initial inflammatory response in rat dental pulp tissue *in vivo* following tooth replantation.

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Figure Legends

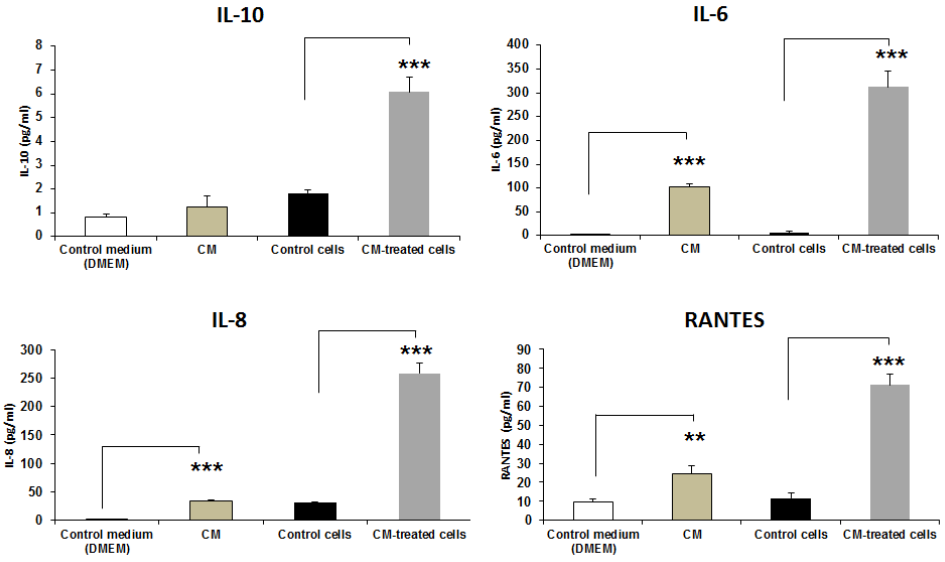

Figure 1 Multiplex assay for IL-10, -6 and -8, and RANTES in control, CM, control cells and CM-treated cells. The data are presented as mean ± standard deviation (**p< 0.01 and ***p<0.001).

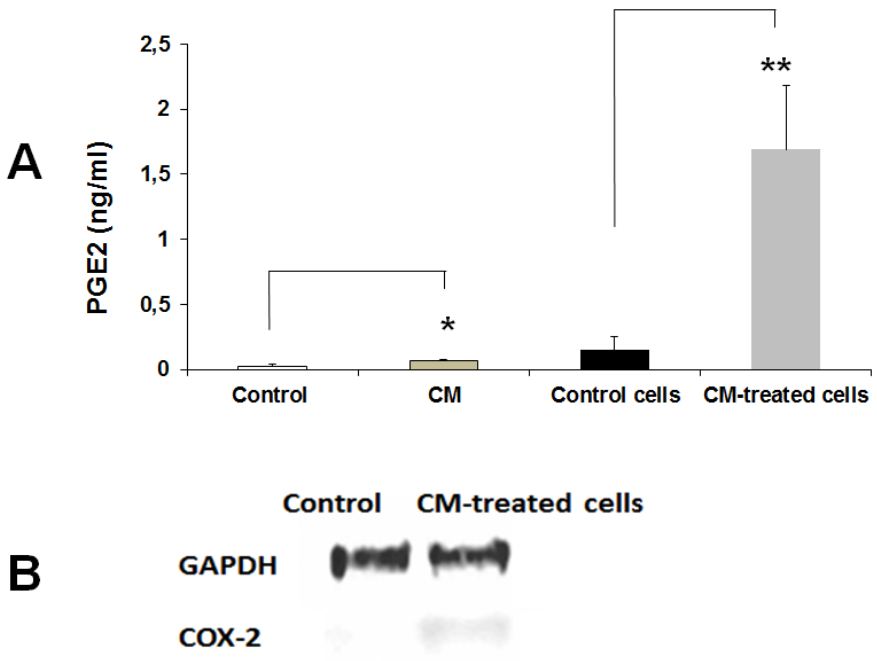

Figure 2 Expression levels of PGE2 and COX-2. (A) ELISA for PGE2 and (B) Western blot analysis of COX-2. The intensity of each band of COX-2 expression was normalised to the internal control GAPDH. The data are presented as mean ± standard deviation (*$p<0.05$, and **$p<0.01$)

