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COMPREHENSIVE EVALUATION OF NOVEL TREATMENT POSSIBILITIES FOR PERIODONTAL HARD- AND SOFT TISSUE RECONSTRUCTION

PhD Thesis

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2. LIST OF ABBREVIATIONS

ALT – alanine transaminase

ADM – acellular dermal matrix

AST – aspartate transaminase

bFGF – basic fibroblast growth factor

BHA - butylated hydroxyanisole

BID – twice a day

BMSC – bone marrow stem cells

BSA – bovine serum albumine

β-TCP – beta tricalcium phosphate

CAF – coronally advanced flap

CAL – clinical attachment level

cAMP – cyclic adenosine monophosphate

CD-34 - human cluster of differentiation 34

CEJ - cementoenamel junction

c-KIT – cellular transmembrane receptor tyrosine kinase

CM – collagen matrix

CRC- complete root coverage

DMEM - Dulbecco's modified Eagle's medium

DMSO – dimethyl sulfoxide

DNA - deoxyribonucleic acid

DPSCs – dental pulp stem cells

EDTA – ethylenediaminetetraacetic acid

ELISA - enzyme-linked immuno sorbent assay

EMD – enamel matrix derivative

ETT – Egészségügyi Tudományos Tanács

FACS – fluorescence activated cell sorting

FAM – carboxyfluorescein

FBS – fetal bovine serum

FMBS – full mouth bleeding score

FMPS – full mouth plaque score

FSH – folliculus stimulating hormone

GFAP - glial fibrillary acidic protein

GGT – gamma glutamil transferase

GR – gingival recession

GRD – gingival recession depth

GRW – gingival recession width

GT – gingival thickness

GTR – guided tissue regeneration

Hb1AC - glycated hemoglobin

HIV – human immunodeficiency virus

HRP - horseradish peroxidase

IBMX - 3-isobutyl-1-methylxanthine

IgG – immunoglobuline G

ITS - insulin-transferrin-sodium

KCl – calcium-chloride

KT – keratinised tissue

KTW – keratinised tissue width

MAGR – multiple adjacent gingival recessions

MCAF – modified coronally advanced flap

MCAT – modified coronally advance tunnel

MedDRA - medical dictionary of regulatory activities

MEM – Eagle's Minimum Essential Medium

MGB - tripeptide minor groove binder

MGJ – mucogingival junction

MRC – mean root coverage

MTT - microculture tetrazolium

NGF – neural growth factor

NSE – neurospecific enolase

NT-3 – neurotubulin 3

OFD – open flap debridement

PD – probing depth

PDLSCs – periodontal ligament stem cells

PFA - paraformaldehyde

PKC – protein kinase C

PBS – phosphate buffered saline

PCR – polymerase chain reaction

RCT – randomised controlled trial

rhGDF – recombinant human growth and differentiation factor

rhPDGF – recombinant human platelet derived growth factor

RPLPO - large ribosomal protein

RNA – ribonucleic acid

RT – room temperature

SCTG subepithelial connective tissue graft

SD – standard deviation

S.E.M. – standard error of the mean

SOC – system of classes

STRO-1 - stromal cell surface marker-1

TID – three times a day

TMB – tetramethyl benzydine

TPA – tissue plasminogen activator

TUKEB – Tudományos Kutatásetikai Bizottság

VAS – visual analogue scale

VIM – vimentin

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3. PREAMBLE

During my undergraduate academic years I had been fascinated by the recent progress of periodontal research on the field of tissue regeneration. After graduation receiving a residency status at the Department of Periodontology my professional interest turned to the biological background of tissue regeneration.

Having been accepted to the Dental Research Programme lead by Professor Gabor Varga of the Semmelweis University School of Clinical Medicine the initial objective of my PhD research was to investigate the cellular events contributing to periodontal tissue regeneration. While preparing for the first in vitro studies I got the opportunity to participate in a literature search and collect data for a literature review on the application of Enamel Matrix Derivatives (EMD) (Study I). That time it became clear to me that the EMD's effect on several cell types in the periodontium had been studied but there had only been relatively limited data available on cellular mechanisms of EMD on pluripotent mesenchymal cells of periodontal ligament origin. It was therefore a challenging opportunity for me to study how EMD act on stem cells of periodontal ligament (Study II). That time stem cell culturing techniques had been elaborated at the Department of Oral Biology, Semmelweis University. According to the later published results of our team it was shown that isolation and culturing of periodontal ligament and pulp derived cell colonies was a suitable approach to study the regenerative and differentiating potential of multipotent adult stem cells. This study lead us to show that stem cells of periodontal origin have the capacity to differentiate not only into different cell lineages of the attachment apparatus but also into cells with neuronal characteristics (Study III). Several signalling factors might play a crucial role in this differentiation process.

The first phase of clinical research was to investigate those modulating/differentiation factors that, like EMD may guide cell differentiation during wound healing and regeneration. At the Department of Periodontology, led by Professor Istvan Gera, an opportunity was given when Professor Peter Windisch invited me to a clinical and histological study by Prof. Ulf Wikesjö and Prof. Anton Sculean on the safety and efficacy of a recently discovered growth and differentiation factor (rhGDF-5). I was involved as study coordinator in the first human clinical and histological data on the regenerative capacity of rhGDF-5 (Study IV)

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Periodontal destruction not only affects the attachment apparatus but might also result in soft tissue defects. Reconstruction of these anomalies is often required to resolve severe aesthetic problems as well as for long-term hard- and soft tissue stability. As a result of this aesthetic mucogingival surgery became again a main focus of clinical research during the turn of this century.

The next phase of my research project was to investigate how connective tissue grafting could be substituted with biomaterials. Since the first studies on Guided Tissue Regeneration (GTR) several attempts had been made to utilise GTR techniques and biological membranes for soft tissue augmentation and root coverage, but without any convincing success. Development of a novel biocompatible xenograft matrix provided a good opportunity to conduct clinical studies in the field of periodontal plastic surgery. Our research group was led by Professor Anton Sculean and Sofia Aroca, we wanted to investigate if the application of the new material to cover denuded root surfaces and improve the biotype of the patients might be comparable to the success rate by the autogenous connective tissue graft. This resulted in two publications (Studies V and VI) in peer reviewed journals, our group was the first to conduct a randomised split mouth clinical trial to evaluate the use of the novel collagen matrix for correction of multiple recessions.

It has always been a great honour for me that I had had the chance to participate in several research projects and to be member of clinical multi-centre studies. Thus I could participate in providing new and relevant data either on adult stem cell research or on clinical studies on the safety and efficacy of certain new and promising growth and differentiation factors as well as some novel biomaterials. In my thesis I would like to give an overview of the relevant literature data on tooth derived stem cell research, novel approaches of periodontal regeneration and also periodontal plastic surgery. I am going to chronologically present the evolution of my doctorate work based on the six phases of my research projects, starting with the summary of the systemic review of the literature on the application of EMD followed by adult stem cell studies. In the clinical part of my studies I am going to summarize and discuss the clinical and histological results from a randomised controlled study on the application of rhGDF-5 and finally finishing with the most recent studies on the clinical applicability of collagen xenograft matrices in periodontal plastic surgery.

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4. INTRODUCTION

/Review of the related literature/

The goal of regenerative therapy for periodontal hard tissue reconstruction is the recreation of the lost periodontal structures (i.e. new formation of root cementum, periodontal ligament and alveolar bone). The approximately 30 years research on GTR and the 60 years history of research on mucogingival surgery and clinical techniques provided tremendous amount of information and knowledge. Nevertheless several questions are still unanswered and there are numerous controversies in the current opinions and trends. It is still not clear what kind of biological factors play crucial roles in wound healing and particularly in complex periodontal regeneration, where three different cell lineages should be re-established.

Many treatment modalities, such as various types of bone grafts, GTR and EMD have been used with varying success during the past to accomplish this goal (Sallum et al. 2003, Donos et al. 2003, Palioto et al. 2004). Results from basic and clinical research have pointed to the predictability, safety and efficacy of the application of EMD and GTR (World Workshop in periodontology 1996, Rincon et al. 2003, Donos et al. 2003) in the periodontal wound healing and regeneration. Nevertheless, current surgical techniques and available biomaterials for hard tissue reconstructions have some well-known limitations in case of advanced attachment loss and unfavourable defect configurations.

Beyond hard tissue reconstruction and gain of new attachment, correction of periodontal soft tissue defects has again become a main focus of clinical research during the past decade. (Hofmänner et al. 2012) Main goals of surgical approaches include prevention of advanced periodontal defects related to mucogingival anomalies as well as fulfilling the increasing aesthetic demands of patients. Periodontal plastic surgery aims at the reconstruction of soft tissue deformities as well as at the modification of unfavourable anatomic conditions, such as thin gingival biotype. Various surgical techniques have been suggested for changing the gingival biotype as well as correction of recession defects. According to literature to achieve optimal predictability and long-term stability connective tissue grafting has been suggested as the standard adjunctive therapy for periodontal plastic surgery (Cairo et al 2008, Chambrone et al 2010, Hoffmänner et al.

2012). Nevertheless, increased patient morbidity and duration of surgery related to a donor surgical site and tissue harvesting are well known drawbacks of autogenous soft tissue grafting (Cairo et al. 2008).

The limitations of currently applied techniques for periodontal hard- and soft tissue reconstruction related to treatment efficacy and patient morbidity have raised a demand to introduce novel treatment approaches as well as biomaterials aiming at increased treatment efficacy as well as reducing duration of treatment and patient morbidity. During the last decade, emerging new research fields have investigated the possibilities of tissue engineering related to the isolation and differentiation of human adult tooth derived stem cells (Gronthos et al. 2000, Miura et al. 2003, Seo et al. 2004) and application of different recombinant growth-factors (Morotome et al. 1998, Sena et al. 2003) for periodontal hard tissue reconstruction as well as application of novel xenogenic materials (Vignoletti et al. 2011) for reconstruction of soft tissue anomalies.

Stem cell research and possibly related tissue engineering applications have become a promising field for tissue regeneration and implementation of regenerative medicine. Since the discovery and characterization of multipotent mesenchymal stem cells from bone marrow, similar populations from other tissues have now been characterized. Postnatal stem cells have been isolated from a variety of tissues including bone marrow, brain, skin, skeletal muscle and the gastrointestinal tract (Kuehnle and Goodell 2002, Javazon et al. 2004, Le Blanc and Pittenger 2005). This obviously influenced and inspired basic research possibly related to future dental applications. Recent studies have revealed the presence of adult stem cells in tissues of dental origin as well. Namely, primary cell cultures containing progenitor cells originating from both adult and deciduous dental pulp as well as periodontal ligament were described (Gronthos et al. 2000, Miura et al. 2003, Seo et al. 2004). Recently, an extraordinary plasticity of postnatal stem cells has been suggested. Bone marrow stem cells may contribute to muscle, liver, and neuronal tissue formation (Miura et al. 2003, Clarke 2003, Seo et al. 2004, Grove et al. 2004). To utilize this potential, it is necessary to gain further insight into the characteristics of postnatal stem cells of dental origin and examine their full developmental potential first in vitro. Since the stem cell cultures of dental origin exhibit mesenchymal stem cell characteristics (Gronthos et al. 2000, Miura et al. 2003, Seo et al. 2004), one of the most plausible direction for differentiation and potential utilization of

these cells in periodontal regeneration is the osteogenic one. Indeed, one important feature of both pulp and periodontal cells is their mineralization potential in response to appropriate pharmacological induction (Gronthos et al. 2000, Miura et al. 2003, Seo et al. 2004). Cells can be induced in vitro to differentiate into cells of osteogenic/odontogenic phenotype, characterized by polarized cell bodies and accumulation of mineralized nodules (Tsukamoto et al. 1992, About et al. 2000, Couble et al 2000). Nevertheless, the exact molecular signalling mechanism for this transition, and also the interaction of various pathways being involved is not completely understood. The dental pulp and the periodontal ligament have also been suggested to harbour cells that are able to differentiate into neuronal direction (Miura et al. 2003, Nosrat et al 2004, Shi et al. 2003, Shi et al. 2005, d'Aquino et al. 2007, Techawattanawisal et al 2007, Widera et al. 2007, Arthur et al. 2008, Koyama et al 2009).

While stem cells research and tissue engineering techniques are not yet available for human application, utilising human recombinant growth factors presents a novel promising treatment option for periodontal hard tissue reconstruction (Morotome et al. 1998, Sena et al. 2003). Nevertheless, literature data are still sparse on treatment safety and efficacy. The 1996 American Academy of Periodontology World Workshop (World Workshop in Periodontology 1996) formulated the following criteria for a treatment modality to be considered a periodontal regenerative procedure: a) controlled histological animal studies demonstrating formation of new cementum, periodontal ligament, and alveolar bone; b) controlled clinical studies demonstrating gain of clinical attachment and alveolar bone; and c) human biopsies demonstrating formation of new cementum, periodontal ligament, and alveolar bone onto a previously "plaque-infected root surface". Fulfilling the first criterion, preclinical studies have pointed to a role of growth/differentiation factor -5, -6, and -7 in the formation of the periodontal ligament (Morotome et al. 1998, Sena et al. 2003). rhGDF-5 exhibits osteoinductive properties in vitro and in vivo (Spiro et al. 2000). Moreover, rhGDF-5 may provide an environment conducive to periodontal wound healing/regeneration affecting extracellular matrix metabolism (Nakamura et al. 2003). Still other studies have shown significant periodontal regeneration in discriminating large animal models following surgical implantation of both rhGDF-5 and rhGDF-7 (Wikesjö et al. 2004, Kim et al. 2009, Lee et al. 2010). An rhGDF-5/β-TCP device has been shown to enhance periodontal regeneration in deep one-

wall intrabony defects in dogs (Lee et al. 2010). The beta-tricalcium-phosphate (β-TCP) carrier matrix exhibits a resorption profile that apparently minimally interferes with bone formation/remodelling and periodontal regeneration; β-TCP, being biocompatible, resorbs and is replaced by bone within weeks of implantation. Indeed, standalone β-TCP technologies have been used for orthopaedic and craniofacial indications for more than 20 years as implantable bone substitutes (Galois et al. 2002). A recent study has shown that rhGDF-5/β-TCP implanted in a rat calvarial defect model enhances local bone formation (Pöhling et al. 2006). Taken together, preclinical data suggest that rhGDF-5 may have a significant potential not only to induce/support periodontal wound healing/regeneration but also to support regeneration elsewhere in the axial and appendicular skeleton (Moore et al. 2010). Beyond preclinical studies, controlled clinical pilot studies are needed to demonstrate clinical potential and safety. However, although rhGDF-5 appears to be promising for enhancing periodontal regeneration, until now, it has not been used in humans to treat periodontal defects and thus, the safety and the clinical potential of the material are unknown.

Mucogingival deformities are often associated to advanced periodontal hard tissue defects but may also occur without the presence of periodontitis. For the correction of periodontal soft tissue defects, application of xenogenic grafting materials has been suggested as a promising alternative for connective tissue grafting (Vignoletti et al. 2011). Since utilising xenografts in regenerative periodontal therapy has been performed on a regular basis in the past, introducing novel xenogenic biomaterials is easily applicable for human use in periodontal plastic surgery, the most important goal being gingival recession coverage. Gingival recession is defined as the exposure of the root surface due to the displacement of the gingival margin apical to the cemento-enamel junction (CEJ). (Wennström 1996, Armitage 1999) As a result, root surface exposure to the oral cavity is frequently associated with aesthetic complaints, root hypersensitivity and difficulties to achieve optimal plaque control (Serino et al. 1994, Lovegrove et al. 2004, Susin et al. 2004, Daprile et al. 2007).

The aetiology of gingival recession is complex, commonly related to over contoured tooth shape and malposition in the dental arch, alveolar bone dehiscence, thin biotype, muscle attachment, obsessive tooth brushing, localized or generalized periodontal disease, iatrogenic dental treatments (Serino et al. 1994, Susin et al. 2004,

Lovegrove et al. 2004, Daprile et al. 2007). As one of the most significant predeterminants, a thin gingival biotype is considered to be the most relevant anatomical factor of gingival recession (Müller et al. 1998), although controversial data have been published on the minimally sufficient width and thickness of keratinised gingiva, needed for long-term stability of marginal soft tissue contours (Kennedy et al. 1985, Aguido et al. 2009). Therefore, most soft tissue augmentation procedures aim not only to obtain complete root coverage (CRC) and natural tissue blending of the exposed surfaces and but also to increase gingiva width and thickness to ensure long-term stability.

Results from systematic reviews indicate that at single Miller (Miller 1985) class I and II gingival recessions CRC can predictably be obtained using different surgical techniques mainly including coronally advanced flap (CAF) with and without soft tissue grafting and/or biologic agents such as an enamel matrix derivative (Cairo et al 2008; Chambrone et al 2010).

On the other hand, predictable coverage of multiple adjacent gingival recessions (MAGR) still represents a challenge for the clinician due to difficulties in managing the soft tissues and poorer wound healing related to factors such as the large avascular surface, blood supply, differences in recession depth and position of the teeth (Hofmänner et al. 2012). From a clinician's point of view treatment of MAGR is a very demanding situation due also to the extent and duration of surgery and patient morbidity. A very recent systematic review evaluating the predictability of various surgical techniques used for the treatment of MAGR has indicated that the modified coronally advanced flap (MCAF) with and without soft tissue grafting and the modified coronally advanced tunnel (MCAT) using soft tissue grafting are the most predictable methods to obtain CRC in Miller Class I and II MAGR (Hofmänner et al. 2012). It is, however, important to point out that on a long-term basis (i.e. up to five years), the use of connective tissue grafts in combination with MCAF yielded more stable outcomes compared to the use of MCAF alone (Pini-Prato et al. 2010). The MCAT has been proposed for the surgical treatment of MAGR since it has several advantages such as: a) it avoids vertical releasing incisions and does not incise the papillae thus improving blood supply, b) due to its coronal displacement, it covers and protects the soft tissue graft thus improving graft survival (Azzi and Etienne, 1998, Zuhr et al. 2007, Aroca et al. 2010). Interestingly, according to the best of our knowledge, at present MCAT in combination with subepithelial connective

tissue grafting is the only technique which has been shown to result in predictable improve coverage of Miller Class III MAGR (Aroca et al. 2010; Hofmänner et al.2012).

Connective tissue graft harvesting is often associated with increase patient morbidity, prolonged surgical time and the possibility of postoperative complications such as bleeding and numbness in the donor area (Hofmänner et al.2012). In order to overcome these inconveniences, attempts are made to develop new materials aiming to replace connective tissue grafts thus, improving patient acceptance and minimizing morbidity. Both the MCAF and the MCAT techniques have been reported applied in combination with biological adjuncts, such as EMD (Pilloni et al. 2006), acellular dermal matrix (ADM) (Modaressi 2009) and platelet rich fibrin (PRF) (Aroca 2009). Nevertheless, according to a recently published systematic review, none of these alternative biological factors have reached or surpassed the effecacy and predictability of connective tissue grafting (Cairo et al. 2008).

A newly developed porcine derived bioresorbable collagen matrix (CM) (Mucograft®, Geistlich Pharma, Wolhusen, Switzerland) has been recently introduced proposed as an alternative to the subepithelial connective tissue graft (SCTG) in periodontal plastic surgery procedures. The safety and efficacy of the CM in root coverage procedures was reported in a histological study of the minipig (Vignoletti et al. 2011), as well as in controlled human clinical studies comparing treatment of Miller Class I and II single recessions by means of CAF with CM or SCTG (McGuire et al. 2010, Cardaropoli et al. 2012). Both randomized controlled clinical studies have indicated that in Miller Class I and II single recessions, CM may yield comparable outcomes in terms of root coverage and tissue blending to that obtained with SCTG. Furthermore, the use of CM was associated with significantly reduced surgical time and patient morbidity compared to the use SCTG (McGuire et al. 2010, Cardaropoli et al. 2012). Taken together, the available data appear to suggest that CM might represent an alternative to SCTG thus warranting further investigations. However, according to the best of our knowledge, until now no prospective, randomized, controlled, clinical studies have compared treatment of MAGR by means of MCAT using either CM or SCTG.

