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Regulation of the Expression of Sclerostin in Periodontal Fibroblasts

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"I have not failed.

I have just found 10,000 ways that won't work."

Thomas A. Edison

Acknowledgment

Writing the diploma thesis was a challenge and a lot of hard work, which I would not have been able to do without support.

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I. Curriculum Vitae

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II. Zusammenfassung

Sklerostin, ein zentraler Regulator der Knochenneubildung, wird nicht nur im Knochen sondern auch im Zahnhalteapparat, dem sogenannten Parodont, produziert. Sklerostin ist daher möglicherweise an Prozessen beteiligt die den Erhalt der Zähne ermöglichen, aber auch pathophysiologische Wirkungen sind nicht auszuschließen. Das Ziel dieser Studie war es den Einfluss von Signalmolekülen auf die Produktion von Sklerostin bei parodontalen Fibroblasten zu untersuchen.

Basierend auf dieser Frage wurden Fibroblasten des parodontalen Gewebes mit verschiedenen Signalmolekülen inkubiert. Untersucht wurde die Veränderung der Produktion von Sklerostin auf mRNA-Ebene mittels PCR, und Proteinebene mittels Westernblot und ELISA. Um eine mögliche autokrine Wirkung aufzuzeigen wurden die Fibroblasten mit Sklerostin inkubiert und der Einfluss auf Zellfunktionen durch Messungen zur Vitalität, Proliferation, Proteinsynthese und Chemotaxis untersucht.

Es konnte gezeigt werden, dass Transforming Growth Factor-beta1 die Produktion von Sklerostin auf mRNA- und Proteinebene steigert. Die Erhöhung erfolgte zeit- und dosisabhängig. Rekombinantes Sklerostin zeigte hingegen keinen Einfluss auf die Zellfunktionen Vitalität, Proliferation, Proteinsynthese und Chemotaxis parodontaler Fibroblasten.

Die Produktion von Sklerostin in parodontalen Fibroblasten unterliegt demnach einer Regulation durch Transforming Growth Factor-beta1. Dass die Regulation von Sklerostin einem autokrinen Mechanismus unterliegt konnte nicht bestätigt werden. Die funktionelle Bedeutung der Regulation von Sklerostin im Parodont ist noch unklar.

III. Abstract

Sclerostin, a key regulator of bone regeneration, is not only expressed in bone but also in the tooth supporting apparatus, the periodontium. Thus, sclerostin may be involved in teeth-preserving processes, but pathophysiological effects cannot be ruled out. The aim of this study was to reveal effects of signaling molecules on the expression of sclerostin in periodontal fibroblasts.

The effect of different signaling molecules was tested on periodontal fibroblasts. Changes in the expression of sclerostin were determined on mRNA level with PCR and on protein level with Western Blot and ELISA. To show a possible autocrine regulatory mechanism, the fibroblasts were incubated with sclerostin and the impact on cell functions viability, proliferation, protein synthesis, and chemotaxis was studied.

It was found that transforming growth factor-beta1 increased sclerostin on mRNA and protein level. This increase was time-dependent and dose-dependent. Recombinant sclerostin, however, showed no effect on cell viability, proliferation, protein synthesis, and chemotaxis.

These findings show that in periodontal fibroblasts sclerostin is regulated by transforming growth factor-beta1. That sclerostin is regulated through an autocrine mechanism could not be confirmed. The functional role of the regulation of sclerostin in the periodontium is still unknown.

IV. List of Abbreviations

$^3\text{[H]}$ thymidine	radioactively labeled thymidine
$^3\text{[H]}$ leucine	radioactively labeled leucine
α -goat HRP	polyclonal rabbit anti-goat Ig with HRP
α -h β Actin	anti-human goat polyclonal β -actin
α -hSOST	anti-human goat monoclonal SOST
α -MEM	minimal enriched medium
β -actin	beta-actin
β -cat	beta-catenin
β -ME	beta-mercaptoethanol
AA-2-P	ascorbic acid-di-phosphate
AA-bis	acrylamid bis solution
AB	alveolar bone
ab	antibody
a.d.	aqua distilled
ALP	alkaline phosphatase
bFGF	basic fibroblast growth factor
BMD	bone mineral density
BMP	bone morphogenic protein
BSA	bovine serum albumin
C	cementum
CB	cementoblast
CC	cementocyte
CMFC	cellular mixed fiber cementum
Co	control
CO ₂	carbon dioxide
CP	cytoplasmic processes
cpm	counts per minute
CT	connective tissue
D	dentine