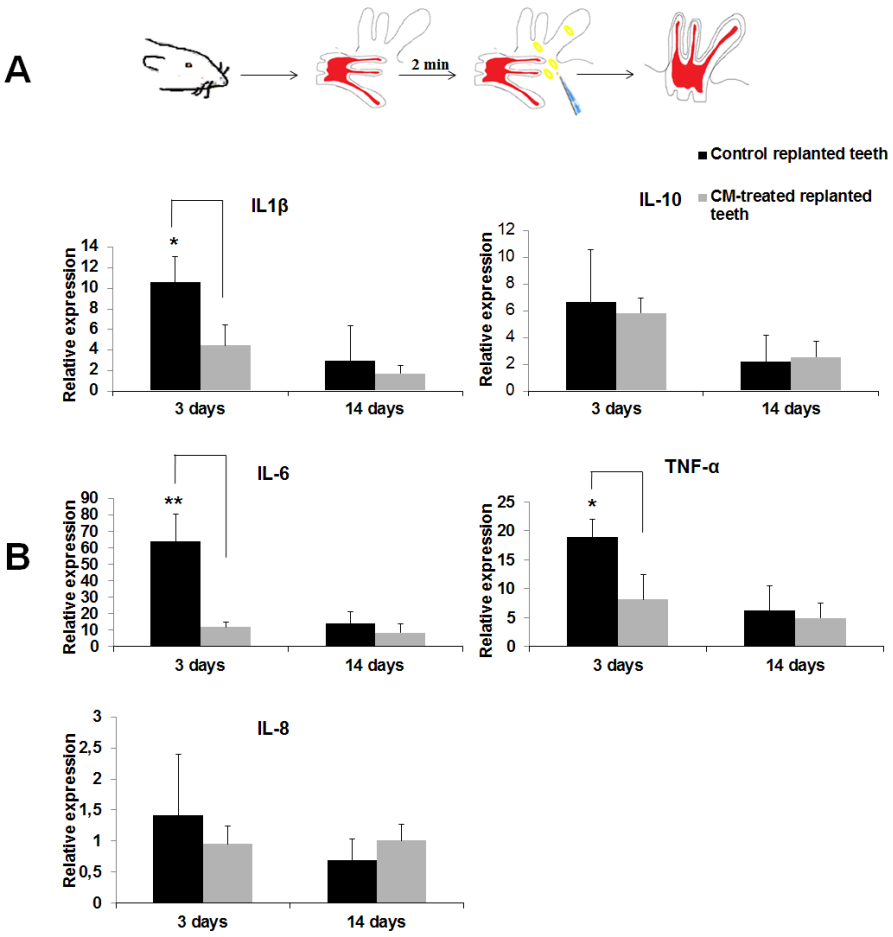

Figure 3 (A) CM treatment of rat first maxillary molars in the rat replantation model. (B) Real-Time qRT-PCR data showing expression of IL-1β, -10, -6, and -8, and TNF-α 3 and 14 days following replantation (*$p<0.05$ and **$p< 0.01$).

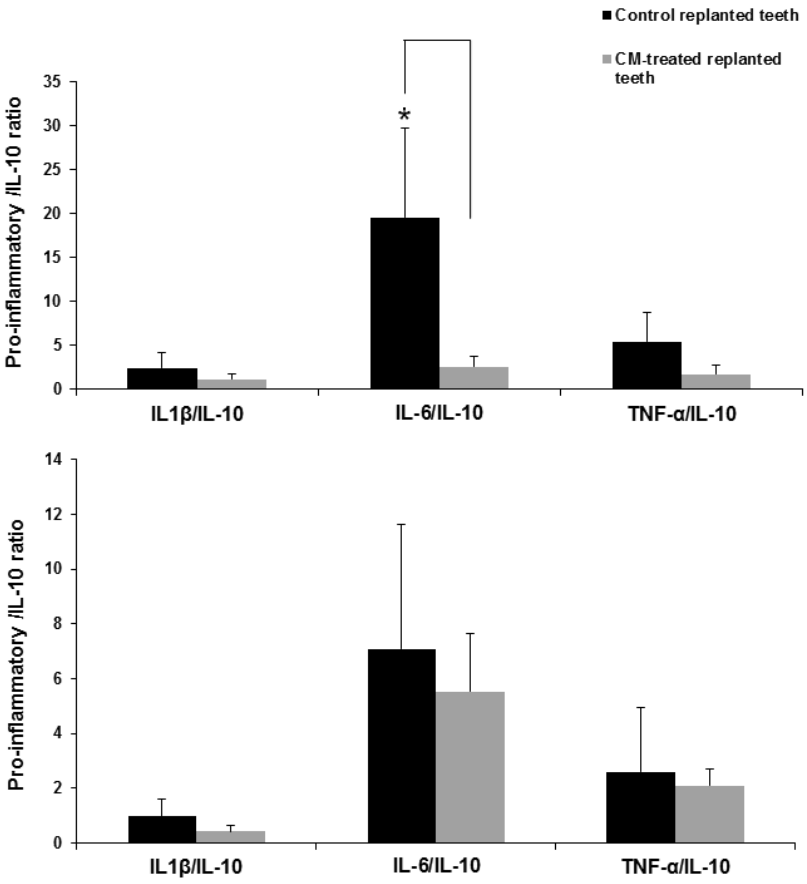

Figure 4 Real-Time qRT-PCR results showing the pro-inflammatory cytokines/anti-inflammatory IL-10 ratios 3 and 14 days following tooth replantation (*$p < 0.05$).

Table 1 Real-Time qRT-PCR probes used in *in vivo* study

Probes	**Codes**
GAPDH	Rn01749022-g1
IL1β	Rn00563409-m1
IL-10	Rn00563409-m1
IL-4	Rn99999010-m1
IL-6	Rn99999011-m1
IL-8	Rn00567841-m1
TNF-α	Rn00562055-m1