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5. OBJECTIVES

The goal of my PhD dissertation was to evaluate - based on the existing evidences available in literature related to adult stem cells, human recombinant growth factors and novel xenogenic biomaterials - the currently available treatment options and the novel materials and techniques that might be the future in periodontal hard- and soft tissue reconstruction.

Available data related to in vitro and clinical research on periodontal regenerative therapy have raised a number of fundamental questions dealing with possible future clinical impact of the above mentioned novel regenerative procedures and biomaterials. These goals focus on establishing the methodological basis to develop future tissue engineering applications, as well as safety and efficacy of currently available prototype biomaterials for human periodontal application. During my PhD research in vitro and clinical studies were conducted to find answers to the main question: how periodontal wound healing and complete regeneration can be improved beyond current therapeutical approaches.

The performed in vitro and clinical studies aimed at:

- Establishing cell cultures of periodontal origin, investigating the effect of EMD on cell proliferation; characterisation of adult stem cells in vitro
- Developing in vitro protocols for osteogenic differentiation of periodontal ligament stem cells (PDLSCs) for future tissue engineering applications
- Investigating the safety and efficacy of a human recombinant growth factor on a β-TCP carrier (rhGDF-5) designed for periodontal hard tissue reconstruction in a pilot clinical study
- Investigating the safety and efficacy of a novel collagen matrix (Mucograft®) for gingival recession coverage of MAGR in a pilot clinical case series
- Comparing the clinical outcome and patient satisfaction related to the application of Mucograft® compared to connective tissue grafting in the treatment of MAGR in a split mouth randomised controlled study

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6. METHODS

In this section the literature research, experimental, surgical and analytical methodology will be described. The present thesis reports on a review article, two in vitro research articles as well as three publications reporting on clinical studies, which are summarised in Table 1.

In vitro research was carried out at the Department of Oral Biology, Semmelweis University. All patients included in the clinical studies were referred to the Department of Periodontology, Semmelweis University for treatment of periodontal soft- or hard tissue defects.

Table 1: Summary of literature review, clinical- and in vitro studies

Study	Description	Appendix
I	Literature analysis on the application of enamel matrix proteins in periodontal regenerative therapy	[I]
II	Isolation and culturing of PDLSCs, investigating the effect of EMD on cell proliferation and viability	[II]
III	Establishing protocols for in vitro differentiation of human periodontal and pulpal stem cells	[III]
IV	Evaluating the clinical safety and efficacy of a novel recombinant growth factor for periodontal hard tissue reconstruction	[IV]
V	Assessing a novel bioabsorbable collagen matrix for soft tissue reconstruction in root coverage procedures	[V]
VI	Comparing the novel collagen matrix and connective tissue grafting for root coverage	[VI]

I. A literature review was performed to collect relevant informations prior to initiating further in vitro and clinical periodontal research. To collect valuable informations, the application of EMD in periodontal regeneration compared to alternative treatment options (e.g. GTR) was analysed based on currently available literature data.

II. In the first in vitro study cell cultures from human periodontal ligament were established and multipotential adult stem cells (PDLSCs) were identified in these cultures. The effect of EMD was also analysed with regards to viability of cells cultures. We established the methodological basis for further in vitro research.

III. The second in vitro study described the introduction of differentiation protocols applicable for maintainable cell cultures containing PDLSCs and dental pulp stem cells (DPSCs). Using optimized pharmacological protocols the potential of periodontal and pulp derived adult stem cell cultures to form mineralized tissues and to undergo neuronal differentiation was analysed.

IV. The first clinical exploratory study was specifically designed to evaluate the clinical and histological outcomes following treatment of intrabony defects with open flap debridement alone or in combination with rhGDF-5 adsorbed onto a particulate β-tricalcium phosphate carrier. The publication reported on the study protocol, safety profile, the early healing phase and the clinical outcomes at 24 weeks while the histological outcomes were presented and discussed in great detail in a subsequent paper (Stavropoulos et al. 2011).

V. The second clinical study presented data from a prospective pilot case series, which was performed to evaluate the safety and efficacy of Mucograft® in the treatment of Miller class I and II MAGR using the MCAT technique.

VI. The third clinical study reported on a prospective, randomized, controlled, split-mouth clinical study. This was conducted to clinically evaluate the treatment of Miller class I and II MAGR using the MCAT technique either in combination with Mucograft® or SCTG.

6.1 Literature review on the application of enamel matrix proteins in periodontal regenerative therapy

In the literature search a protocol of review was set out with the following eligibility criteria for study inclusion to collect valuable information on the application of EMD in periodontal regeneration. A technique or a material must have fitted in the following categories to be classified as "regeneration-related article"

- In vitro studies, which investigated the cellular and molecular mechanisms of EMD
- Controlled histological animal studies, which evaluated the formation of new root cementum, periodontal ligament and alveolar bone.
- Human biopsies, which assessed the formation of root cementum, periodontal ligament and alveolar bone on a plaque-infected root surface.
- Controlled clinical studies, which measured the magnitude of gain of clinical attachment and radiographical new bone formation. In the literature overview, the existing evidence regarding the clinical use of EMD was provided.

6.2 In vitro isolation and differentiation of periodontal ligament stem cells

In our in vitro studies different methodologies were applied to establish and maintain periodontal and pulpal cell cultures and to achieve differentiation of adult stem cells into different cell lineages.

6.2.1. Cell isolation and culturing of periodontal ligament stem cells

Our protocol to isolate and culture dental pulp stem cells is based on a procedure described previously (Gronthos et al. 2000), with some modifications. In brief, normal human impacted third molars were collected from adults (18-26 years of age) at the Department of Periodontology, Semmelweis University, under approved ethical guidelines set by the Ethical Committee of the Hungarian Medical Research Council. Tooth surfaces were cleaned and the periodontal tissue was removed with a sterile scalpel and was collected. The tooth was cut around the cemento-enamel junction by sterile dental fissure burs to expose the pulp chamber, and the pulp tissue was removed from the

crown and root. Both pulp tissue and periodontal tissue were then separately digested in a solution of collagenase type I (3 mg/ml, Sigma-Aldrich, St. Louis, USA) and dispase (4mg/ml, Roche, Basel, Switzerland) for 1 hour at 37°C. Single-cell suspensions were obtained by passing the cells through a 70 μm strainer (Falcon). Cells were seeded into 6-well plates (Costar) in alpha modification of Eagle's medium (a-MEM, GIBCO/BRL) supplemented with 15% Fetal Bovine Serum (FBS, GIBCO/BRL), 100 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, USA), 2 mM L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin (GIBCO/BRL), and then incubated at 37°C in 5% CO^2. To assess colony-forming capability, 14 day old cultures were fixed with 4% paraformaldehyde, and then stained with 2% Giemsa. Aggregates of 50 cells were scored as colonies

6.2.3. Cell viability studies and treatment with enamel matrix derivative

The influence of FBS and EMD (Emdogain, Straumann, Basel, Switzerland) containing media as well as osteogenic and neuronal differentiation protocols on primary cell cultures was assessed by Microculture Tetrazolium (MTT) assay. To test the effect of these conditions on culture growth, DPSCs and PDLSCs were cultured in 96-well plates for 24 hours. In each well 3×10^3 DPSC cells or 5×10^3 PDLSC cells were grown in their regular media for 24 hours. Afterwards, cells were serum-starved for another 24 hours. Then, 15% (PDLSC) or 20% (DPSC) FBS containing medium, or serum free medium (control) was added for 24 hours. Thereafter, 100 μl MTT solution (0.2 mg/ml, Sigma-Aldrich, St. Louis, USA) diluted in a-MEM was added into each well until formazane crystal formation occurred. 100 μl DMSO (99.5%, dimethyl-sulfoxid) was added into wells to dissolve formazane crystals. Then the intensity of staining was determined by a microplate reader (Model 3550, Biorad, Hercules, USA) at 595 nm (measurement wavelength) and 650 nm (reference wavelength). Under these circumstances, the level of optical density is proportional to the number of living cells in the culture. The proliferative effect was expressed as a ratio between optical density of treated cells and serum-free cultured control cells and given in percent.

6.2.4. Immunocytochemistry

To identify the mesenchymal stem cell marker “stromal cell surface marker-1“ (STRO-1) in our cultures, cells were grown on glass coverslips in 24-well plates (Costar, Cole-Parmer, Vernon Hills, Illinois, USA) ($5x10^4$ cells per well) and fixed with 4% PFA in phosphate buffered saline (PBS) for 20 min. To block non-specific binding, fixed cultures were incubated in PBS containing 7.5% FBS for 90 min and incubated with an anti-STRO-1 primary antibody (1/200, a generous gift from Prof Richard Oreffo, University of Southampton, Southampton, UK) overnight at 4°C. Subsequently, the cells were incubated with Alexa 488 conjugated goat anti-mouse IgG (1:1000, Molecular Probes, Invitrogen, Carlsbad, CA, USA) for 1 hour. Nuclei were counterstained with 10 mg/ml bisbenzimide (Sigma-Aldrich, St. Louis, USA) for 30 minutes.

To evaluate protein expression during differentiation experiments, cells grown on poly-L-lysine-coated glass coverslips were fixed with 4% PFA in PBS for 20 min at room temperature (RT), then 0.1% Triton X-100 (in PBS) was added for 8 min to permeabilise them. Fixed cultures were incubated in PBS containing 4% bovine serum albumin (BSA; 90 min at RT) to block non-specific binding, then reacted with primary antibodies at 4°C overnight. Antibodies were diluted in 4% BSA as follows: anti-NSE 1/200, anti-NF-M 1/200. IgG anti-mouse and anti-rabbit Alexa Fluor 488 conjugated (Molecular Probes, Invitrogen, Carlsbad, CA, USA) secondary antibodies were diluted 1/750 and applied for 1 h at RT. Nuclei were counterstained with 10 mg/ml bisbenzimide (Sigma-Aldrich, St. Louis, USA) for 30 minutes. Labelled preparations were examined by a fluorescent microscope (Nikon Eclipse E600, Nikon Instruments, Tokyo, Japan), and images were captured with a cooled CCD camera (SPOT RT Color 2000, Spot Imaging Solutions, Sterling Heights, Michigan, USA) connected to a PC running an image acquisition software (SPOT Advanced, Spot Imaging Solutions, Sterling Heights, Michigan, USA.). Adobe Photoshop® was used to merge the digitized images of bisbenzimide and specific staining.

6.2.5. FACS analysis

Fluorescence Activated Cell Sorting (FACS) analysis was performed to identify cells expressing STRO-1, CD34 and c-kit mesenchymal stem cell markers as described

previously (Gronthos et al. 1994, Laino et al. 2005). Single cell suspension were prepared from the cell cultures of 0,2% EDTA content, and subsequently incubated with STRO-1/CD34/c-kit antibody or with isotype matching negative controls for 1 hour on ice. Cells were washed with 5% PBS solution of FBS, then fluorescent stain-conjugated secondary antibodies were added to the samples. Following repeated rinsing with 5% PBS solution of FBS, cell suspensions were fixated with 4% paraformaldehyde. FACS analysis was performed subsequently.

6.2.6. Osteogenic induction

Osteogenic differentiation was induced by modifications of a previously reported protocol (Kemoun et al. 2007). In brief, DPSCs and PDLSCs were cultured with 1% FBS, 100 mg/ml streptomycin, 100 U/ml penicillin, 2 mML-glutamine, 10^{-8}M dexamethazone, 50 mg/ml L-ascorbic acid 2-phosphate, 10 mmol/l b-glycerophosphate in aMEM for 20 days without passaging. The medium was replaced twice a week. After 3 weeks of treatment calcium accumulation was detected by 2% Alizarin red S (pH 4.2, buffered with ammonium hydroxide) staining. Similar culture media without dexamethazone and ß-glycerophosphate was used as control condition.

6.2.7. Neuronal induction

For neuronal differentiation, cultured morphologically homogeneous DPSCs and PDLSCs, (passage 1-4) were plated (~2×10^4 cells/well) into a 24 well plate containing poly-L-lysin coated glass coverslips. After 24 hours, cells were treated with 3 different protocols:

6.2.7.1 Protocol 1.

Cells were differentiated as previously described by Scintu et al. (Scintu et al. 2006) with 10 ng/ml FGF-1 (R&D, Minneapolis, MN), 200 nM 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich, St. Louis, USA), 250 μM IBMX (Sigma-Aldrich, St. Louis, USA) and 50 μM forskolin (Sigma-Aldrich, St. Louis, USA), in Dulbecco's modified Eagle's medium/F12 1:1 (DMEM/F12) (Sigma-Aldrich, St.

Louis, USA) supplemented with ITS Liquid Media Supplement (Sigma-Aldrich, St. Louis, USA). The cells were fixed for immunocytochemistry right before and 24 h post-induction.

6.2.7.2. Protocol 2.

This protocol was also based on a method recently reported by Choi et al. (Choi et al. 2006). Cells were preinduced for 1 day with DMEM/F12, with 20% FBS, and 10 ng/ml basic fibroblast growth factor (bFGF; Sigma-Aldrich, St. Louis, USA). The preinduction medium was removed, cells were washed with PBS and then changed to serum-free induction medium that consisted of DMEM containing 2% DMSO, 200 μM BHA, 25 mM KCl, 2 mM valporic acid, 10 μM forskolin, 1 μM hydrocortisone and 5 μg/ml insulin (Sigma-Aldrich, St. Louis, USA). The cells were fixed for immunocytochemistry right before and 24 h post-induction.

6.2.7.3. Protocol 3.

A three-step differentiation method was developed in our own laboratory since Protocols 1 and 2 did not yield satisfactory results. DPSCs or PDLSCs were seeded onto poly-L-lysin coated glass coverslips in DMEM/F12, 2.5% FBS, 100 mg/ml streptomycin, and 100 U/ml penicillin, and cultured for 24 h. Step 1: epigenetic reprogramming was performed using 10 mM 5-azacytidine in DMEM/F12 containing 2.5% FBS and 10 ng/ml bFGF for 48 h. Step 2: neural differentiation was induced by exposing the cells to 250 mM IBMX, 50 mM forskolin, 200 nM TPA, 1 mM dbcAMP, 10 ng/ml bFGF, 10 ng/ml NGF and 30 ng/ml NT-3, supplemented with ITS Liquid Media Supplement in DMEM/F12 for 3 days. Step 3: at the end of the neural induction treatment, cells were washed with PBS and then neuronal maturation was performed by maintaining the cells in Neurobasal A media supplemented with 1 mM dbcAMP, 1% N2, 1% B27, and 30 ng/ml NT-3 for 3-8 days. Solutions 168 were freshly prepared immediately prior to use. The cells were fixed for immunocytochemistry before treatment, on the first day neuronal induction (step 2) and on the third day of maturation (step 3).

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6.2.8. Real-time PCR

Total RNA from DPSCs and PDLSCs was isolated using an RNeasy Plus Micro Kit (Qiagen) with on-column DNase digestion. The concentration of the RNA was determined by the Ribogreen method (Invitrogen, Carlsbad, CA, USA). The integrity of the RNA was verified by electrophoresis on a 1% agarose gel and 200 ng total RNA was used per sample for cDNA synthesis, using random primers (High-Capacity cDNAArchive Kit, Applied Biosystems, Invitrogen, Carlsbad, CA, USA) in a total volume of 50 µl. For quantitative PCR amplification, 5% of the cDNA synthesis reaction was used with real time PCR primers and a target-specific fluorescence probe (FAM-labelled MGB probe). The probes and primers were selected from the Applied Biosystem Assay on Demand database for the specific markers vimentin (VIM) and neurospecific enolase (NSE) and for the human acidic ribosomal phosphoprotein P0 (RPLP0), which was used as an internal control. Universal Mastermix (Roche, Basel, Switzerland) containing AMP-erase was used for amplification in a total volume of 20 µl. For detection of fluorescence signal during the PCR cycles, a (StepOne® Real-Time PCR System, Applied Biosystem, Invitrogen, Carlsbad, CA, USA) was used with the default setting (50°C for 2 min, 95°C for 10 min, 45 cycles: 95°C for 15 s, 60°C for 1 min). Each treatment was repeated five times and each sample was measured in duplicate. Changes in gene expression levels were estimated by calculating the relative expression values normalized to the RPLP0 level from the same sample.

6.2.9. Statistical analysis

Data were presented as means ± S.E.M. For statistical comparisons, analysis of variance was followed by Bonferroni post-hoc test (Instat, GraphPad Software).

6.3 Clinical studies

In our clinical studies hard- and soft tissue regenerative procedures were investigated using similar preoperative protocol and postsurgical care. Standardised clinical measurements were taken for evaluation of treatment safety and efficacy. Surgical protocols varied throughout the studies.

6.3.1 Hard tissue regeneration following treatment with rhGDF-5/β-TCP

This pilot, phase IIa study used a stratified randomized, open, controlled, two-arm, parallel group design. The overall design and patient treatment allocation is summarized in Fig. 1. The study was conducted at the Department of Periodontology, Semmelweis University, Budapest, Hungary between July 2007 and August 2008. The study protocol was approved by the Hungarian National Institute of Pharmacy and the Institutional Ethics Committee (application no. 32579/40/06) of the Semmelweis University, Budapest, Hungary (TUKEB no. 20/2007). All patients received oral and written explanations of the research protocol. Patients signed a consent form providing the possibility of withdrawing from the study at any time. The study was planned and conducted in compliance with the Declaration of Helsinki of 1975 as revised in 2000, Good Clinical Practice, and relevant local laws.

The total study duration was 175–182 days, in all ten visits/patient. After screening, selected patients received flap surgery (control) or flap surgery combined with implantation of rhGDF-5/β-TCP at the qualified defect site (Visit 2). They then returned for general and oral health evaluations as well as professional tooth cleanings following a set schedule (Visits 3 through 8). Blood samples were collected at screening (Visit 1), and at weeks 2 and 24 (Visits 3 and 9) to evaluate routine haematology and clinical chemistry, rhGDF-5 plasma levels, and antirhGDF-5 antibody formation.

Randomization was performed using a computer-generated randomization list via block randomization. Ten patients were randomized to each treatment group. A separate random scheme was generated. The investigators were masked to the block length. The sponsor retained the randomization scheme for control purposes. The investigator implemented the predefined randomization by opening a randomization envelope at the appointment for surgery (Visit 2). The randomization code was opened only after the

defect site was fully prepared. The defects were randomly assigned to receive: rhGDF-5/β-TCP following the manufacturer's instructions (test), or no additional treatment (control). All randomized patients completed the study. Masking of treatment was not applicable because the test group received rhGDF-5/β-TCP whereas the control group was treated by periodontal surgery only without additional treatment.