DAN	diff. screening-selected gene aberrative in neuroblastoma
DEXA	dexamethasone
Dkk	Dickkopf
DMSO	dimethyl sulfoxide
DTT	DL-dithiothreitol
ELISA	enzyme-linked immunosorbent assay
ES	enamel space
FAK	focal adhesion kinase
FCS	fetal calf serum
FDA	Food and Drug Administration (U.S.A.)
FGF	fibroblast growth factor
GF	gingival fibroblasts
GF 1/2/3	gingival fibroblasts from donor 1/2/3
GP	glycerophosphate
GSK3 β	glycogen synthase kinase 3 β
hMSC	human mesenchymal stem cells
hPRS	human platelet-released supernatant
HRP	horseradish peroxidase
Ig	immunoglobulin
IGF	insuline-like growth factor
JE	junctional epithelium
KO	knock-out
L	lacuna
LW	lacuna wall
LEF	lymphoid-enhancer factor
L-Mimo	L-mimosine
LRP 4/5/6	low-density lipoprotein receptor related protein 4/5/6
MIM	Mendelian Inheritance of Men identification number
MM	master mix
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
O	osteoid layer
OB	osteoblast

OC	osteocyte
OE	oral epithelium
OD	optical density
OD-medium	osteoblast differentiation medium
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor (synonymous for PDGF-BB)
PDL	periodontal ligament
PDLF	periodontal ligament fibroblasts
PDLF 1/2/3	periodontal ligament fibroblasts from donor 1/2/3
PMSF	phenylmethanesulfonyl fluoride
P/S	penicillin/streptomycin
P/S/A	penicillin/streptomycin/amphotericin
PTH	parathormone
qRT-PCR	quantitative real time polymerase chain reaction
rhbFGF	recombinant human basic fibroblast growth factor
rhPDGF-BB	recombinant human platelet-derived growth factor
rhPTH	recombinant human parathormone
rhSclerostin	recombinant human sclerostin
rhTGF- β 1	recombinant human transforming growth factor-beta1
rhBMP-6	recombinant human bone morphogenic protein-6
S ^(-/-)	homozygous knock-out of both alleles in the SOST gene
SC-medium	serum-containing medium
SDS	sodium dodecyl sulphate
SE	sulcus epithelium
SF-medium	serum-free medium
Sfrp	secreted frizzled-related protein
SOST	gene encoding sclerostin; synonymously for sclerostin
TAE-buffer	tris base-acetic acid-EDTA-buffer
TCF	T-cell factor
TBS	tris-buffered saline
TBS-T	tris-buffered saline – Tween20®

TE	trypsin-EDTA (ethylenediaminetetraacetic acid)
TGF- β	transforming growth factor (synonymously for TGF- β 1)
UV	ultra violet light
w/o	without; unstimulated sample
Wnt	morphogenic ligand; protein of the Wnt-signaling pathway
WT	wild-type

1 Introduction

The first part of the following chapter shall give an introduction of the anatomical, histological and physiological make-up of the tooth. Then take a closer look at the periodontium; its structures, functions, tissues, and development. In the second part sclerostin, a key regulator of bone formation, is described – structure, function, and history. Then the present scientific knowledge of sclerostin follows with the reasoning and need for this diploma thesis along with the study's objectives.

1.1 The Tooth

The teeth play an important role in the nourishment; they are one of the first steps of digestion. (1-3) Through mastication food is crushed and mixed with saliva to a mash before it is further digested. (4, 5) But the teeth have other functions as well. They are important for a clear articulation, as a tool or as a weapon for defense in phylogenic development (3) and in modern times aesthetic plays an important role in social attractiveness. (6)



Figure 1: Permanent dentition set. The different forms and occlusal surfaces are shown. There are four different types of teeth in human dentition incisors, canines, molars and premolars. (7)

Human teeth are heterodontic – the dentition is made up of different specialized set of teeth, and dyphodontic - two complete set of teeth. The primary dentition, with 20 deciduous teeth, is replaced by the secondary dentition, with 32 permanent teeth. (3, 8) The form of the teeth differs depending on their function. Incisors and canines have a slimmer figure with a cuspid formed occlusal surface for cutting. Molars and premolars are stockier, broader in form and have a wider blunt occlusal surface for grinding. (2, 3, 8)

1.1.1 Anatomy

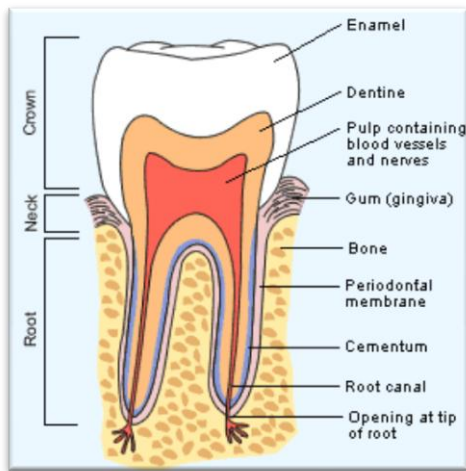


Figure 2: Tooth in cross-section. The tooth can be divided into three layers - crown, neck, and root. On top dentine is coated by enamel, the lower part by cementum. Underneath the dentine is the pulp with its apical root canal that leads blood and nerve vessels into the bone. The PDL anchors the tooth via cementum to the alveolar bone. The gingiva encloses and fixates the tooth at the neck level. (9)

Figure 2 shows the different layers of the tooth in cross section: functional structures and surrounding tissues. The tooth can be structured into three main parts: crown, neck and root. The crown is the upper visible part of the tooth above the gingiva. The root is the lower part that lies in the alveolar bone. The neck lies between crown and root at the point of attachment of the gingiva.

The crown is coated with enamel, underneath is dentine. At the crown dentine is covered by enamel and at the root by cementum. Under the dentine is the pulp cavity containing the pulp. Apical the pulp goes over into the root canal that leads blood vessels and nerves into the surrounding bone tissue. The PDL connects with the cementum on the tooth and anchors it within the alveolar bone of the maxilla or mandible at the root. The gingiva encloses and fixates the tooth at the neck level and seals the periodontal gap off to the oral cavern. (1-3)

Anatomically enamel, dentine, pulp and cementum are structures of the tooth. PDL, gingiva and alveolar bone form the periodontium, the so-called tooth-holding apparatus. Although anatomically cementum is put with the tooth it functionally belongs to the periodontium. (3)

1.1.2 Histology

Enamel coats the tooth from crown to neck. It is the hardest tissue in the human body and consists to 97% of minerals in form of hydroxyapatite. (1, 3) The enamel is produced by ameloblasts during tooth development. (1) The hard tissue is unable to regenerate; it declines with age due to attrition and abrasion. (1, 2)

Dentine encloses the pulp cavity. It is highly mineralized, up to 70% inorganic components, and has a composition similar to bone only harder. (1-3) The main organic components are collagen fibers, type I, and different matrix proteins. (1, 3) Dentine producing odontoblasts are lined near the pulp cavity. (1, 2) They produce dentine during teeth development and keep this function postnatal. (1)

Cementum covers the dentine from neck to root. (1-3) It is a hard tissue, consisting to 65% of minerals, hydroxyapatite; organic components are collagen fibers type I and cells. (1, 3) Cementum has a low turnover rate, but is produced by cementoblasts lifelong. Cementoblasts form a thin layer on the outside of the cementum, can differentiate to cementocytes, and connect to the PDL. (1, 10)

Pulp – a loose fine-fibrous connective tissue - is contained within the pulp cavity. (1-3) The pulp consists of a glycosaminoglycans-rich matrix with imbedded fibers – collagen type I/III and elastic fibers. (1) There are three main types of cells into the pulp: odontoblasts, fibroblasts and free-moving cells. The odontoblasts lie on the pulp-dentine-border, are responsible for the production of dentine and impulse conduction. The fibroblasts can differentiate to osteoblasts as well as regulate the turnover of the matrix and stem cells. Free-moving cells are mainly lymphocytes, macrophages or dendritic cells for immunological functions. Nerve and blood vessels are connected to surrounding tissue through the root canal and nourish the tissue. (1, 3)

1.2 The Periodontium

The periodontium consists of soft tissues – gingiva, PDL - and hard tissues – cementum, alveolar bone. The structures are responsible for different functions. (10) For example: anchoring the tooth in the alveole, as immune defense due to the lymphatic cells and sensoric functions - pain, pressure, and tactile sensitivity (1-3, 10) - as well as a nutritive function. (11, 12) Further the periodontium has a high physiological and functional remodeling rate which enables it to regenerate defects caused by mechanical occlusal pressure, human anterior tooth movement, aging or dental treatment. (1-3, 10) The periodontium absorbs the pressure put on the tooth, converts it into traction and moves the tooth back into its original position. The traction stimulates bone formation in the alveolar bone. (13) If the periodontium is damaged, the pressure on the bone causes bone resorption; as is observed after tooth loss. (8)

1.2.1 Anatomy and histology

The Periodontium consists of four parts - cementum, PDL, gingiva, and alveolar bone - that form a functional unit. (1-3, 10)

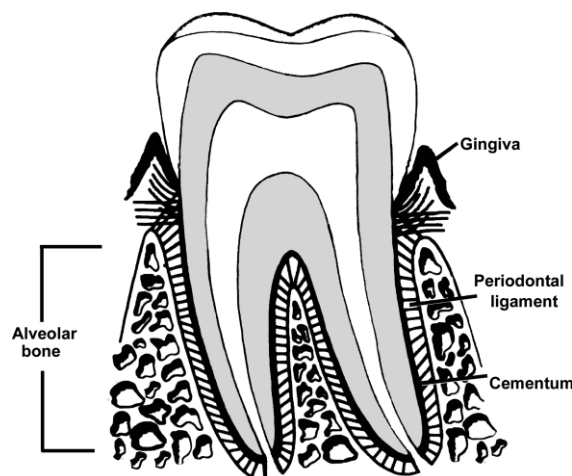


Figure 3: Periodontium. Cementum, PDL, alveolar bone, and gingiva form a functional unit that anchors the tooth in the alveole. (14)