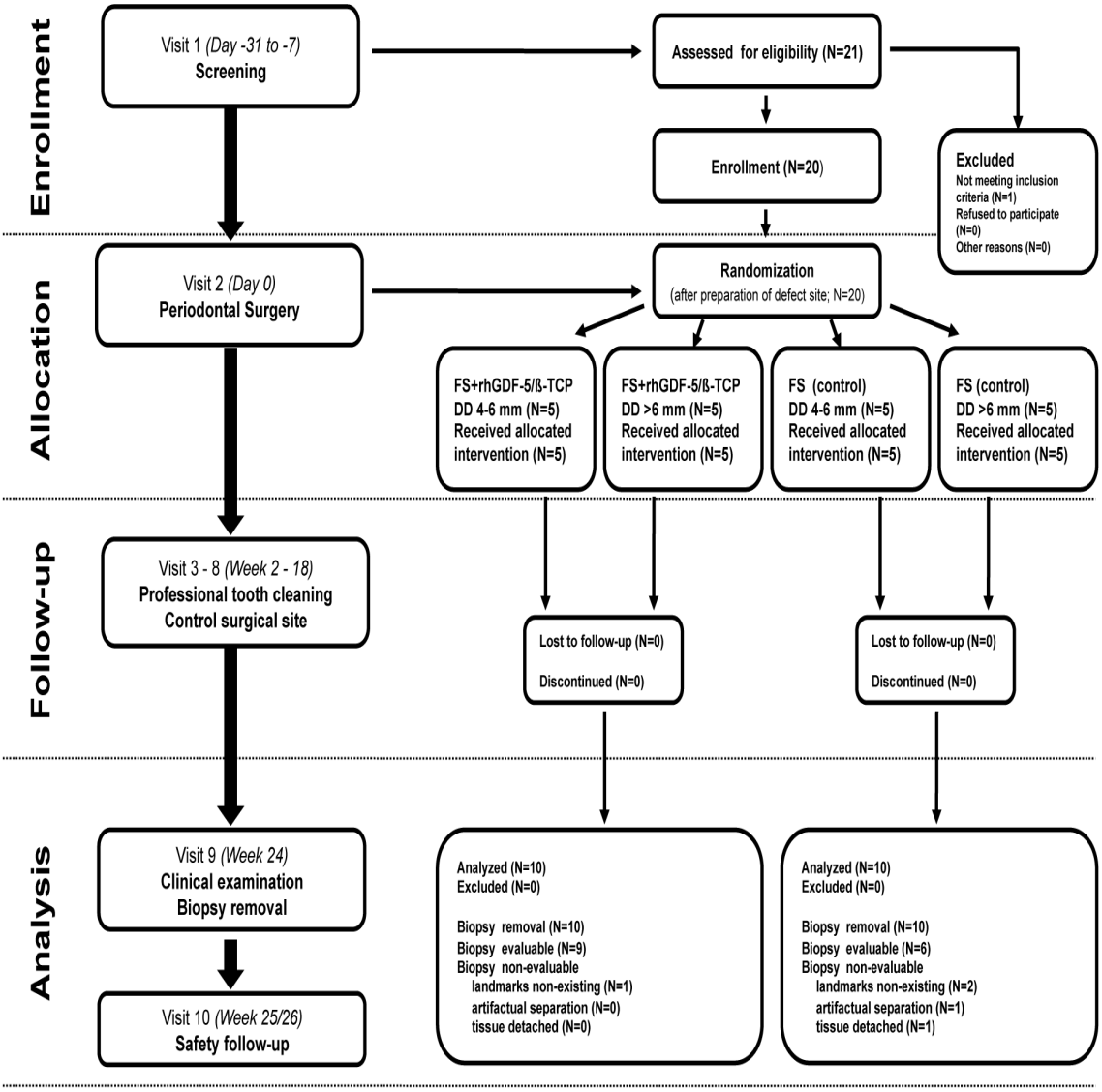

Fig. 1 Study IV flow chart including patient enrolment, treatment allocation, follow-up and analysis (FS: flap surgery; DD: intrasurgery defect depth).

6.3.1.1 Subject selection, preoperative protocol

Twenty Caucasian male and female patients, non-smokers, in good general health, volunteered to participate in this study. They all exhibited advanced chronic periodontitis with one deep intrabony defect located at a maxillary or mandibular single-rooted tooth without root concavities/ furrows or at the mesial or distal aspect of a mandibular molar without contacting teeth (Fig. 1). Mandibular incisors and teeth with furcation involvements were excluded. Only teeth with a probing depth ≥6 mm and an intrabony component ≥4 mm as estimated from long cone parallel technique radiographs confirmed during surgery were considered (Fig. 1). Moreover, the patients were expected to meet oral hygiene standards encompassing full mouth plaque and bleeding scores <20% after completion of basic periodontal therapy (O'Leary et al. 1972, Ainamo and Bay 1975). Each patient contributed one tooth subject to the study treatment. Main exclusion criteria were: a) women of childbearing potential (FSH level <25 IU/L and menstrual bleeding within 6 months)/pregnant or lactating women; b) tobacco smoking; c) evidence of acute/chronic infection at the study site; d) previous (<2 months)/current treatment with systemic corticosteroids of a prednisone equivalent >5 mg/day; e) previous (<12 months)/current treatment with drugs influencing bone metabolism including calcitonin, parathormone, bisphosphonates, or fluoride; f) common contraindications for periodontal surgery; and g) clinically relevant cardiovascular, hepatic, and renal diseases. Due to the explorative type of this study, a sample size of ten patients/group was selected.

All patients had completed basic periodontal therapy (individual oral hygiene instructions, supra- and subgingival scaling and root planing) 8 weeks before screening. If necessary, composite splinting of mobile teeth or eventually fixed temporary restorations were completed.

6.3.1.2 Study material

The rhGDF-5/β-TCP device (Scil Technology GmbH, Martinsried, Germany) comprises rhGDF-5 coated onto a synthetic inorganic carrier, β-TCP, at a concentration of 500 μg/g β-TCP [13]. The β-TCP carrier consists of particles of 500 to 1,000 μm in size with interconnecting porosity. It comprises microporous and macroporous irregular granules of a phase purity >95%. The results of porosity analysis have shown 43.7%

microporosity, an average pore diameter of 2.12 μm, and a total pore area of 0.647 m2/g. The pore size of the macropores ranges between 100 and 400 μm. The surface area is estimated at 1.2 m2/g (Pöhling et al. 2006). The rhGDF-5 protein was coated onto the carrier using Scil Technology's proprietary technology. One vial rhGDF-5/β-TCP contained 250 μg rhGDF-5 and 0.5 g β-TCP (Pöhling et al. 2006). In vitro analysis of the carrier used in this study has shown that almost the entire amount of rhGDF-5 was released from the carrier within the first 7 days (Pöhling et al. 2002).

6.3.1.3 Surgical procedures

One experienced periodontist (PW) performed all surgeries using local anaesthesia, microsurgical instrumentation, and appropriate magnification. (Fig. 2. Fig. 13) The surgical technique was exactly the same for both the test and control groups. An intracrevicular incision was made on the buccal and lingual aspects of the surgical site. The flap was horizontally extended to accommodate the defect location and configuration, and ensured tension-free wound closure for primary intention healing. Vertical releasing incisions were not used. Granulation tissue removal and root instrumentation followed elevation of the mucoperiosteal flaps. In the test group, six patients received one-half vial rhGDF-5/β-TCP, one patient received three-fourth vial rhGDF-5/β-TCP, and three patients received one vial rhGDF-5/β-TCP (one vial rhGDF-5/β-TCP contains 250 μg rhGDF-5 and 0.5 g β-TCP). The mucoperiosteal flaps were then adapted and closed using vertical or horizontal holding mattress sutures and interrupted closing monofilament sutures (5/0 Dafilon; B. Braun Melsungen AG, Melsungen Germany).

6.3.1.4 Postoperative care

Postsurgery care included pain control (Nurofen, 200 mg, 3–4 times per day, Reckitt Benckiser, Slough, UK), systemic (Augmentin 625 mg, GlaxoSmithKline, London, UK London, UK; TID/7 days) and local (twice daily 0.2% chlorhexidine; Curasept, Curadent International AG, Kriens, Switzerland; rinses for 1 min, BID/4 weeks) antimicrobial control. Antibiotic therapy started immediately after surgery. Sutures were removed at day 14. A series of control and recall appointments were

scheduled (biweekly, the first 6 weeks and then monthly until the end of the study) including reinforcements of oral hygiene and professional supragingival tooth cleaning.

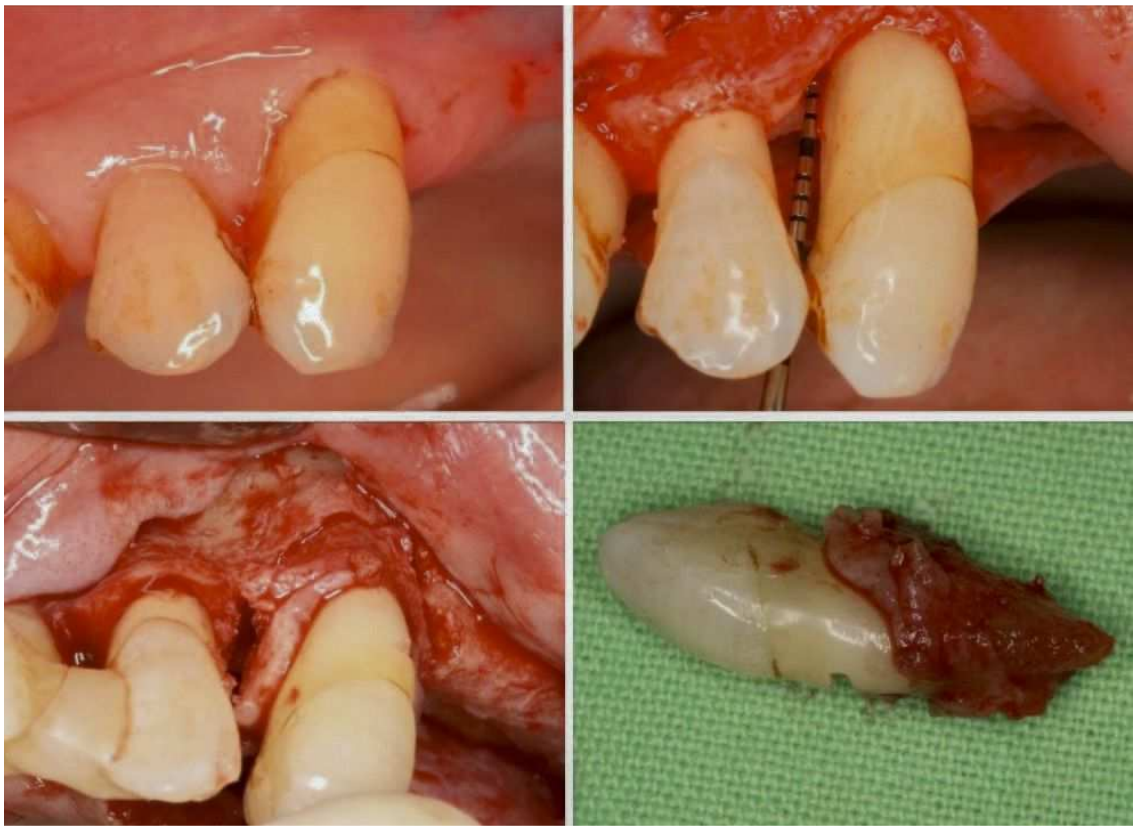

Fig. 2 Flap surgery (control): Presurgery (top left); intrasurgery defect morphology (top right); the biopsy event at 24 weeks postsurgery (bottom left); and biopsy including defect site (bottom right). Histological outcomes were published elsewhere (Stavropoulos et al. 2011).

6.3.1.5 Clinical assessment

Clinical outcomes were evaluated at baseline and at 24 weeks postsurgery. Probing depth (PD), gingival recession (GR) and clinical attachment level (CAL) were recorded using a standard periodontal probe (UNC 15, Hu-Friedy, Chicago, IL, USA). Intraoral radiographs were taken with the long cone parallel technique at baseline and at 24 weeks postsurgery. However, due to the design of the study (i.e. no grafting in the control group), the radiographs were not evaluated. Full mouth plaque and bleeding scores were recorded as a percentage of total surfaces (four surfaces/tooth) with the presence of plaque/bleeding on probing, respectively (O'Leary et al. 1972, Ainamo et al. 1975). One calibrated examiner, masked to the patients' treatment protocol, performed

all clinical recordings. At 24 months postoperatively, biopsy removal was performed. Histological outcomes were published elsewhere (Stavropoulos et al. 2011).

6.3.1.6 Safety assessment

Adverse events were monitored and recorded throughout the study, as well as laboratory values, vital signs, and physical status. Adverse events were coded using the Medical Dictionary of Regulatory Activities (MedDRA) [http://www.meddramsso.com/index.asp]. Summaries and tabulations by severity and relationship to therapy were based on the preferred terms and the primary system organ classes (SOCs). Blood samples were collected at screening (Visit 1), 2 weeks postsurgery (Visit 3), and prior to conclusion of study (Visit 9) to determine laboratory values (clinical chemistry, haematology), rhGDF-5 plasma levels, and antirhGDF-5 antibodies.
The determination of rhGDF-5 in human plasma (EDTA) samples was carried out by Elisa over a quantitation range of 40 pg/ml to 1,250 pg/ml. A monoclonal antibody specific for rhGDF-5 has been precoated on a 96-well plate. Standards/QCs and samples were then pipetted into the wells and any rhGDF-5 present was bound by the immobilized antibody. After washing away any unbound substances, a biotinylated monoclonal antibody specific for rhGDF-5 was added to the wells. After a second washing step, PolyHRP Streptavidin was added that bound to the biotinylated antibody. After a third washing step, peroxidase bound in the complex was visualized by TMB (3,3′,5,5′-Tetramethylbenzidine) substrate solution. After stopping the enzymatic reaction with sulphuric acid, the intensity of the resulting colour was determined at 450 nm. The colour intensity was proportional to the concentration of rhGDF-5 in the sample.

6.3.1.7 Statistical analysis

The statistical analysis was conducted on an intent-to-treat basis. All randomized patients with periodontal treatment were included in the intent-to-treat population. Paired sample t-test and Wilcoxon signed-rank test were used to evaluate the impact of surgical interventions on the various clinical parameters. Mann–Whitney U test (rank sum test) was used to analyse differences among the various outcome variables between treatment groups. No formal statistical comparisons were made related to safety data.

6.3.2 Soft tissue regeneration following treatment with Mucograft®

a) In the pilot case series, 8 adult patients (3 males and 5 females, aged from 18 to 39 years, mean 29 years) presenting Miller class I-II MAGR displaying a total of 42 recession were recruited. All patients presented MAGR defects, which were treated by means of MCAT technique using a bioresorbable collagen matrix (Mucograft®, Geistlich, Wolhusen, Switzerland). The primary outcome variable was the assessment of CRC. The secondary outcome variables included the assessment of mean root coverage (MRC), keratinised tissue width (KTW) and gingival thickness (GT).

b) The randomised controlled study was performed according to a split-mouth design. Thus, in each patient, one side of the jaw served as control while the contralateral side served as test (Fig. 3.) Randomisation was performed by using a computer-generated programme. Recessions were treated by means of MCAT technique using either a bioresorbable collagen matrix (Mucograft®, Geistlich, Wolhusen, Switzerland) (test) (Fig. 12) or SCTG harvested from the palate (control) (Fig. 13). Both surgeries (test and control site) were performed during one single session by the same experienced surgeon (S.A.). The primary outcome variable was the assessment of CRC. The secondary outcome variables included the assessment of MRC, KTW, GT and patient-centred outcomes.

6.3.2.1 Subject selection, preoperative protocol

a) In the pilot case series, patients were treated after having completed preliminary professional tooth cleaning and having received individual oral hygiene instructions. The study was performed between July 2009 and June 2010 at the Department of Periodontology, Semmelweis University Budapest, Hungary in accordance with the Helsinki Declaration of 1975, as revised in 2000 and following approval of the Regional Bioethical Committee (Approval number: ETT TUKEB/365/PI/10/). Inclusion criteria for participation in the study were as follows: (1) at least 18 years of age (2) systemically healthy without any signs of periodontal disease (3) presence of at least three adjacent gingival recessions in the maxilla or mandible, (4) a full-mouth plaque score (FMPS) < 20%[18]; (5) full-mouth bleeding score (FMBS) < 20%[19] (6) non-smoker; (7) not pregnant.

Before enrolment, written informed consent forms were obtained from all patients participating in the study.

b) In the split mouth randomised, controlled study 22 patients with multiple Miller Class I and II MAGR (Miller 1985) with evidence of CEJ were enrolled in the study after having signed an informed consent. The study protocol was in accordance with the Helsinki Declaration of 1975, as revised in 2002 and was submitted to and approved by the ethical committee of the Semmelweis University Budapest, Hungary (protocol: 5242-0/2010-101SEKU; 365/PI/10). The study was performed between July 2010 and November 2011 in the Department of Periodontology of the Semmelweis University Budapest. One month before surgery, individualized oral hygiene instructions were given for each of the included patients accompanied by full mouth supragingival scaling and polishing. The following inclusion criteria were applied: 1) Age ≥ 18 years, 2) Absence of relevant medical conditions, 3) Patients with healthy or treated periodontal conditions. 4) Presence of ≥ 3 adjacent Miller class 1 and 2 gingival recessions on both sides of the maxillary or mandibular arch with an apico-coronal extension (i.e. recession depth) > 2 mm, 5), Full-Mouth Plaque Score (FMPS) ≤ 25% (O`Leary et al. 1972). Patients were excluded on the basis of the following criteria:1) Pregnant or lactating females, 2), Tobacco smoking, 3) Uncontrolled medical conditions, 4) Untreated periodontal conditions, 5) Use of systemic antibiotics in the past 3 months, 6) Use of systemic antibiotics for endocarditis prophylaxis, 7) Patients treated with any medication known to affect gingival conditions (e.g. hyperplasia), 8)Infectious diseases such as hepatitis, tuberculosis and HIV, Drug and alcohol abuse, (9) Failure to sign written informed consent

6.3.2.2 Study material

The CM (Mucograft®, Geistlich Pharma, Wolhusen, Switzerland) has a bilaminar structure, consisting of two adherent layers: a superficial, compact, cell occlusive membrane-like layer incorporating collagen fibres, and an underlying three dimensional spongious collagen matrix designed to serve as scaffold conducing the ingrowth of blood vessels and cells and to enhance blood clot stability.