1.2.1.1 Cementum

Cementum has already been partly discussed in the former chapter. The cementum covers the root dentine, tightly interconnected, and is thickest at the apical end of the root. It consists of mineralized matrix, cells and collagen fibers. For the periodontium the cementum is important for the attachment to the PDL and the anchoring of the tooth to the alveole. (1-3) The make-up of the matrix is similar to that of bone. There are two different collagen fiber nets; fine intrinsic fibers, Ebner fibrils, that spiral around the root and extrinsic fibers, Sharpey fibers, radially running periodontal fibers. The extrinsic fibers are most common on the outside and middle of the cementum. (3) Further there are four different types of cementum depending of the composition of cells, cementocytes, and fibers. (1, 3, 10)

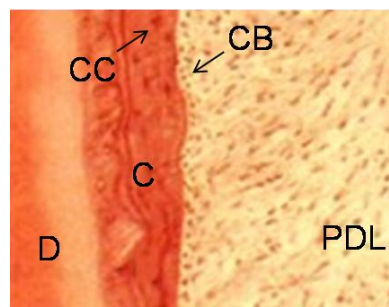


Figure 4: Cementum. The cementum (C) lies between dentine (D) and PDL. It consists of cementoblasts (CB) lined on the PDL-near surface and built-in cementocytes (CC). (15)

1.2.1.2 Gingiva

The gingiva is a specialized type of the oral mucosa with a high turnover and regeneration rate. It consists of collagen connective tissue – collagen type I/III – blood vessels, nerves, lymph, as well as fibroblasts, fibrocytes and free moving cells. (1, 3) The gingiva surrounds the tooth at the neck and closes the periodontium at the coronal end off. (1-3, 8) It stabilizes the tooth supporting the anchoring within the alveole. (1-3) It is distinguished between three epitheliums. (i) oral epithelium is interconnected with the connective tissue and lines the outer part of the gingiva facing the oral cavern. (ii) junctional epithelium surrounds the neck of the tooth. It is unkeratinized, undifferentiated, and not interconnected with the connective tissue. (iii) sulcular epithelium lies between

oral and junctional epithelium. Subepithelial lies connective tissue consisting of collagen fibers with fibrocytes and free cells; blood vessels and nerves are also present. Also a supraalveolar fiber apparatus that consists of fiber bundles which insert in the root surface. The fiber apparatus surrounds the root, connects the gingival tissue with the tooth neck and the alveolar ridge. (1, 3, 10)

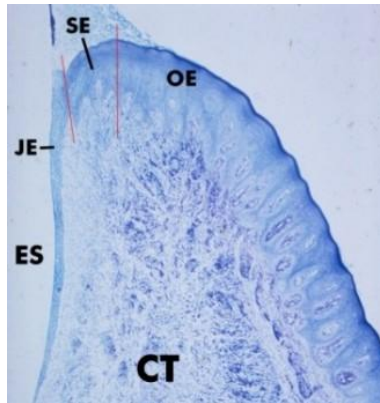


Figure 5: Gingiva. The gingival structure consists of subepithelial connective tissue (CT) covered by oral epithelium (OE), and junctional epithelium (JE) by the enamel space (ES). Sulcular epithelium (SE) is oral epithelium at the gingival sulcus. (16)

1.2.1.3 PDL

The PDL is a fibrous attachment apparatus that anchors the tooth at root level to the alveolar bone. (1, 10) It lies in a gap of 0.1 – 0.2 mm, periodontal gap, between tooth and alveolar bone (1) and functions as a syndesmosis. (3) The PDL consists of firm collagen and elastic fibers that run from crown to root and Sharpey fibers inserting and spanning between cementum and alveolar bone. (1, 3, 10) The ligamentous apparatus absorbs the pressure put in the tooth caused by mechanical loading. Cells, nerve, blood, and lymph vessels are also present in the PDL. The blood vessels help to distribute the occlusal pressure hydrodynamically. (1, 3) The cells, mainly fibroblasts, are responsible for the regeneration of the periodontium. They produce new collagen fibers and can differentiate to cementoblasts and osteoblasts. (1, 3)