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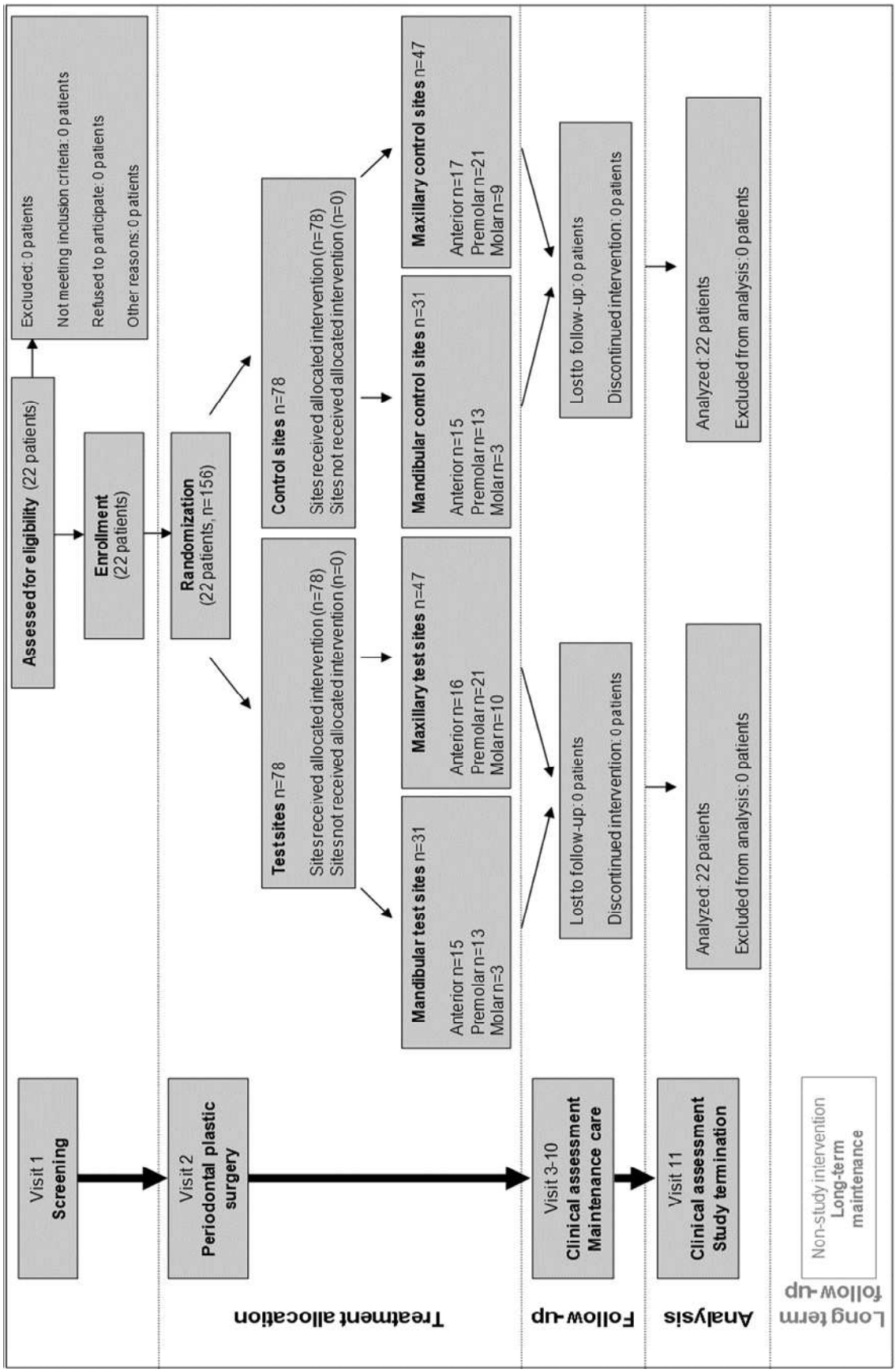

Fig. 3 Study VI flow chart including patient enrolment, treatment allocation, follow-up and analysis

6.3.2.3 Surgical procedures

a) Patients recruited to the pilot case series and MAGR defects on the test sides of patients recruited to the split mouth trial were consecutively treated as follows using the MCAT technique (Azzi, Étienne, 1998). Preoperatively, resin bonding of adjacent contact points at the operation site was performed in order to enable suspended suturing. Following local anaesthesia (Ultracain DS Forte, Sanofi Aventis, Paris, France) root planing of the exposed root surfaces was performed with Gracey curettes (Hu-Friedy, Chicago, IL, USA). Intrasulcular incisions around involved teeth were performed using microsurgical tunnelling knives (Stoma, Liptingen, Germany). Mucoperiosteal envelope flap elevation was performed via the same instruments up to the level of the mucogingival junction at each recession site, leaving interdental papillae intact (Fig. 2). Separate mucoperiosteal envelopes were subsequently interconnected, resulting in tunnel preparation. Mucoperiosteal tunnel elevation was extended by full thickness preparation apically from the mucogingival junction utilising tunnelling knives. Attaching muscles and inserting collagen fibres were separated and released from the inner aspect of the alveolar mucosa by means of Gracey-curettes (Stoma, Liptingen, Germany). As a result of this, the tunnelled flap could be mobilised and advanced coronally without tension. To achieve complete mobilisation of the flap, interdental papillae were gently undermined using microsurgical elevators. Special attention was paid not to disrupt the interdental papillary tissues. Subsequently, the CM was trimmed and adapted to the recipient site with gentle wetting. The CM was carefully advanced into the subperiosteal tunnel through the widest recession using horizontal mattress sutures at the mesial and distal aspects of the matrix (Fig. 12). At surgical sites extending to more than 3 teeth, the CM was cut to multiple segments. The compact membrane-like layer was directed towards the inner side of the flap. The CM was gradually moisturised by sterile saline during this procedure to avoid detaching the underlying spongious layer by overwetting. Having reached the desired position with the coronal margin positioned at the level of the cemento-enamel junction (CEJ), the CM was fixed to gingiva via previously inserted horizontal mattress sutures (Fig. 12). Finally, suspended sutures (i.e. crossed horizontal mattress sutures, anchored over the preoperatively placed interproximal resin splints) were placed into interdental gingiva to coronally advance the fully mobilized mucoperiosteal tunnel,

resulting in complete coverage of the CM and the recessions (Fig. 12). In cases where complete CM coverage could not be obtained with the first sutures, additional vertical mattress sutures were placed interdentally to enable coronal displacement of the tunnel slightly over the CEJ.

b) MAGR defects on control sides of patients recruited to the split mouth trial were treated with the MCAT technique as described above, in combination with connective tissue grafting. A SCTG was immediately harvested after tunnel preparation by using either a modified distal wedge procedure (Azzi & Etienne 1998) or the single incision technique (Hürzeler & Weng 1999) depending on anatomical considerations. If needed, the harvested graft was trimmed using a N°15 blade to achieve an optimal thickness of 1-1.5 mm. Immediately after SCTG harvesting, the donor site was closed with either a cross-mattress suture or with a modified mattress suture (Monnet-Corti et al. 2006) (5-0 polyglactin 910, Vicryl, Ethicon, Johnson & Johnson, USA). SCTG was always inserted under the tunnelled flap by starting at the deepest recession (Fig 13). Subsequently, the grafts were pulled laterally towards each end of the tunnel by means of mattress sutures (Azzi & Etienne 1998) . Finally, the flaps were positioned coronally to the cemento-enamel junction (CEJ) by means of suspended sutures placed above the contact point (Azzi & Etienne 1998) (Fig. 4, 14, 15).

6.3.2.4 Postoperative care

Patients attending either of the trials were given postoperative analgesics (3 X 50 mg Cataflam, Budapest, Hungary) for 3 days and antibiotics (3x625 mg Augmentin, Pfizer KFT, Budapest, Hungary) for 7 days due to university regulation for implantable biological materials. Patients were instructed to rinse with a 0.2% chlorhexidine solution, two times a day for one minute for 3 weeks. Patients avoided brushing in the operated area until suture removal. Patients underwent manual supragingival tooth cleaning twice a week until suture removal. At suture removal two weeks after surgery, patients were instructed in mechanical tooth cleaning of the operated areas using a soft tooth brush and a roll technique. The interproximal resin splints were removed at 21 days. All patients were recalled after 28 days, 3, 6 and 12 months and received one session of prophylaxis, including reinforcement of oral hygiene, supragingival debridement, and tooth polishing.

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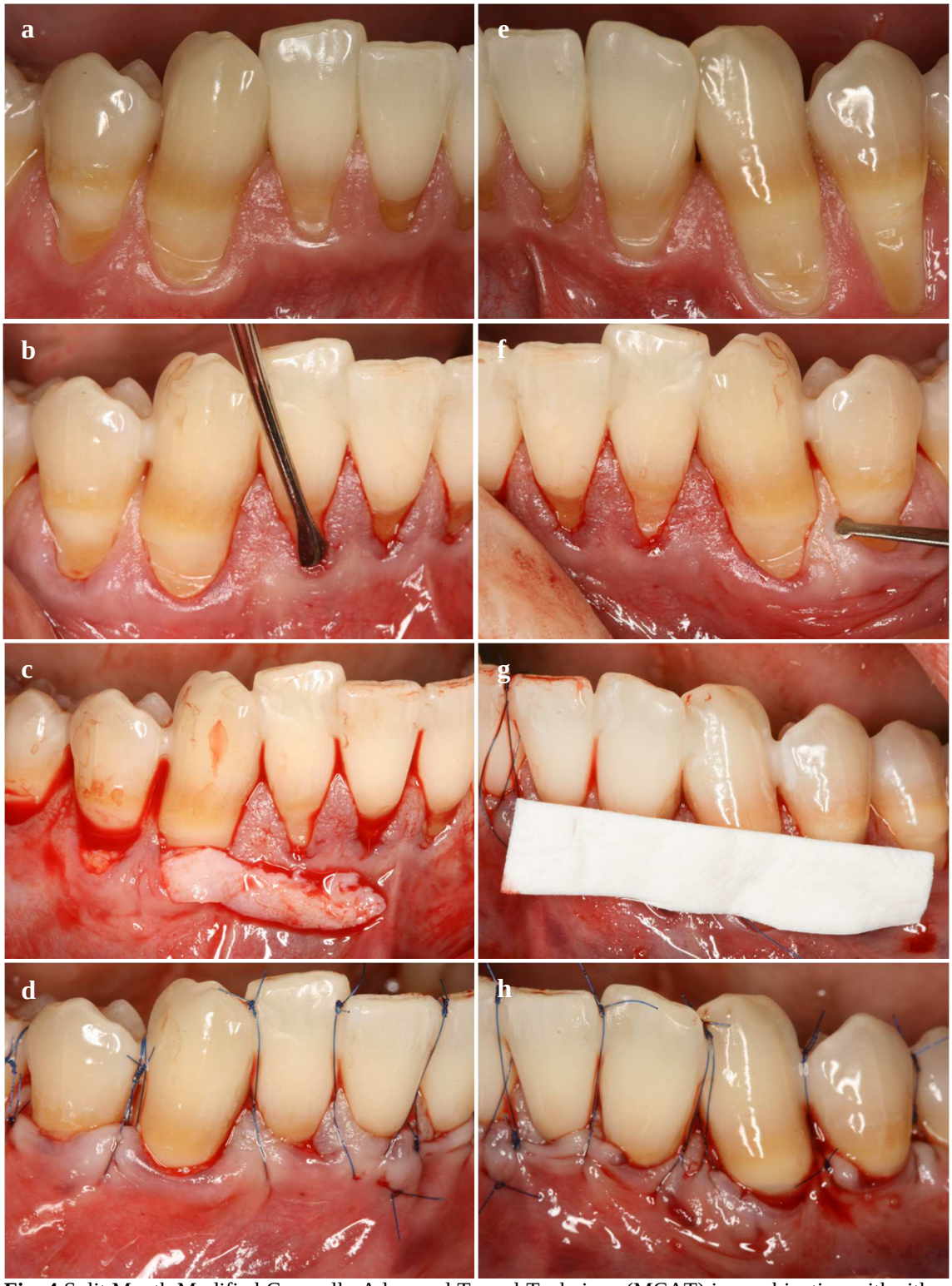

Fig. 4 Split Mouth Modified Coronally Advanced Tunnel Technique (MCAT) in combination with either subgingival connective tissue graft (SCTG) or Mucograft® (CM) (a) Control side prior treatment (b) Control side tunnel preparation (c) Control side SCTG insertion (d) Control side suspended suturing (e) Test side prior treatment (f) Test side tunnel preparation (g) Test side CM insertion (h) Test side suspended suturing

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6.3.2.5 Clinical assessments

In both studies following measurements were made at the mid-buccal point of the involved teeth at baseline (prior to surgery) 6, and at 12 months by the same blinded investigator (B.M.) using the same type of periodontal probe (UNC 15, Hu-Friedy, Chicago, IL, USA): 1) Gingival Recession Depth (GRD in mm) measured as the distance from the CEJ to the Gingival Margin, 2) Gingival recession width (GRW in mm) measured at the CEJ 3), keratinised tissue width (KTW in mm), measured as the distance from the mucogingival junction (MGJ) to the gingival margin.

To avoid interference with wound healing, the following clinical parameters were only registered at baseline, 6 and 12 months postoperatively: 4) Gingival thickness (GT in mm) measured 3 mm apically from the free gingival margin at the mid buccal aspect of the tooth, 5) pocket probing depth (PPD in mm) at the distobuccal, midbuccal, mesiobuccal aspects of surgical sites, 6) clinical attachment level (CAL in mm). At surgery, the length of time of the full procedure was evaluated (in minutes).

Intra-examiner reproducibility: In both trials, the same calibrated investigator performed all clinical measurements using a standard periodontal probe (PCP-UNC 15, Hu-Friedy, Chicago, IL, USA). Five patients, not related to the study and each showing a pair of contralateral single-rooted teeth (with recession depth > 2 mm on the mid-buccal aspect) were used to calibrate the examiner. The examiner evaluated the patients on two occasions 24 hours apart. Calibration was accepted if 90% of the recordings could be reproduced within a difference of 1.0 mm (Pilloni et al. 2006).

6.3.2.6 Evaluation of patients' satisfaction

In the split mouth trial, at suture removal, both procedures were evaluated by the patient for discomfort, duration and difficulty on a visual analogue scale (VAS). At 12 months, the aesthetic outcome of both treatments was appreciated by the patient on a VAS scale.

6.3.2.7 Statistical analysis

a) For the evaluation of data obtained in the pilot case series, statistical analysis was performed using Instats 2000 (Version 3.05, GraphPad Software Inc., San Diego, CA, USA). The primary outcome variable was CRC. A subject level analysis was performed for each parameter. Mean values and standard deviations (mean ± SD) for the clinical variables were calculated for each treatment. The Kolmogorov and Smirnov test was used to confirm that the data were sampled from a Gaussian distribution. The significance of the difference within group before and after treatment was evaluated with the paired samples t-test. Differences were considered statistically significant when the p-value was <0.05. **b)** In the randomised controlled trial sample size calculation was performed based on root coverage outcomes. Using root coverage percentage as the primary outcome variable and assuming that the standard deviation (SD) of the differences in the paired measurements would not exceed 30%, the sample size for paired continuous data were calculated to be 18 subjects per group. This would provide 80% power to detect a true difference of 20% between test and control (Julious et al. 1999). To allow for possible dropouts, 22 patients were finally recruited. Our null hypothesis of the randomised controlled trial was: No statistically significant differences are observed with respect to the clinical parameters CRC, MRC, KTW and GT and patient-centred outcomes between the two treatment modalities (i.e. MCAT technique with CM or palatal SCTG). Statistical analysis was performed using Instats 2000 (Version 3.05, GraphPad Software Inc., San Diego, CA, USA). A patient-level analysis was performed for each parameter. Therefore, mean values and SD for the clinical variables were calculated for each patient per treatment. The method of Kolmogorov-Smirnov analysis was used to confirm that the data were sampled from a Gaussian distribution. The Kolmogorov–Smirnov test of baseline data showed a homogeneous distribution of the data as parameters' values passed the normality test with p>0.05. Accordingly, the significance of the difference within each group and between groups before and after treatment was evaluated with the paired samples t-test regarding all numerical data (GRD, GRW, KTW, GT, PD, CAL, %root coverage, VAS scale). Finally, Fisher's exact test was applied for categorical data (frequency of CRC). Differences were considered statistically significant when the p-value was <0.05.

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7. RESULTS

7.1 Literature review on the application of enamel matrix proteins in periodontal regenerative therapy

Based on the presented evidences the following conclusions could have been drawn:

a) Surgical periodontal treatment of deep intrabony defects with EMD promotes periodontal regeneration. The application of EMD in the context of non-surgical periodontal therapy has failed to result in periodontal regeneration.

b) Surgical periodontal therapy of deep intrabony defects with EMD may lead to significantly higher improvements of the clinical parameters than open flap debridement alone. The results obtained following treatment with EMD are comparable to those following treatment with GTR and can be maintained over a longer period.

c) Treatment of intrabony defects with a combination of EMD + GTR does not seem to additionally improve the results compared to treatment with EMD alone or GTR alone.

d) The combination of EMD and some types of bone grafts/bone substitutes may result in certain improvements in the soft and hard tissue parameters compared to treatment with EMD alone. However, further studies are needed in order to definitively clarify the possible advantage of a combination therapy of EMD and bone grafts/bone substitutes in relation to the single therapies.

e) Treatment of recession-type defects with coronally repositioned flaps and EMD may promote formation of cementum, periodontal ligament and bone and may significantly increase the width of the keratinized tissue. Application of EMD seems to provide better long-term results than coronally repositioned flaps alone.

f) Application of EMD may enhance periodontal regeneration in mandibular class II furcations. The clinical results are comparable to those obtained following GTR.

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7.2 Isolation and in vitro differentiation of periodontal ligament stem cells

7.2.1 Isolation and primary cultures

PDL derived cells from third molars attached to plastic surfaces of culturing dishes within a couple of hours, cell proliferation was observed under standardised conditions. As it has been described, osteogenic stem cells can be isolated from aspirates of bone marrow by their ability to adhere to a plastic surface, and with appropriate stimulation these cells start to proliferate. Under these circumstances, each colony originates from a single progenitor cell and displays a wide variation in cell morphology and growth potential.

We were able to show the ability of both periodontal ligament-derived and pulp-derived cells to form adherent clonogenic cell clusters of fibroblast-like cells, similar to those recorded for other mesenchymal stem-cell populations. These colony-forming cell populations, which we termed PDLSCs and DPSCs had high proliferation rate, as demonstrated by the doubling of the cell number during culture in about two days. A mean of 25-30 colony forming units were detectable within 10^5 cells, this number is well in line with data in literature referring to mesenchymal stem cells. For this reason, cells were passaged once a week until they reached confluence. Cells of primary single cell suspensions and previously attached cells treated with trypsin were rounded. Both PDLSC (Fig. 5) and DPSC cultures showed typical fibroblast-like morphology and high clonogenic activity similar to the progeny of human bone marrow colony forming units. Monolayers were usually formed 2 weeks following tissue preparation and isolation (Fig 3.), at the time point of reaching confluence, proliferation rate of cells in monolayers decreased due to contact inhibition. We managed to maintain isolated PDL cells within monolayer cultures, in several cases well over 20 passages.

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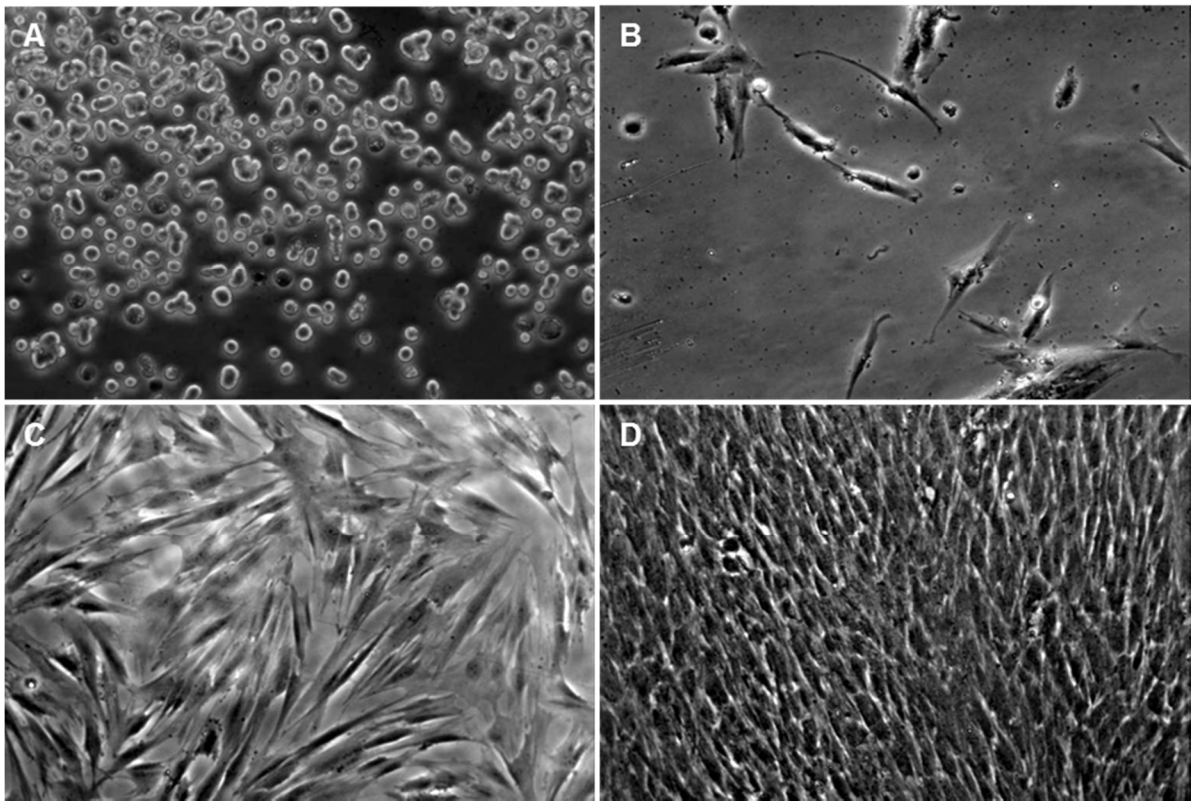

Fig. 5 Morphology of Periodontal Ligament Stem Cells (PDLSCs) following the second passage. (A) Day 1, immediately after trypsin-EDTA passage, single cell suspension (B) 3 hours later, adhering cells (C) Day 3, subconfluent culture (D) Day 5, cells have reached full confluence /200x magnification/

7.2.2 Cell viability studies and treatment with enamel matrix derivative

As mentioned above, both DPSC and PDLSC containing primary cultures of pulp cells were growing continuously and had to be passaged frequently because of the fast doubling rate. To test the importance of serum in the test medium, cells were serum-starved for 24 h and then either received 20% FBS or were left in serum-free a-MEM medium. To validate our assay system, as an internal control, we plated only half of the cells into some wells. Our data revealed that FBS stimulated cell proliferation compared to serum-free controls. When 50% less cells were initially plated, MTT assay also showed about 40% less optical density than in control after 24 h incubation. DPSC cells showed a 209%±9% proliferation rate in 20% FBS supplemented medium relative to the control without FBS. PDLSC cells grown in medium with 15% FBS had a proliferation rate of 142%±8% compared to control.

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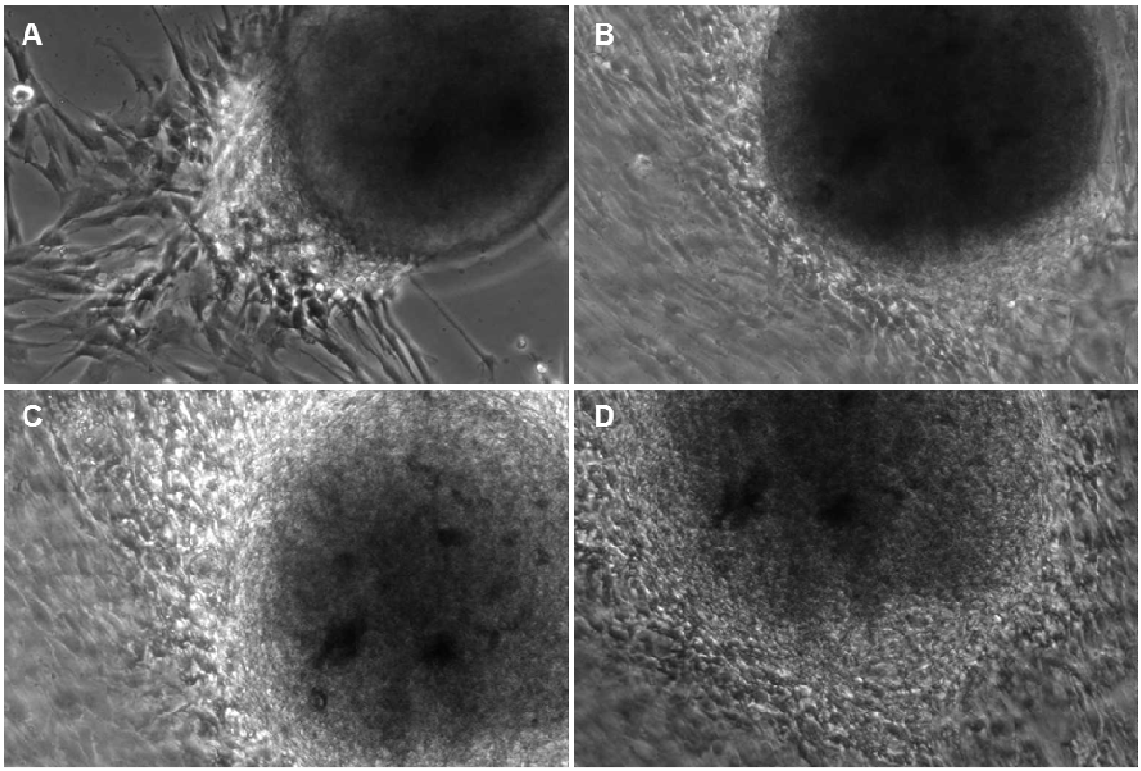

Fig 6. Periodontal Ligament Stem Cells (PDLSCs) following the second passage on an Enamel Matrix Derivative (EMD) treated surface. (A) Day 2, EMD initiates migration of cells (B) Day 5, a group of cells formed around an EMD droplet (C) (D) Day 6-7 cell proliferation is continued /200x magnification/

According to our results, effect of EMD compared to serum starving at the concentration of 25, 50, 100, 200, 400 μg/ml was not linear. EMD yielded a significantly proliferative effect at concentrations of 200 and 400 μg/ml on the viability of PDL derived primary cultures (Fig. 7). Migration of PDL cells was observed following the treatment of plastic culturing surfaces with EMD towards EMD droplets (Fig. 6). This was confirmed via phase contrast light microscopy. PDL cells with remote localization seemed to connect with these droplets via cytoplasmic pedicles (Fig. 6).

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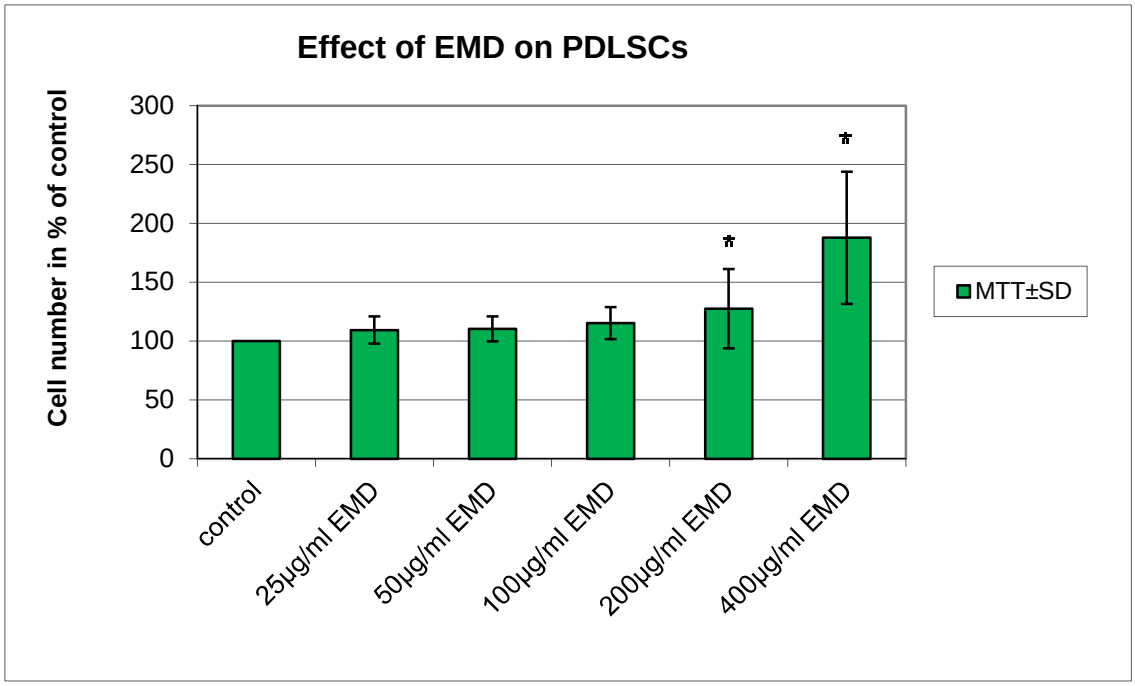

Fig 7. Effect of Enamel Matrix Derivative (EMD) on Periodontal Ligament Stem Cells (PDLSCs), MTT analysis. The enamel matrix derivative concentrate had a significantly positive effect on cell viability in 200 and 400 µg/ml concentrations $p<0,05$.

7.2.3 Immunocytochemistry and FACS analysis

A fraction of the cells in DPSC (Fig. 8) and PDLSC cultures expressed the cell surface molecule STRO-1, a mesenchymal stem cell marker, which is also present in bone-marrow derived and periodontal ligament derived stem cell cultures. STRO-1 immuno-reactivity gradually decreased with increasing passage numbers, but 8,47% of the cells were still STRO-1 positive even at higher passage numbers (Fig. 8.). CD34, a characteristic marker of haemopoetic stem cells showed a prevalence of 21%, while c-kit embryonic stem cell marker positivity was detected in 18% of the cells in PDL cultures. Nevertheless, according to our representative findings these numbers decreased gradually, similar to STRO-1 positivity over time following several passages.

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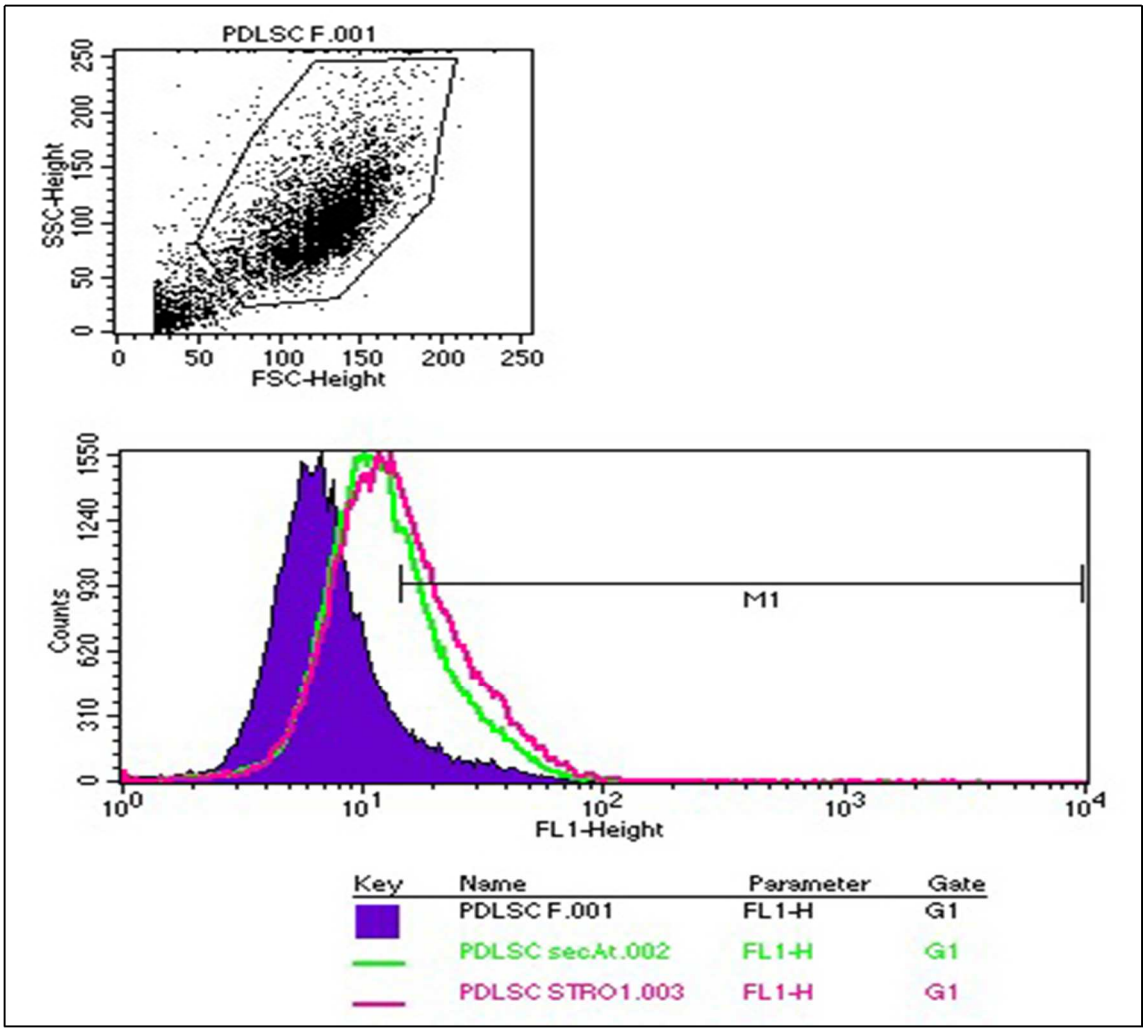

Fig 8. 8,47 % of periodontal ligament (PDL) cultures showing STRO-1 immuno-positivity following the second passage

7.2.4 Osteogenic differentiation

In these experiments DPSC and PDLSC (Fig. 9) cultures were grown in the presence of osteogenic differentiation cocktail consisting of dexamethazone, L-ascorbic acid 2-phosphate and b-glycerophosphate. Under these conditions cultures uniformly demonstrated the capacity to form Alizarin red S positive condensed nodules with high calcium content. The deposits were sparsely scattered throughout the adherent layer as single mineralized zones. Control cultures formed adherent layers without any sign of calcium deposition. Thus, this observation confirms the previous findings that DPSCs and PDLSCs are capable of differentiation to mineralized tissue in vitro in response to appropriate pharmacological stimulation.

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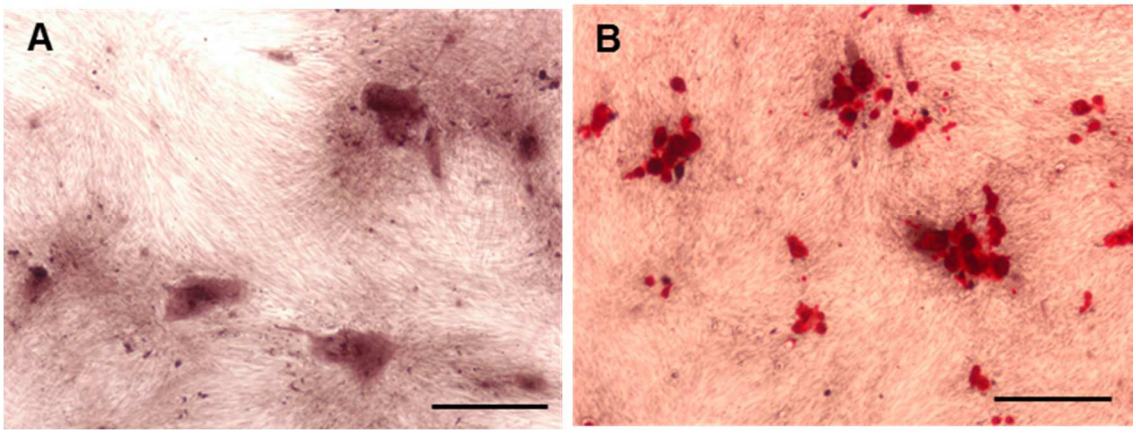

Fig. 9 (A) Dental Pulp Stem Cells (DPSCs) produced mineralized deposits under osteogenic conditions as detected by 2% Alizarin Red S staining. (B) Control staining of non-induced DPSCs. The length of scale bars indicate 100 μm.

7.2.5 Neuronal differentiation

7.2.5.1 Partial differentiation induced by Protocols 1 and 2

When PDLSC and DPSC cells were treated using Protocol 1 with a mixture containing substances such as TPA, IBMX and forskolin, the morphology of cells observed by phase contrast microscopy changed rapidly (Fig. 10, data shown for DPSC). After 4 hours of treatment initiation, the cytoplasm of the cells retracted toward the nucleus in many cells, taking a more spherical shape and extending processes. The percentage of cells with modified morphology decreased within 24 hours. When differentiation factors were removed from the medium, cell morphology reverted to the original. Following the application of Protocol 2 (bFGF, KCl, forskolin, DMSO and BHA) neuronal morphology changes were temporary, followed by an irreversible distraction of the culture: after 48 hours, the majority of cells were seen to have a rounded morphology followed by the death of almost the entire culture, except for a few spindle shaped cells expressing none of the neuronal markers investigated by us (Fig. 10, data shown for DPSC). These morphological changes were very similar for DPSC and PDLSC after both treatments.

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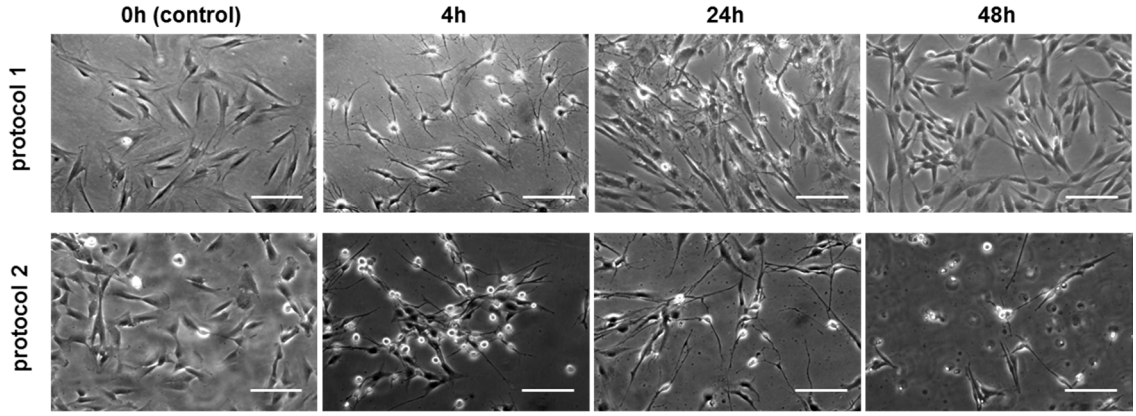

Fig. 10 Morphological changes during neuronal differentiation to protocols 1, 2 in Dental Pulp Stem Cells (DPSCs). (Protocol 1) Control DPSCs had spindle-shaped morphology on poly-L-lysine coated plastic surface. After culturing for 4 hours with Protocol 1, cytoplasm was retracted towards the nucleus, resulting more rounded cell body with long, extending processes. The ratio of cells with modified morphology decreased by 24 hours. When differentiation factors were removed from medium, cell morphology reverted to the original shape (48 hours). (Protocol 2) Control DPSCs had spindle-shaped morphology. After treatment for 4 hours with Protocol 2, cells showed similarly to protocol 1 rounded cell body with long, extending processes. However, the ratio of cells with modified morphology has not changed by 24 hours. Extensive cell death were observed by 48 hours, the surviving cells does not show neuronal morphology.