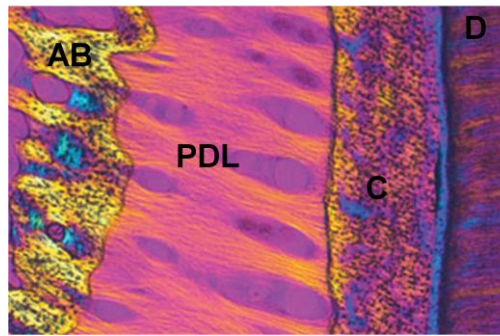


Figure 6: PDL. The attachment of the PDL to the alveolar bone (AB) and the root - cementum (C) connected to dentin (D) - via collagen fiber bundles is shown. (17)

1.2.1.4 Alveolar bone

In the alveole the bone is made of compact bone tissue with small perforations, Volkmann canals, for nerve, lymph, and blood vessels. Adjacent is spongy bone tissue that undergoes steady remodeling. (1, 3) For example due to the tendency of the anterior movement of human teeth the mesial alveole wall undergoes resorption and the lateral wall bone formation. (1) The PDL-near-surface of the alveole is interlaced with Sharpey fibers from the PDL. This guarantees a tight connection of the PDL to the bone. (1, 10)

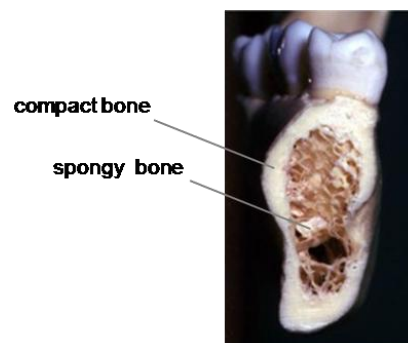


Figure 7: Alveolar bone. The outer side of the alveolar bone is made of compact bone, followed on the inside by spongy bone containing Volkmann canals for nerve, lymph, and blood vessels. (18)

1.2.2 Cells – origin, differentiation and function

1.2.2.1 GF

The gingiva is of mesodermal (19) and ectodermal (10) origin. Due to heterogeneity of the fibroblasts they derive from different sources, e.g. perifollicular mesenchyme or undifferentiated perivascular cells. The GF are involved in stabilizing the tooth and keep it from rotation. (11) The turn-over rate of the gingiva is lower than in PDL, but higher than in bone or skin. (10)

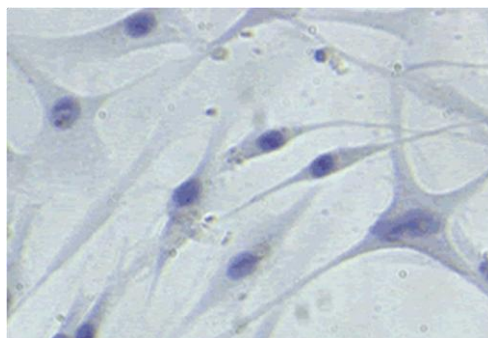


Figure 8: Oral fibroblasts. The cells were taken from incisive vestibules of a rabbit. (20)

1.2.2.2 PDLF

In the PDL the most common type of cells are PDLF. (11) They are of ectomesenchymal origin. (10, 19) Periodontal fibroblasts derive from precursor cells of the dental follicle. (10) Different subpopulations of fibroblasts are known. One of them are osteoblast-like fibroblasts that are able to differentiate to cementoblasts and osteoblasts. (11) In the PDL undifferentiated stem cells remain with the potential to turn into fibroblasts, cementoblasts and osteoblasts. (11) PDLF are needed for production of components of the extracellular matrix (11) and are responsible for the remodeling of the tissue. (1) The turnover of the PDL is higher than that of gingiva and skin. (10)

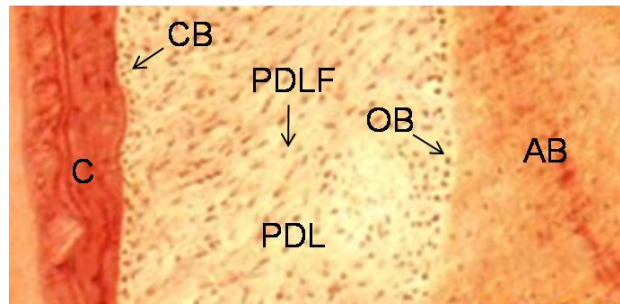


Figure 9: PDLF. Fibroblasts of the PDL (PDLF) can differentiate into cementoblasts (CB) and osteoblasts (OB). Cementoblasts lie near the cementum (C) and osteoblasts near the alveolar bone (AB). (15)

1.2.2.3 Cementoblasts

Cementoblasts origin from ectomesenchymal cells. (10) Precursor cells from the dental follicle are able to differentiate to cementoblasts. (10, 12) The cells form a thin layer on the outside of the cementum. (1) Mature cementoblasts are built into mineralizing cementum matrix and become cementocytes. (1, 3) Cementocytes lie in lacunae, like osteocytes, and are connected with cell extensions via canaliculi. (1, 3) Also cementoblasts and cementocytes participate in the production of intrinsic fibers. Extrinsic fibers, Sharpey fibers, are secreted by PDLF. (1)