7.2.5.2 Robust neuronal differentiation induced by Protocol 3

While Protocol 1 and Protocol 2 resulted in short term and reversible neuronal differentiation or even death of the cells after 48 h, Protocol 3, our three step differentiation procedure over 9 days resulted in a robust differentiation of both pulp cultures and periodontal cells towards neural lineages in essentially all surviving cells that initially showed the characteristics of dental fibroblasts. The originally fibroblast-like PDLSC and DPSC cells (Fig. 11, data shown for DPSC) become more rounded after pre-treatment with 5-azacytidine and bFGF for 48 h (epigenetic reprogramming). As early as after 2 h of treatment with the inducing mixture, PDLSCs and DPSCs grew processes and started moving towards the high cell-density areas. During the 3 days of induction, cells anchored their position in the network structure, the previously developed processes disappeared, and their morphology reverted to flat and round cell shapes observed during earlier stages of differentiation. In the final maturation step, cells diverging radially from the centres began to grow neurite-like processes. After 9 days of differentiation, the vast majority of cells, derived from either the dental pulp or the periodontal ligament,

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displayed complex neuronal morphology, expressing both bipolar and stellate forms. However, a small portion of cells retained their flat shape, and were attached beneath the processes of the neuronal cells. These elements are presumably committed towards glial fates, or serve as stanchions for the developing neuronal cells. Therefore, they might be indispensable for neuronal survival.

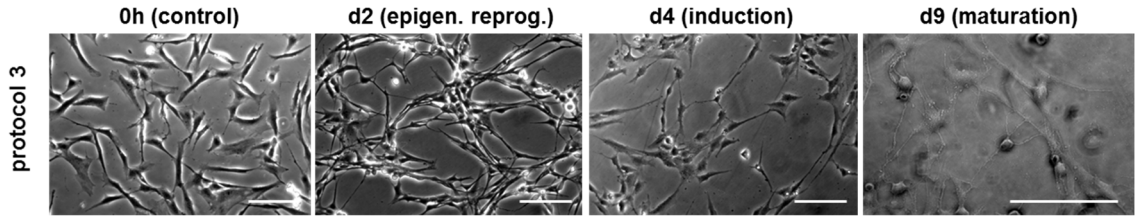

Fig. 11 Morphological changes during neuronal differentiation to protocol 3 in Dental Pulp Stem Cells (DPSCs). (Protocol 3) Control DPSCs had spindle-shaped morphology on poly-L-lysine coated plastic surface. After 2 days of epigenetic reprogramming, cells formed clusters. After 4 days (2 days of induction), cells had morphological features typical of neurons, showing complex neuronal processes. After 9 days (4 days of maturation), most of the cells displayed either multi- or bipolar forms. The length of scale bars indicate 100 μm

The time-dependent changes of neuronal marker gene expression in DPSC and PDLSC cultures undergoing neural development were evaluated by real time PCR (Fig. 12). Total RNA was harvested at four different time points during this period: at time 0 (non-induced control DPSCs and PDLSCs), on the first day of induction (d3), on the third day of induction (d5), and after three days of maturation (d8). The expression of each target gene was normalized to that of the RPLP0 housekeeping gene, and expressed as fold change relative to the non-induced sample.

DPSCs and PDLSCs showed a very similar expression pattern during neuronal differentiation treatment. There was a sharp decrease in the expression of the mesenchymal marker vimentin in response to neurogenic induction. This very striking decrease became less pronounced during maturation. The expression of the neuronal marker NSE progressively increased under maturing conditions and remained increased during maturation. The protein expression pattern investigated by immunocytochemistry corresponded well to the gene expression data.

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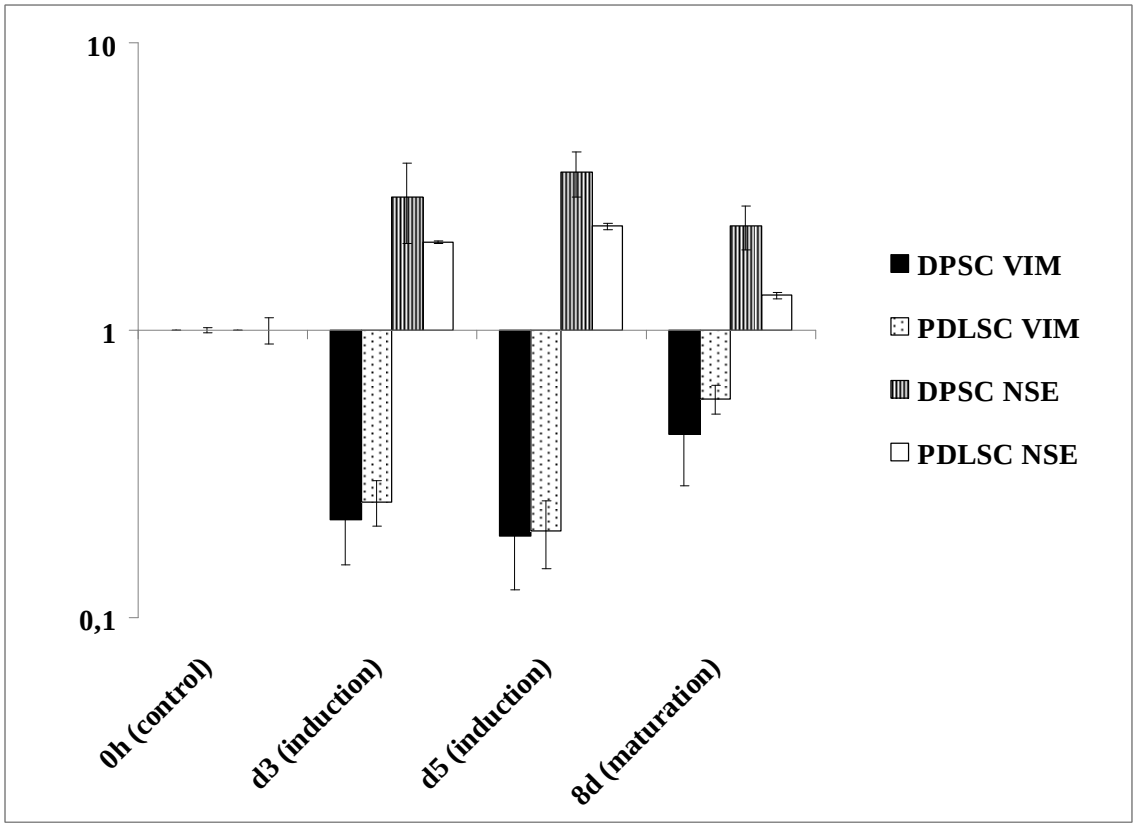

Fig. 12 mRNA expression pattern of mesenchymal, neuronal markers in Dental Pulp Stem Cells (DPSCs) and Periodontal Ligament Stem Cells (PDLSCs) during neuronal differentiation with protocol 3. Total RNA of DPSCs isolated from five independent normal human donors was used. RNA was harvested at four time points during the differentiation of each sample: from non-induced DPSCs (0 day), after the first day of induction (3rd day), on the third day of induction (5th day) and on the third day of maturation (8th day). Target gene expressions were normalized to RPLP0 housekeeping gene expression levels and evaluated as fold change relative to the non-induced sample. mRNA expression of the mesenchymal vimentin appeared to be significantly suppressed by the treatment, while the mRNA expression of the neuron specific glycolytic enolase NSE was significantly elevated by the by the treatment. DPSCs and PDLSCs showed similar gene expression pattern. Data are reported as mean ± S.E.M.

Non-induced DPSCs and PDLSCs (Fig. 12) showed weak, nonspecific expression pattern of NFM. The neuronal induction resulted in an increased proportion of cells with an expression pattern specific to mature neurons in both DPSC and PDLSC (Fig. 12) cultures. After 8 days cells also showed positive staining for NFM and NSE (Fig. 12), markers typically expressed by mature neurons.

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7.3 Healing following rhGDF-5/β-TCP treatment

7.3.1 Patient demographics and baseline defect distribution

Twenty-one patients were screened, 20 patients fulfilling the inclusion criteria were randomized into two groups of ten patients each (Fig. 1). One female patient was not enrolled due to a low FSH level. Sixteen patients were male and four, female. One female patient was randomized to the control group and three to the rhGDF-5/β-TCP group. All four female patients were postmenopausal. Patient mean age was 48.8±10.6 years (range 33–74 years). Age did not differ considerably between treatment groups; median age was 50.0 years for both groups. No dropouts occurred; all randomized patients completed the study. The depth of the intrabony component measured 6.7±2.8 mm in the test and 6.4±2.1 mm in the control group, respectively (Tables 2 and 3).

Full mouth plaque scores at Visit 2 averaged 7.3±6.7% for the rhGDF-5/β-TCP and 8.9±4.8% for the control group; and full mouth bleeding scores 11.2±4.1% for the rhGDF-5/β-TCP and 12.7±5.4% for the control group. Probing depths averaged 9.1±1.3 mm for the rhGDF-5/β-TCP and 9.2±2.3 mm for the control group. Clinical attachment level averaged 12.2±1.5 mm for the rhGDF-5/β-TCP and 11.4± 3.5 mm for the control group. The depth of the intrabony component as measured during surgery measured 6.7±2.8 mm for the rhGDF-5/β- TCP and 6.4±2.1 mm for the control group. The defects displayed predominantly one- and two-wall configurations (Tables 2 and 3).

7.3.2 Clinical assessments

Healing following surgeries progressed without major complications. Primary intention healing was observed in nine of ten patients in the rhGDF-5/β-TCP group, and in all ten patients in the control group. One patient in the rhGDF-5/β-TCP group experienced a slightly delayed epithelialization of the interdental papilla resulting in complete wound closure within 3 weeks. At 6 months, both treatments resulted in significant improvements in terms of PD reduction and CAL gain compared to baseline. Treatment with rhGDF-5/β-TCP resulted in higher, but statistically not significant, PD reduction (3.7±1.2 vs. 3.1±1.8 mm; p=0.26) and CAL gain (3.2±1.7 vs. 1.7± 2.2 mm; p=0.14) compared to the control (Fig. 13.) (Tables 2 and 3).

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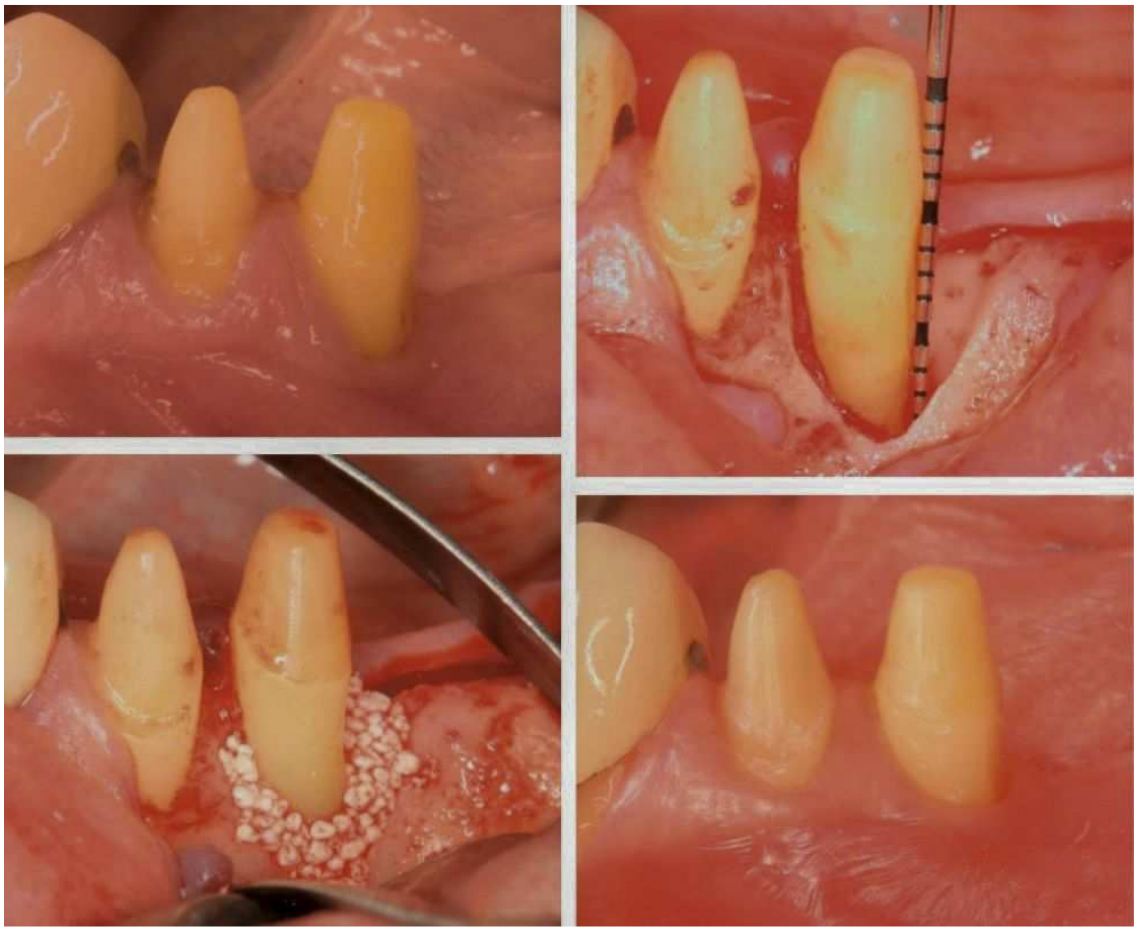

Fig. 13 Flap surgery plus rhGDF-6/ß-TCP: Presurgery (top left); intrasurgery defect morphology (top right); site following implantation of rhGDF-6/ß-TCP (bottom left); and clinical view at the biopsy event 24 weeks postsurgery (bottom right). Histological outcomes were published elsewhere (Stavropoulos et al. 2011).

Table 2 Patients, experimental teeth and surfaces, predominant defect morphology, intrasurgery defect depth, initial pocket probing depth (PD), clinical attachment level (CAL) and gingival recession (GR), and changes in primary outcome variables (mm) for defect sites receiving flap surgery combined with rhGDF-5/ß-TCP.

Patient	Tooth	Defect Morphology	Defect Depth	PD	CAL	GR	ΔPD	ΔCAL	ΔGR
2	35d	1-wall	7	8	12	4	5	5	0
3	36d	2-wall	5	9	13	4	4	4	0
6	11m	2-wall	4	9	12	3	3	4	1
10	34d	circumferential	5	9	11	2	4	3	-1
13	23p	1-wall	11	11	15	4	6	6	0
14	23d	2-wall	11	11	12	1	4	3	-1
16	21m	1-wall	7	10	12	2	3	2	-1
17	21m	1-wall	4	7	10	3	2	0	-2
18	12d	1-wall	4	8	11	3	3	2	-1
19	45d	circumferential	9	9	14	5	3	3	0
Mean±SD			6.7 ± 2.8	9.1 ± 1.3	12.2 ± 1.5	3.1 ± 1.2	3.7 ± 1.2	3.2 ±1.7	0.5 ± 0.8

Table 3 Patients, experimental teeth and surfaces, predominant defect morphology, intrasurgery defect depth, initial pocket probing depth (PD), clinical attachment level (CAL) and gingival recession (GR), and changes in primary outcome variables (mm) for defect sites receiving flap surgery alone (control).

Patient	Tooth	Defect Morphology	Defect Depth	PD	CAL	GR	ΔPD	ΔCAL	ΔGR
1	21m	circumferential	4	9	10	1	2	1	-1
4	12m	2-wall	7	9	11	2	4	4	0
7	11m	1-wall	7	12	13	1	6	4	-2
8	23m	1-wall	5	8	9	1	3	1	-2
9	22m	1-wall	5	8	10	2	3	0	-3
11	35d	2-wall	5	7	8	1	1	-1	-2
12	44d	2-wall	9	13	17	4	6	5	-1
15	34d	1-wall	9	8	15	7	3	3	0
20	35d	circumferential	9	12	15	3	2	1	-1
21	11m	1-wall	4	6	6	0	1	-1	-2
Mean±SD			6.4 ± 2.1	9.2 ± 2.3	11.4 ± 3.5	2.2 ± 2.0	3.1 ± 1.8	1.7 ± 2.2	1.4 ± 1.0

7.3.3 Safety findings

The safety profile was comparable for patients receiving rhGDF-5/β-TCP and control treatments. All abnormal haematology and clinical chemistry values were judged as clinically non-relevant. Most common abnormal values were GGT, followed by cholesterol, glucose, and white blood cell count. Summary statistics did not show remarkable trends from Visits 1 to 9 for any of the parameters analysed. All patients showed negative anti-rhGDF-5 antibody levels at screening and Visits 3 and 9. No relevant rhGDF-5 plasma levels were detected in any patient. Two patients, both belonging to the rhGDF-5/β-TCP group showed rhGDF-5 plasma levels at screening (Visit 1) and postsurgery (Visits 3 and 9). One patient showed a plasma level of 46.7 pg/mL rhGDF-5 at baseline (before implantation of rhGDF-5/β-TCP) vs. 47.3 pg/mL at Visit 3, and 57.9 pg/mL at Visit 9.The other patient showed a plasma level of 514 pg/mL rhGDF-5 at baseline vs. 427 pg/mL at Visit 3, and 690 pg/mL at Visit 9. Due to the fact that the measured rhGDF-5 plasma levels were already positive at the baseline (before periodontal surgery) and did not increase throughout the study, a causal relationship to study medication can be excluded. For all other samples, rhGDF-5 plasma levels were below the limit of quantification (<40.0 pg/mL). It may, thus, be anticipated that these results were false positive.

7.3.4 Adverse events

All adverse events were judged as mild or moderate. Seventy-two events were reported; 54 in seven patients from the rhGDF-5/β-TCP group (42 of which for one single patient), and 18 in eight patients from the control. As these were always single isolated events, one adverse event was recorded for each episode. Most events were related to back pain (14 events) and headache (26 events). The following two events, in two different patients, were judged to be possibly related to the regenerative surgery but not necessarily related to the rhGDF-5: delayed epithelialization and secondary healing of the interdental papilla. Both were judged as mild and the patients recovered without sequel. No withdrawals due to an adverse event were recorded. There were also no meaningful changes in vital signs that would raise a safety concern. Healing following surgeries progressed without major complications. Primary intention healing was observed in nine of ten patients in the rhGDF-5/β-TCP group, and in all ten patients in the control group. One patient in the rhGDF-5/β-TCP group experienced a slightly delayed epithelialization of the interdental papilla resulting in complete wound closure within 3 weeks.

7.3.5 Protocol deviations

A few patient visits occurred out of schedule. Blood collection Visit 9 was completed 1–5 days early in five patients. Two patients showed increased liver enzyme values at screening (ALT=123 U/L, AST=107 U/L, GGT=142 U/L and ALT=101 U/L, GGT=382U/L) that decreased to normal range within 2weeks (Visit 3) except GGT. No chronic liver disorder was reported for either patient. One patient had insulin independent diabetes (Hb1Ac=10.3% at screening), which was medically controlled. None of these protocol deviations was considered as major violation, and therefore, all patients were included in the analyses.

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7.4 Healing following treatment with Mucograft®

7.4.1 Patient demographics and baseline defect distribution

7.4.1.1 Pilot study

Five patients were treated with in the maxilla, three patients in the mandible. Five patients presented recessions involving only anterior teeth (four in the maxilla and one in the mandible). One patient presented a recession site on a bicuspid in the maxilla, two patients in the mandible. Recession sites of two mandibular molars were treated in one of the cases. The baseline values and the 12 months results are shown in Table 4.

7.4.1.2 Split mouth randomised controlled study

A total of 156 recessions were treated in 22 patients (i.e. 78 received SCTG and 78 CM). Location and distribution of the treated defects is depicted in Table 2. Thirteen patients had maxillary and nine mandibular recessions. Upper molars were treated in eight patients while lower molars were included in two patients. None of the treated molars displayed a furcation involvement. No statistically significant differences ($p>0.05$) were observed within and between groups for FMPS values between baseline and 12 months measurements.

7.4.2 Clinical assessments

7.4.2.1 Pilot study

The postoperative healing was uneventful in all 8 cases. No complications such as allergic reactions, matrix exfoliations, abscesses or infections were observed throughout the entire study period. All patients completed the study and no patient was lost during follow-up. All patients expressed improvement in root sensitivity. At 12 months CRC was obtained in 2 out of the 8 patients and in 30 out of the 42 recessions (71%). MRC was 84%. Mean GRD, GRW, GT and KTW improved statistically highly significantly

($p<0.0001$) compared to baseline while PD did not show statistically significant differences (Table 4).

Table 4 Mean ± standard deviation (SD) at baseline and at 12 months of all evaluated parameters. GRD: gingival recession depth, GRW: gingival recession width, KTW: keratinized tissue width, GT: gingival thickness, PD: probing depth, MRC: mean root coverage, CRC: complete root coverage. $p<0.05$ indicates statistically significant differences *

Parameters	Baseline	12 months post op	P value
GRD (mm)	2.0 ± 0.5	0.3 ± 0.3	0.0001 *
GRW (mm)	3.4 ± 0.8	1.0 ± 1.3	0.0001 *
KTW (mm)	2.9 ± 1.3	3.4 ± 1.3	0.0006 *
GT (mm)	1.0 ± 0.3	1.3 ± 0.4	0.0051 *
PD (mm)	1.5 ± 0.1	1.4 ± 0.1	0.0692

MRC (%) patient level	84% ± 15
CRC (%) patient level	2/8 (25%)
CRC (%) tooth level	30/42 (71%)

7.4.2.2 Split mouth randomised controlled study

All patients completed the study and attended all recall visits. Exposure of the CM was not observed in any of the cases. No adverse events related to both treatment modalities were recorded. At 12 months, KGW increased on average from 2.1 ± 0.9mm to 2.4 ± 0.7 mm on test sites and from 2.0 ± 0.7 mm to 2.7 ± 0.8 mm on control sites. The difference between the two treatments was not statistically significant (Table 5). At 12 months, there was no difference in the mean value of PD on test sides compared to control sides (Table 5). Both treatment groups showed significant post-surgical improvement in GRD and clinical attachment (CAL) gain, when compared to baseline (Table 5) (Fig. 14, Fig. 15). In the test group, mean GRD decreased significantly from 1.9 ± 0.6 mm at baseline to 0.6 ± 0.5 mm at 12 months while in the control group the corresponding values were 1.8 ± 0.5 mm and 0.2 ± 0.3 mm, respectively (Table 5). Both treatments resulted in statistically significant CAL gain (1.9 ± 0.6mm mm and 1.4 ± 0.4 mm for test and control groups, respectively) (Table 5).

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Table 5 Mean ± standard deviation (SD) at baseline and at 12 months of all evaluated parameters. GRD: gingival recession depth, GRW: gingival recession width, KTW: keratinised tissue width, GT: gingival thickness, PD: probing depth, CAL: clinical attachment level. $p<0.05$ indicates statistically significant differences *

	Baseline (mm)	12 months (mm)	p-value
	Mean ± SD	Mean ± SD	Baseline vs. 12 months
GRD Test	1.9 ± 0.6	0.6 ± 0.5	0.0001 *
GRD Control	1.8 ± 0.5	0.2 ± 0.3	0.0001 *
p-value	0.7565	0.0009 *	-
GRW Test	3.8 ± 0.8	1.4 ± 1.2	0.0001 *
GRW Control	3.8 ± 0.9	0.5 ± 1.0	0.0001 *
p-value	0.9195	0.0002 *	-
KTW Test	2.1 ± 0.9	2.4 ± 0.7	0.0309 *
KTW Control	2.0 ± 0.7	2.7 ± 0.8	0.0001 *
p-value	0.5802	0.0796	-
GT Test	0.8 ± 0.2	1.0 ± 0.3	0.0019 *
GT Control	0.8 ± 0.3	1.3 ± 0.4	0.0001 *
p-value	0.6591	0.0001 *	-
PD distal Test	1.8 ± 0.3	1.8 ± 0.2	0.7334 *
PD distal Control	1.8 ± 0.4	1.8 ± 0.2	0.5203 *
p-value	0.2732	0.7344	-
PD midline Test	1.4 ± 0.3	1.4 ± 0.2	0.3744 *
PD midline Control	1.3 ± 0.2	1.3 ± 0.3	0.9453 *
p-value	0.1388	0.3652	-
PD mesial Test	1.9 ± 0.3	1.8 ± 0.2	0.0572 *
PD mesial Control	1.8 ± 0.3	1.8 ± 0.2	0.4223 *
p-value	0.1298	0.5151	-
CAL Test	3.2 ± 0.6	1.9 ± 0.6	0.0001 *
CAL Control	3.1 ± 0.5	1.4 ± 0.4	0.0001 *
p-value	0.3198	0.005 *	-

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When results were expressed as percentage of root coverage at 1 year, both treatment modalities resulted in a statistically significant percentage of root coverage amounting to 71% ± 21 % in the test group and 90% ±18 % in the control group, respectively. The difference between the two groups was statistically significantly greater for the control treatment (p= 0.0004) (Table 6). At 12 months, CRC was recorded in 5 patients in the test and in 13 patients in the control group, respectively and was statistically significantly greater for the control treatment (p= 0.0305) (Table 6). In both groups GRW decreased statistically significantly between baseline and 6 months and 12 months. The differences between the two groups were not statistically significant (p>0.05) (Fig. 14, Fig. 15) (Table 6). Mean surgery time was significantly lower (p <0.0001) in the test (i.e. 42.5 ± 4.8 min) compared with control (i.e. 58.6 ± 6.6 min) (Table 7). Postoperative complaints on the VAS scale were lower for CM. All patients reported a decrease in root sensitivity. The number of 100% satisfaction was higher in the test group compared with the control one, but was not statistically significant (p>0.05).

Table 6 Complete- and percentage (%) of root coverage at 12 months. CRC: complete root coverage. p<0.05 indicates statistically significant differences *

	12 months (mm)
CRC Test	5/22
CRC Control	13/22
p-value	0.0305 *
% root coverage Test	71 ± 21 %
% root coverage Control	90 ± 18 %
p-value	0.0004 *

Table 7. Duration of surgery, patients' complaints and satisfaction at 12 months. VAS: visual analogue scale p<0.05 indicates statistically significant differences *

	Duration of surgery (min)	Patient complaints (VAS)	Patient satisfaction (VAS)
Test	42.5 ± 4.8	7.3 ± 3.4	92.9 ± 8.4
Control	58.6 ± 6.6	12.8 ± 7.5	90.6 ± 7.9
p-value	0.0001 *	0.0020 *	0.203

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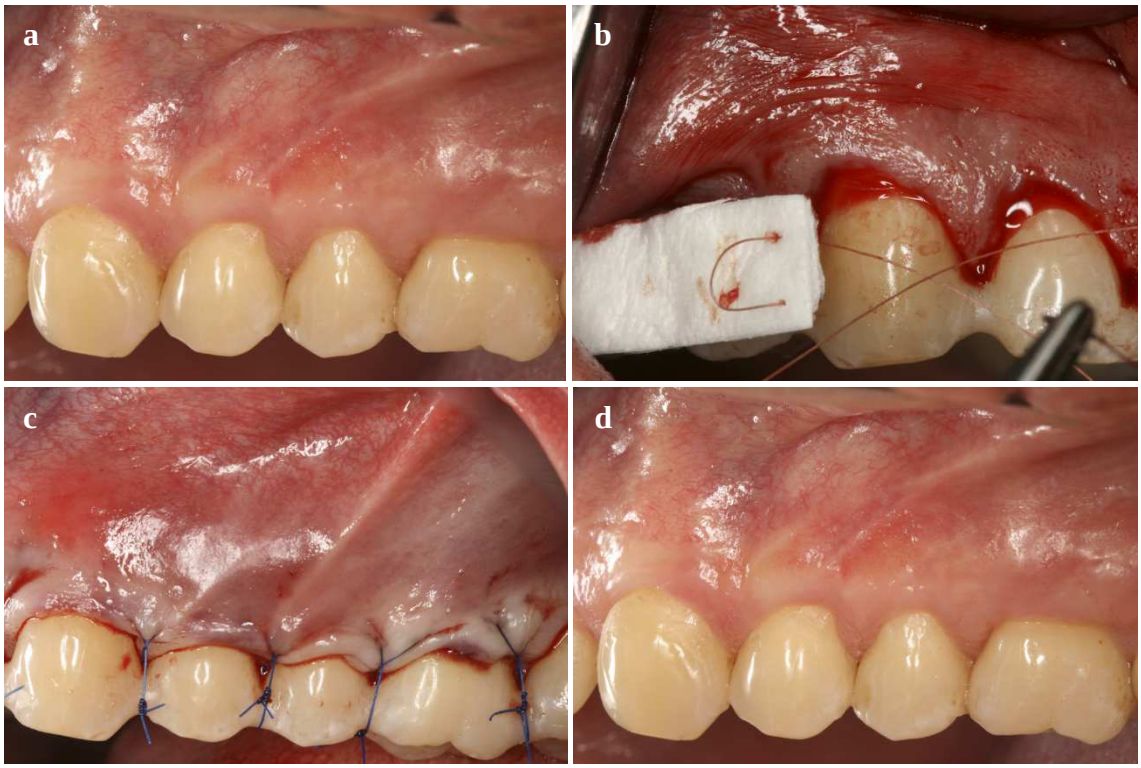

Fig. 14 Clinical results – Mucograft ® (a) Test side prior to treatment (b) intraoperative view (c) immediate postoperative view (d) twelve months outcome

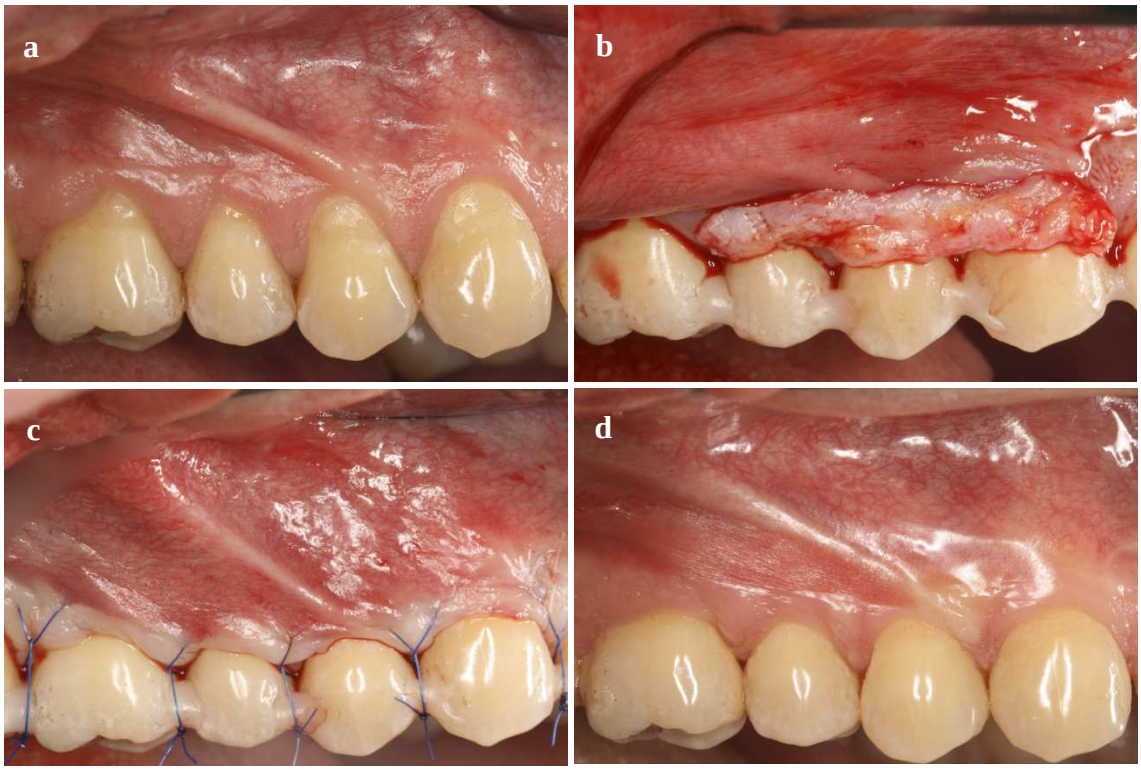

Fig. 15 Clinical results – SCTG: subepithelial connective tissue graft (a) Control side prior treatment (b) intraoperative view (c) immediate postoperative view (d) twelve months outcome

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8. DISCUSSION

The results of our literature review, in vitro and clinical studies allow us to discuss our findings with respect to the goals formulated in Chapter 2. The conclusions will be compared with results available in literature. Results from basic research have pointed to the important role of EMD in periodontal wound healing. Histological results from animal and human studies have shown that treatment with EMD promotes periodontal regeneration. Moreover, clinical studies have indicated that treatment with EMD positively influences periodontal wound healing in humans (World Workshop in periodontology 1996, Rincon et al. 2003, Donos et al. 2003).

Nevertheless, certain limitations experienced in cases with advanced hard- and soft tissue defects have raised the demand for safe reconstructive procedures, surpassing current techniques in terms of efficacy and predictability. Novel approaches should be ideally characterised by reduced duration of treatment and limited patient morbidity. Improved efficacy of periodontal regenerative therapy might be achieved by increased activation of cellular elements contributing to the re-establishment of hard- and soft tissues. Previous studies have suggested that human bone marrow and dental pulp as well as periodontal ligament tissue contain postnatal stem cells that are capable of differentiating into various cell types including osteoblasts, odontoblasts, cementoblasts, adipocytes (Gronthos et al. 2000, Miura et al. 2003, Seo et al. 2004). In accordance to our aims, we established, maintained and characterized cell cultures of human PDL. Isolated cells showed fibroblast morphology in monolayer cultures, we have obtained similar results to BMSC with regard to cell proliferation patterns (Bianco et Gahron 2000). The presence of clonogenic cells in the primary cultures was confirmed, the number of colony forming units was comparable to those data available from literature. These results confirm the in vivo evidence for PDL cells initiating periodontal regeneration. Using immunocytochemistry and FACS analysis, we identified PDLSCs, which are similar to other mesenchymal stem cells in that they are highly clonogenic and show expression of the STRO-1 mesenchymal progenitor cell marker. This expression could be detected in 8,47% of the whole cell population. We also confirmed the presence of CD34 and c-kit stem cell marker positivity within the primary cultures. Our results are well in line with those obtained by investigating DPSC cultures (Laino et al 2005). These findings might

allow for establishing more homogenous cell cultures, which contain PDLSCs in higher numbers thus allowing for establishing future in vitro or in vivo models of periodontal regeneration.

Our in vitro findings related to the use of EMD indicate that the observed beneficial clinical effects reported in literature might be related with promotion of cell proliferation and cell viability (Chong et al. 2006, Rodrigues et al. 2007). The well-known triggering effect of EMD in periodontal regeneration is supported by the phenomenon that PDL cells showed increased activity and positive chemotaxis related to EMD droplets in the cultures. Cell viability was assessed via MTT assay, we investigated the effect of culturing media containing 5, 10, or 15% FBS. We confirmed the presence of vital cells in a significantly higher number following FBS treatment compared to serum free conditions. MTT assay was used to confirm the significantly positive effect of EMD on vital cells within primary PDL cultures in the concentrations of 200- and 400 μg/ml compared to serum free cultures. This might indicate the importance of a certain minimal dosage necessary to achieve regenerative treatment outcomes during clinical application in specific tissue conditions.

The mechanisms controlling the development of teeth are largely unknown (Parner et al. 2002), in particular with respect to how craniofacial components including bone and soft tissues surrounding teeth, participate in the process of tooth development. DPSCs clearly have the ability to regenerate dentin, at least in experimental animals (Batouli et al. 2003, Iohara et al. 2004) and therefore have a high potential for tooth regeneration as odontoblast progenitors. However, based on current information, DPSCs and PDLSCs may have a broader capacity for differentiation than originally thought (Gronthos et al. 2002). Growing evidence suggests that they are able to differentiate into several different cell types (Gronthos et al. 2000, Gronthos et al. 2002, Miura et al. 2003, Seo et al. 2004). Our data emphasize that optimizing in vitro conditions may soon lead to successful serum-free culture of DPSCs and PDLSCs, which are potentially applicable for human transplantation. The osteogenic differentiation cocktail that we used induced similar and well reproducible mineralization in both DPSC and PDLSC cultures. Several recent studies reported that DPSCs and PDLSCs were also capable of osteogenic differentiation (Gronthos et al. 2002, d'Aquino et al. 2007). In our neurodifferentiation experiments, when we applied Protocol 1 both DPSC and PDLSC cells exhibited a

transient neurodifferentiation. Very similar observations were made recently. However, the expression change was transient, and the cells reverted to the original fibroblastic BMSCs within 48 hours (Scintu et al. 2006). When we used Protocol 2, based on another recently described method (Choi et al. 2006), again we observed only transient neurodifferentiation followed by the death of most of the cells. Our newly developed neurodifferentiation method, Protocol 3, is based on the elements of previously used methods in a special temporo-spatial arrangement. The first element aims at dedifferentiating the cells by the application of 5-azacytidine, a cytidine analogue where nitrogen replaces a carbon at the 5^{th} position of the pyrimidine ring. 5-azacytidine is reported to promote the maturation of neurons generated from neural or bone marrow derived stem cells (Schinstine and Iaconitti 1997, Kohyama et al. 2001). The second step of our new protocol was a robust, combined activation of the PKC and PKA pathways in order to activate pathways redirecting the cells to a neuronal fate. Human bone marrow stromal stem cells (BMSCs) were also shown to differentiate into neural progenitors in response to increased intracellular cAMP levels (Deng et al. 2001). The final step after the dedifferentiation and induction steps was the use of a mixture of conventional neuronal differentiation factors to promote neurodifferentiation. The importance of neurotrophin-3 (NT-3) and nerve growth factor (NGF) in the induction of advanced neuronal development has already been described (Tatard et al. 2007). Our three step differentiation procedure resulted in a robust differentiation of both DPSC and PDLSC cultures towards neural lineages. At the end of the differentiation, most cells displayed complex neuronal morphology showing both bipolar and multipolar forms. In both pulp and periodontal derived cultures morphological changes were accompanied by a similar increase in the expression of the neuronal marker NSE, and a sharp decrease in the expression of the mesenchymal marker vimentin. Our immunocytochemical observations corresponded well with the real time RNA expression data. These clearly suggest that both DPSC and PDLSC cultures are capable of massive cell differentiation at least as far as cell morphology, gene expression profile, and molecular marker expression is concerned.