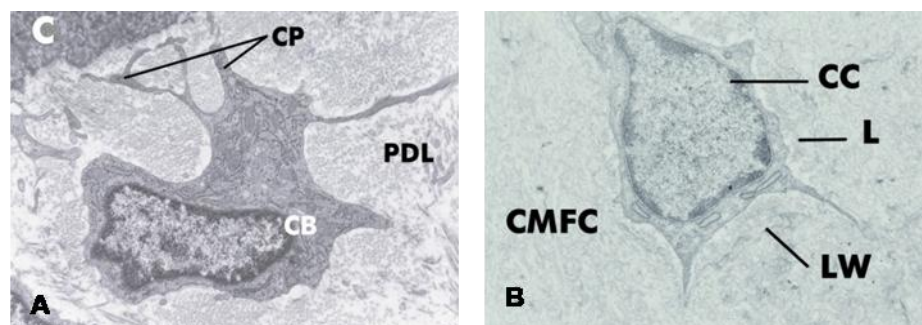


Figure 10: Cementoblast and cementocyte. (A) Lying on the PDL-cementum (C) border is a cementoblast (CB) with cytoplasmic processes (CP). (21) (B) A cementocyte (CC) lying in a lacunae (L) with surrounding lacuna wall (LW) in cellular mixed fiber cementum (CMFC). (22)

1.2.2.4 Osteoblasts

The alveolar bone is of ectomesenchymale origin. Osteoblasts derive from periodontal progenitor cells of the dental follicle and are able to further differentiate to osteocytes. (10, 12) Osteoblasts build new bone through imbedding osteoblast into new mineralizing bone matrix. (10, 23) The built in osteoblasts differentiate into osteocytes, lie in lacunae and are connected via cell extensions. (10) Further there are osteoclasts in the bone. They are multinuclear giant cells lying in Howship's lacunae on the bone surface. Osteoclasts evolve from fusioning of hematopoietic mononuclear marrow progenitor cells and are responsible for the resorption of bone. (10, 23)



Figure 11: Osteoblasts. Osteoblasts (OB) lie on the border of a bone forming layer, osteoid layer (O). Inside the alveolar bone is a built-in osteocyte (OC). (24)

GF and PDLF are established models for in vitro studies (25) and were used in this research project.

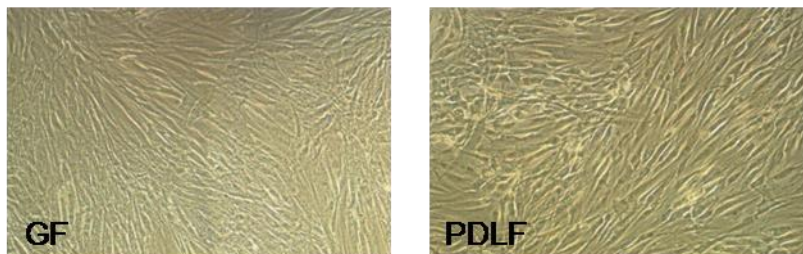


Figure 12: GF and PDLF in vitro. (magnification x10)

1.2.3 Regulation and regeneration

The periodontal tissues are continually regenerated to repair damages; e.g. caused by mechanical loading due to mastication, but also local inflammation. Therefore the cells have to be able to react to local signaling molecules, which are released upon damage of the tissue. (10) Under physiological conditions a cellular control mechanism regulates the remodeling of the periodontium and subsequently the anchoring of the tooth in the alveolar bone. Under pathological conditions the repair mechanisms can be disrupted or overstressed and the resorption can take overhand. (26)

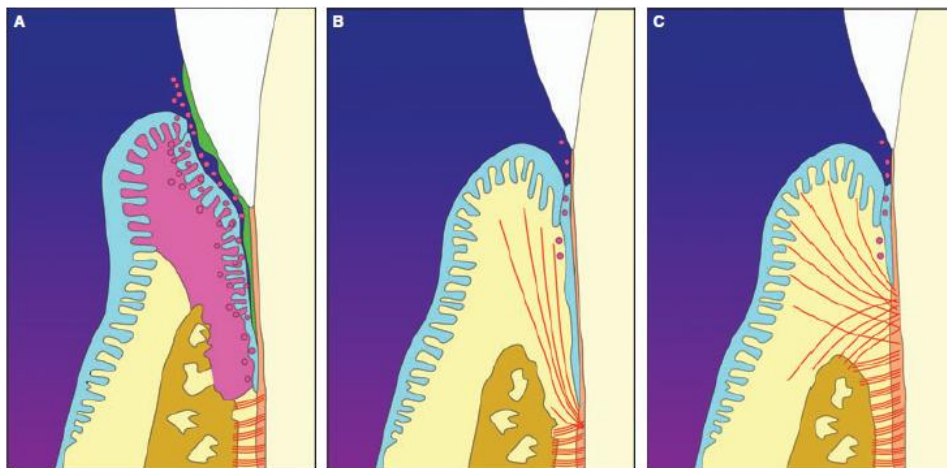


Figure 13: (A) In periodontitis inflammation causes loss of soft tissue and alveolar bone. (B) Periodontal repair is characterized by formation of a long junctional epithelium. (C) In regeneration the functional structures of the periodontium are fully restored. (17)

One of the most common chronic inflammatory diseases in adults is periodontitis. (10) Periodontitis is caused by bacteria that accumulate at the gingival sulcus, forming plaques, and cause inflammation within the periodontium. (10) Without treatment it comes to destruction of soft tissue in the periodontium and to bone resorption. (10, 17) The resorption leads to the loss of attachment and subsequently to the loosening and loss of the tooth. (10, 17) Periodontitis is therefore a serious clinical problem that affects large parts of the adult population. (10, 26)

For the treatment of attachment loss two strategies of healing can be followed: periodontal repair and regeneration. In repair, the aim is to heal the defect, but without restoration of the involved tissues. Characteristically for repair is the formation of a long junctional epithelium. On the other side, the aim of regeneration is to fully restore the functional structures of the periodontium – gingiva, cementum, PDL and alveolar bone. Another goal is to ensure the attachment between root and connective fibers as well as between tooth and junctional epithelium. (Figure 13) (17)

Bosshardt and Sculean presented an overview of recent studies in the field of regenerative techniques – the different methods and their potential. (17) Conditioning of the root surface could be of clinical interest for enhancing the attachment and expanding of cells onto the surface. Bone grafts and bone substitute materials, depending on the type used, can support new bone formation. In combination with barrier membranes in guided tissue regeneration their regenerative effect is known. Also of interest are enamel matrix proteins. Studies gave evidence that these proteins assist in the production of new periodontal structures and wound healing. Of clinical interest the enamel matrix protein Emdogain® which showed positive impact in the treatment of periodontitis in clinical studies. (17) Although growth and differentiation factors-like IGF, FGF, PDGF, PTH, TGF- β , and BMPs showed high potential in preclinical studies, they are still not yet used as standard therapeutics. (14, 17)

All in all regeneration of the periodontal structures is possible. Although with the present available methods only partial reconstruction of the functional tissues is achieved. (17) Consequently, there is a high demand for further studies.

1.2.4 Growth factors in the periodontium

As mentioned in the previous chapter, TGF- β 1 and PDGF-BB are growth factors used in periodontal regeneration, clinically as well as in studies, and show a lot of potential.

1.2.4.1 TGF- β 1

TGF- β 1 is a cytokine belonging to the highly conserved TGF- β family and is one of three forms - β 1, β 2, β 3. The growth factor is a dimeric polypeptide consisting of a disulfide bond connecting two amino acid chains. (30, 31) TGF- β 1 binds to the corresponding receptors TGF β - type I and II, which can be found in many cells. TGF- β 1 is involved in regulatory functions of cells like adhesion, migration, differentiation, proliferation, (31) and immune regulation. (30) It has dual functions, for example it can inhibit or stimulate proliferation in the same or different cells types. (30) Also it regulatory effects different growth factors and causes stimulation in mesenchymal cells. (31) TGF- β 1 is present in tissues of the PDL and may be important for the preservation of PDL structures. Also TGF- β 1 may promote differentiation of progenitor cells to fibroblasts. (32) Further it may regulate fibroblast differentiation via periostin, through FAK-dependent pathways. (33) In tooth morphogenesis signaling of TGF β -1 via type II receptors play a major role in the formation of dentine and maturing of odontoblasts. (34) TGF- β 1 can modulate the eruption of teeth in adolescent rats. The growth factor was found in osteoblasts, PDLF and cementoblasts in the periodontium. (35)

1.2.4.2 PDGF-BB

PDGF-BB is a homodimer with an eight cysteine motif and is mitogenic for mesenchymal cells, like osteoblasts or their progenitor cells. (27, 28) In PDLF it positively effects proliferation and chemotaxis. (29, 30) PDGF-BB was one of the first growth factors clinically used – mostly combined with guided tissue regeneration. The FDA approved rhPDGF-BB, in therapeutical use for regeneration of periodontal defects, as secure and successful. (29)