Culturing of pluripotent tooth derived adult stem cells may lead to promising in vivo tissue engineering applications in the future in combination with proper carrier materials. Nevertheless, these techniques are not yet available for human use. This is due

to certain safety concerns and lack of information on possible treatment efficacy. On the other hand, application of recombinant growth- and differentiation factors have been verified to be safe and efficient in animal experiments, thus allowing pilot human trials. The presented pilot randomized, controlled study was the first to investigate the influence of GDF-5 on periodontal wound healing/regeneration in humans. An important finding was that no safety concerns related to rhGDF-5/β-TCP were encountered. None of the patients exhibited antiGDF-5 antibody levels while elevated GDF-5 plasma levels were observed in two patients receiving rhGDF-5/β-TCP. Overall laboratory evaluations indicated that the rhGDF-5 formulation appears safe. The observation that healing was uneventful in both groups also indicates that the rhGDF-5/β-TCP treatment was well tolerated and did not elicit any local adverse reactions. The clinical observations suggest that the rhGDF-5/β-TCP construct did not appear to exert any detrimental influence on periodontal wound healing/regeneration. The clinical evaluation has indicated that both open flap debridement (OFD) combined with rhGDF-5/β-TCP and OFD alone may result in statistically significant probing depth reductions and clinical attachment gains compared to baseline. Application of rhGDF-5/β-TCP however, resulted in greater, although statistically not significant probing depth reduction and clinical attachment gain compared to the control. These findings are in agreement with those reported in previous preclinical studies indicating a beneficial effect of GDF-5 on periodontal wound healing/regeneration (Kim et al. 2009, Lee et al. 2010, Kwon et al. 2010). Nevertheless, our results failed to demonstrate significant differences in terms of clinical improvements between test and control groups. The resorption rate of the β-TCP carrier material and the possibly impaired blood clot stabilisation might have contributed to these observations. The magnitude of clinical improvements appeared to be in the range of those obtained with other regenerative materials such as a recombinant platelet derived growth factor (rhPDGF BB) on a β-TCP carrier, an enamel matrix protein derivative alone or guided tissue regeneration either alone or combined with grafting materials (Pontoriero et al. 1999, Sculean et al. 2001, Tonetti et al. 2002, Sculean et al. 2003, Tonetti et al. 2004, Nevins et al. 2005, Kuru et al. 2006, Yilmaz et al. 2010). Furthermore, it is also important to note that the results observed in the control group compare favourably with previous studies evaluating treatment of intrabony defects using flap surgery alone. This indicates that substantial clinical improvements may be achieved with this treatment modality if an

optimal level of plaque control is maintained (Pontoriero et al. 1999, Sculean et al. 2001, Tonetti et al. 2002, Sculean et al. 2003, Tonetti et al. 2004). This might also indicate limitations related not only to applied biomaterials but surgical techniques, especially flap designs. On the other hand, it should be kept in mind that the present study was not only designed to evaluate the safety of rhGDF-5/β-TCP but also the histological outcomes (Stavropoulos et al. 2011). The histological evaluation has indicated that treatment with OFD+ rhGDF-5/β-TCP resulted in 2- to 3-fold higher amount of new bone and new cementum formation compared to OFD alone without differences in frequency of root resorption and ankylosis between the two groups (Stavropoulos et al. 2011). The amount of residual β-TCP carrier juxtaposed to the root surface in the present group of biopsies was generally small (mean 8.4%), suggesting that this carrier would completely degrade and/or resorb within a relatively short interval (LeGeros RZ 1993).

As far as safety is concerned, xenogenic materials present an even more established alternative for tissue reconstruction compared to growth- and differentiation factors. A novel xenogenic CM (Mucograft®) was recently proposed for the correction of periodontal soft tissue anomalies (Vignoletti et al. 2011). The application of these biomaterials has gained particular significance since novel minimally invasive surgical techniques were introduced for the treatment of MAGR (MCAT, Azzi, Etienne 1998). This technique allows for simultaneous root coverage of MAGR, nevertheless the extent of donor areas for connective tissue grafting is usually limited. Predictable coverage of MAGR represents a challenge for the clinician and the data on the literature are still limited. Until now, the most predictable in terms of CRC and MRC were reported following the use of either MCAF or MCAT combined with, SCTG. These techniques appear to yield the most predictable outcomes on both short (6 months to 1 year) and long-term (up to 5 years) basis (Hofmänner et al. 2012). Since the main drawback in this approach is related to SCTG harvesting which increases patient morbidity, postoperative complication rate and surgical time, it is logical that various attempts have been made to develop new soft tissue replacement materials.

The presented prospective case series was the first to evaluate the possibility to use the newly developed CM in the treatment of Miller Class I and II MAGR in combination with the MCAT technique. The used CM was excellently tolerated as confirmed by other authors (Vignoletti et al. 2011, McGuire and Scheyer 2010,

Cardaropoli et al. 2012). The safety and efficacy of the CM in the treatment of single recessions in conjunction with CAF was evaluated histologically in minipigs (Vignoletti et al. 2011). The results have shown that both techniques resulted in similar histological and similar clinical outcomes. In a randomised, controlled, split mouth-study McGuire and Scheyer (McGuire and Scheyer 2010) have treated single Miller Class I and -II recessions with CAF + CM (test) or CAF + SCTG (control). At 12 months, the percentage of root coverage averaged 88.5% in the test group and 99.3% in the control group, respectively. Both treatments resulted in comparable gains of keratinized tissue width, while there were no statistically significant differences between subject-reported values for aesthetic satisfaction, and subject`s assessments of pain and discomfort. Comparable outcomes were also very recently reported in another study (Cardaropoli et al. 2012). The results reported in the two aforementioned studies are in line with those from the present investigation where at 12 months following surgery, CRC was obtained in 2 out of the 8 patients (i.e. in 71% of the total number of recessions) while MRC amounted to 84%. From a clinicians point of view the present results are even more valuable when one considers that the present patient population comprised not only anterior teeth located in the maxillary arch, but also mandibular teeth and molars. This is an important aspect to be considered since data on treatment of mandibular MAGR are extremely scarce (Hofmänner et al. 2012). Moreover, the clinical relevance of using CM in the treatment of MAGR is further substantiated by the fact that in all 8 patients a statistically significant increase in KTT and KTW was observed.

The subsequently performed randomised controlled study was the first to evaluate the treatment of Miller Class I and II MAGR by means of MCAT using either CM or SCTG. The results indicated that compared to baseline, both treatments resulted in statistically significant root coverage but CM yielded lower CRC compared to SCTG. The present study comprised a total of 156 recessions (i.e. 78 in the test group and 78 in the control group, respectively). To analyse the data, a patient level analysis was chosen since this approach may allow to appreciate the overall outcomes following the surgery, and thus, increase the clinical relevance of the results (Aroca et al. 2010). The reason to also include bicuspids and molars was due to the fact that these posterior sites may be of concern for patients exhibiting root hypersensitivity or for patients with high lip lines and compromised aesthetics. It has to be pointed out that the inclusion of molars has, most

likely, influenced the overall results since recession coverage at molars is still a major challenge for the clinician. A recent systematic review has evaluated the predictability of various surgical techniques for obtaining CRC in MAGR (Hofmänner et al. 2012). The findings indicated that in Miller class I and II MAGR the use of CAF or MCAF with or without SCTG may lead to predictable CRC while MACT in combination with SCTG represented a valuable technique in Miller class III MAGR. The fact that the MCAT has been shown to lead to predictable root coverage even in Miller class III recessions (Aroca et al. 2010) corroborate the present results and points to the clinical relevance of this surgical technique. Thus, these surgical principles may be also applied when not only SCTG but also other soft tissue grafts such as CM are used for the treatment of Miller class I and II MAGR. One advantage of the applied MCAT is that this technique avoids the use of vertical releasing incisions, thus maximizing the chance for obtaining complete defect coverage by enhancing blood supply and decreasing the risk of graft exposure (Aroca et al. 2010). The postoperative level of the flap, flap tension and complete graft coverage are also important aspects to be considered for obtaining predictable root coverage (Pini Prato et al. 2000, 2005). The obtained results with both CM and SCTG in MAGR may be explained as follows: a) the mucoperiosteal tunnelled flap was released beyond the muco-gingival line, b) the SCTG or the CM were completely covered which, in turn, may favour revascularisation of the recipient site (Guiha et al. 2001) since their stabilization coronal to the gingival margin and the papillae is ensured c) the papillae were also released interproximally from the underlying bone, and d) the use of suspended sutures around the contact points allowed for an extended coronal stabilization of the flap. In the present study, at 12 months, CRC was observed in 5 out of the 22 patients (i.e. in 22%) in the group and are in line with the findings reported by (Molnár et al. 2013). In the control group, however, CRC was observed in 13 out of the 22 patients (i.e. in 59%). It needs to be kept in mind that CRC may be affected by factors such as statistical analysis (evaluation of multiple sites) and surgical technique and may explain the differences between the present outcomes and those reported by (Zucchelli et al. 2009). In that study, treatment of MAGR with CAF resulted in 77.7% CRC when releasing incisions were used, while it amounted to 89.3 % in the group treated without vertical releasing incisions (Zucchelli et al. 2009). In the present study, the MRC amounted to 71% ± 21% in the test and 90% ± 18% in the control group, respectively, and is in line with the outcomes of

recent reports (Hofmänner 2012, Molnár et al. 2013). These differences might be related to more favourable clinical handling of the applied SCTG when used in combination with the MCAT technique. The fact that both treatments yielded comparable improvements in terms of KT is in agreement with previous studies using CAF for the treatment of MAGR and may be attributed to the natural tendency of the mucogingival junction to regain its original, genetically determined position (Zucchelli & De Sanctis 2005, Zucchelli et al. 2009). At 12 months, GT was statistically significantly increased in the control group compared to the test group. Furthermore, it is interesting to note that CRC was observed in 9 patients with 0.5 mm GT (test or control sites) and in 5 patients with 1 mm GT (test and control sites). On the other hand, these findings appear to contradict the outcomes from other studies reporting a MRC of 64.3% for seven recessions with a flap thickness of ≤ 0.5mm and a CRC only at sites with a flap thickness > 0.8 mm following the use of CAF (Baldi et al. 1999). The importance of GT for root coverage with CAF was emphasized in systematic reviews on single recessions (Hwang & Wang 2006, Cheng et al. 2007) but limited information is available for MAGR (Paolantonio et al. 2002, Aroca et al. 2009, 2010) and even less information comparing the influence of soft tissue thickness of MACF and MCAT in multiple recessions. In a long-term study over five years (Zuchelli & De Sanctis 2005) reported that the soft tissue margin stability was correlated to the patient's individual tendency to develop gingival recession, although they did not quantify soft tissue thickness. It thus appears that the issue on the importance of flap thickness and the possibility of obtaining CRC in MAGR is still not completely elucidated and warrants further studies.

Taken together, our findings stress the clinical significance of possible tissue engineering applications and application of novel biomaterials such as human recombinant growth factors and xenogenic materials. A combination of these might be considered since PDLSCs were shown to initiate periodontal regeneration in vivo on a proper carrier (Seo et al. 2004). Therefore, biomaterials as rhGDF-5/β-TCP or Mucograft® might be applied as possible carriers for PDLSCs in future tissue engineering applications to increase treatment efficacy. On the other hand, current treatment modalities for periodontal hard- and soft tissue reconstruction seem to be limited among other factors by the applied surgical techniques. Thus, these novel treatment approaches should be used in combination with innovative, minimally invasive surgical techniques.

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9. CONCLUSIONS

In this section the major conclusions from in vitro and clinical investigations will be drawn. These conclusions refer to the respective impact of publications indicated in table 1. The following statements are related to a literature review (I), in vitro research providing the methodological basis for future models of periodontal regeneration (II) as well as possible in vivo applications (III). Also conclusions of clinical studies on the human application of rhGDF-5 (IV) as well as Mucograft® (V, VI) will be summarised.

Study I

Surgical periodontal treatment of deep intrabony defects with EMD promotes periodontal regeneration. The application of EMD in the context of non-surgical periodontal therapy has failed to result in periodontal regeneration. Surgical periodontal therapy of deep intrabony defects with EMD may lead to significantly higher improvements of the clinical parameters than open flap debridement alone. The results obtained following treatment with EMD are comparable to those following treatment with GTR and can be maintained over a longer period. Nevertheless, in cases with advanced attachment loss a different treatment approach may be needed to overcome limitations of current regenerative techniques.

Study II

Isolation, culturing and characterization of stem cell cultures periodontal ligament origin was successful. Our in vitro model allows for further in vitro and in vivo investigations of periodontal regenerative procedures. Our present findings establish the methodological basis for future research on proliferation and differentiation of PDLSCs. The identified adult stem cells of periodontal origin might be used in future tissue engineering applications aiming at periodontal regeneration.

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Study III

Our data demonstrated that both DPSC and PDLSC cultures may differentiate into either osteogenic or to a neuronal fate in response to appropriate pharmacological treatments. Further investigations are still necessary to optimise these procedures, enabling the utilisation of the differentiation potential of dental human stem cell cultures. Nevertheless, it is already clear that both the human dental pulp and the periodontal ligament is a potential source for tissue engineering not only in aspects related to dental bone regeneration, but also for neuroregenerative applications.

Study IV

The findings of this study indicate that in the tested application the use of rhGDF-5/β-TCP appeared to be safe and the material possesses a sound biological rationale. Thus, further, adequately powered, randomised controlled clinical trials are warranted to confirm the clinical relevance of this new approach in regenerative periodontal therapy.

Study V

Within their limits, our results indicate that treatment of Miller Class I and II multiple adjacent gingival recessions by means of MCAT and Mucograft® may result in statistically and clinically significant root coverage. Further studies are thus warranted to evaluate the performance of Mucograft® compared to connective tissue grafting and other treatment alternatives.

Study VI

The presented findings indicate that the use of Mucograft® may represent an alternative to connective tissue grafting by reducing surgical time and patient morbidity but yielded lower root coverage than connective tissue grafting in the treatment of Miller Class I and II multiple adjacent gingival recessions when used in conjunction with the MCAT technique.

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10. SUMMARY

Several treatment approaches have been suggested in the past for periodontal hard- and soft tissue reconstruction. Based on our literature review (Study I) surgical periodontal therapy of intrabony defects with EMD may lead to significantly higher improvements than open flap debridement alone. These results are comparable to GTR and can be maintained over a longer period.

The limitations of current techniques for periodontal hard- and soft tissue reconstruction have raised a demand to introduce novel treatment approaches and biomaterials aiming at increased treatment efficacy as well as reducing duration of treatment and patient morbidity. Recently, the possibility of tissue engineering related to PDLSCs and recombinant growth-factors for periodontal hard tissue reconstruction as well as xenogenic materials for reconstruction of soft tissue anomalies were suggested.

In vitro (Study II) EMD was shown to promote proliferation of PDL cultures in vitro, which may be a useful model for further in vitro and in vivo investigations of tissue engineering applications. We showed both DPSCs' and PDLSCs' regenerative potential confirming in vitro mineralisation induction and neuronal differentiation (Study III).

In an RCT (Study IV) we were the first to clinically and histologically investigate that rhGDF-5 can be safely used for periodontal regenerative therapy in humans. It was shown that the tested concentration of rhGDF-5 /β-TCP might be a promising new approach in regenerative periodontal regeneration.

In a pilot case series (Study V) it was reported that treatment of Miller Class I - and II MAGR by means of MCAT and Mucograft® may result in statistically significant root coverage.

In a subsequent split-mouth RCT (Study VI) it was verified that Mucograft® may be a useful alternative to SCTG by reducing surgical time and patient morbidity for root coverage procedures. Nevertheless, the use of Mucograft® yielded lower CRC than SCTG in the treatment of Miller Class I and II MAGR when used in conjunction with MCAT.

Future clinical applications with increased efficacy in periodontal regenerative therapy might be introduced by combining tissue engineering therapeutical approaches and growth factors or xenogenic biomaterials utilising improved, minimally invasive surgical techniques.

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11. ÖSSZEFOGLALÁS

A múltban számos kezelési módszert leírtak a parodontális kemény- és lágyszövetek rekonstrukciójára. Irodalomkutatásunk alapján (I. vizsgálat) az intraosszer defektusok sebészi kezelése EMD-vel szignifikánsan eredményesebb lehet, mint a műtéti tisztítás önmagában. Az eredmények hosszú távon hasonlóak a GTR technikához.

A jelenlegi kemény- és lágyszövet rekonstrukciós technikák korlátai szükségessé tették, hogy újszerű kezeléseket alkalmazzunk, új bioanyagokkal a nagyobb hatékonyság, valamint a rövidebb kezelés és a páciensek kisebb terhelése érdekében. A közelmúltban felmerült a PDLSC sejtek alkalmazása szövettenyésztésben, ill. humán rekombináns növekedési faktorok alkalmazása a parodontális keményszövetek helyreállításában, valamint xenogén anyagok felhasználása a parodontális lágyszövetek rekonstrukciójára.

In vitro kimutattuk (II. vizsgálat), hogy az EMD fokozza a PDL sejtkultúrák proliferációját, melyek szövettenyésztési alkalmazások további in vitro és in vivo modelljei lehetnek. A DPSC-k és PDLSC-k regeneratív potenciálját is kimutattuk in vitro mineralizáció indukció, valamint neurogén differenciáció segítségével. (III. vizsgálat).

Egy randomizált kontrollált vizsgálatban (IV. vizsgálat) elsőként igazoltuk klinikailag és hisztológiailag, hogy az rhGDF-5 biztonságosan alkalmazható humán regeneratív terápiában. Igazoltuk, hogy az alkalmazott koncentrációban az rhGDF-5 /β-TCP ígéretes új regeneratív anyag lehet a parodontális regenerációban.

Egy bevezető esettanulmányban (V. vizsgálat) kimutattuk, hogy a Miller I-II osztályú többszörös ínyrecessziók kezelése MCAT technikával és Mucograft® segítségével statisztikailag szignifikáns gyökérfelszín fedést eredményezhet.

Egy kapcsolódó split-mouth randomizált kontrollált vizsgálatban (VI. vizsgálat) igazoltuk, hogy a Mucograft® alternatívát kínálhat a kötőszövet átültetéssel szemben a műtéti idő és a páciensek terhelésének csökkentésével. Mindemellett a Mucograft® alkalmazása alacsonyabb teljes gyökérfelszín fedési arányt eredményezett kötőszövet átültetéshez képest Miller I- II többszörös ínyrecessziók kezelésében MCAT technikával.

A jövőben elképzelhető hatékonyabb parodontális regeneratív technikák kifejlesztése szövettenyésztési alkalmazások segítségével és növekedési faktorok, vagy xenogén anyagok továbbfejlesztett, minimálinvazív műtéti technikákkal történő kombinációjával.

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13. BIBILIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

13.1 Related publications

(I) Sculean A, Windisch P, Döri F, Keglevich T, Molnár B, Gera I. (2007) Emdogain in regenerative periodontal therapy. A review of the literature. Fogorv Sz, 100(5): 220-32, 211-9.

(II) Molnár B, Kádár K, Király M, Porcsalmy B, Somogyi E, Hermann P, Grimm WD, Gera I, Varga G. (2008) Isolation, cultivation and characterisation of stem cells in human periodontal ligament. Fogorv Sz, 101(4): 155-61.

(III) Kádár K, Király M, Porcsalmy B, Molnár B, Rácz GZ, Blazsek J, Kállo K, Szabo EL, Gera I, Gerber G, Varga G. (2009) Differentiation potential of stem cells from human dental origin - promise for tissue engineering. J Physiol Pharmacol, 60 Suppl 7: 167-75. **IF: 1.489**

(IV) Windisch P, Stavropoulos A, Molnár B, Szendröi-Kiss D, Szilágyi E, Rosta P, Horváth A, Capsius B, Wikesjö UM, Sculean A. (2012) A phase IIa randomized controlled pilot study evaluating the safety and clinical outcomes following the use of rhGDF-5/β-TCP in regenerative periodontal therapy. Clin Oral Investig, 16(4): 1181-9. **IF: 2.364**

(V) Molnár B, Aroca S, Keglevich T, Gera I, Windisch P, Stavropoulos A, Sculean A. (2013) Treatment of multiple adjacent Miller Class I and II gingival recessions with collagen matrix and the modified coronally advanced tunnel technique. Quintessence Int, 44(1): 17-24. **IF: 0.762**

(VI) Aroca B, Molnár B, Windisch P, Gera I, Salvi GE, Nikolidakis D, Sculean A. (2013) Treatment of multiple adjacent Miller class I and II gingival recessions with a Modified Coronally Advanced Tunnel (MCAT) technique and a collagen matrix or palatal connective tissue graft. A randomized, controlled clinical trial. J Clin Periodontol, 40(7): 713-20.**IF: 2.996**

13.2 Not related publications

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