1.3 Sclerostin – a key regulator of bone remodeling

1.3.1 Sclerosteosis and Van Buchem

In 2001 different mutations in a new gene, *SOST* (MIM 605740), were identified. A nonsense mutation in the *SOST* gene is responsible for sclerosteosis (MIM 269500). Causes are problems with the splicing or a premature break off of the encoding due to an early stop codon. (36, 37) A 52-kb deletion in the enhancer region downstream of *SOST* causes the disease Van Buchem (MIM 239100), also called hyperostosis corticalis generalisata. (37-39) Sclerosteosis and Van Buchem are sclerosing bone dysplasias, with a characterizing hyperostosis. (40, 41) Both mutations are autosomal recessive; meaning only in homozygous form a phenotypic manifestation develops. (41) Sclerosteosis is the extremer form of the two; clinical manifestations are more severe than in patients with Van Buchem - milder form. (42, 43)

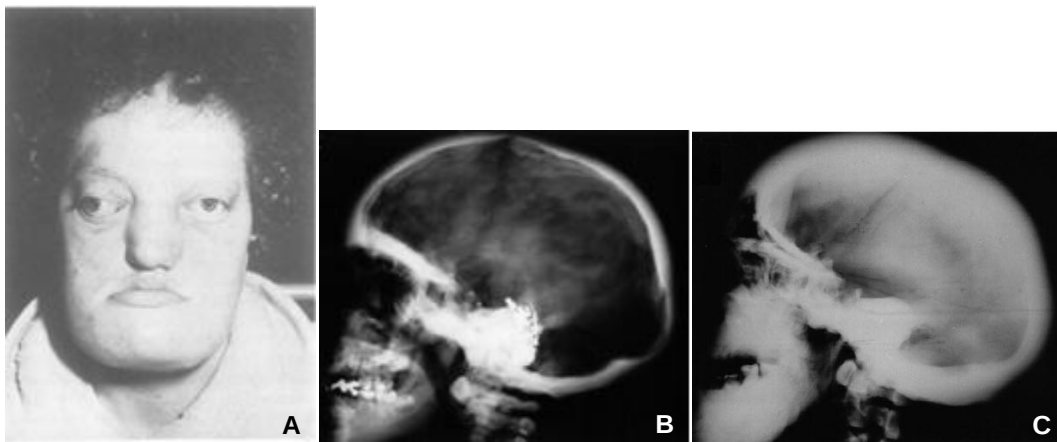


Figure 14: Clinical manifestations of sclerosteosis and Van Buchem. (A) The characteristic broadening of the jaw and deformation of facial structures in sclerosteosis. (41) (B+C) X-rays of patients with Van Buchem – (A) child and (B) adult – with increased bone mass in the adult. (44)

The pathological bone overgrowth leads to clinical symptoms that are rather prominent at the mandible and the skull. Typical manifestations are the enlargement of the facial bones and the jaw that lead to distortion of facial structures. The thickening bones can compress nerves and lead to facial palsy as well as loss of sight, smell, and hearing. (43) Also the thickening of the skull

can cause an increase of intracranial pressure, which can be lethal. (41-43) Further syndaktyly and a tall stature have been reported. Clinical manifestations can also be found in the dental region. (42) Position and shape of teeth can be anormal, malocclusion, belated eruption of teeth as well as partial anodontia have been observed. (41, 45) Often the extraction of teeth are rather difficult. (45) This observations led to the conclusion that the protein of the SOST gene, sclerostin (MIM 605740), must play a role in the control of bone formation. (37)

1.3.2 Sclerostin

Sclerostin is encoded by the SOST gene in humans. SOST lies at the locus 17q12-q21. (37-39) The gene consists of two exons and one intron with a total size of 5 kb. Sclerostin is a secreted glycoproteine that belongs to the Cerberus/DAN-family, a family of BMP antagonists characterized by a C-terminal cysteine knot-like domain. (46) The sclerostin protein consists of 213 amino acids. (47)

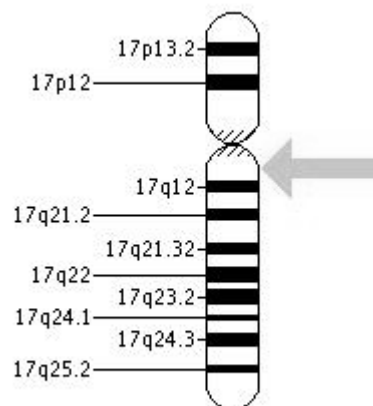


Figure 15: Locus of the SOST gene in humans. The gene lies at 17q12-q21, consists of two exons and one intron, and has a size of 5 kb. (40)

```

1   mqlplalclv cllvhtafrv vegqgwqafk ndateiipel geypepppel ennktmnae
61  nggrpphhpf etkdvseysc relhftryvt dgpcrsakpv telvcsggcg parllpnaig
121 rgkwwrpsgp dfrcipdryr aqrvqlpcpg geaprarkvr lvasckckrl trfhnqselk
181 dfgteaarpq kgrkprprar sakanqaele nay

```

Figure 16: Amino acid sequence of sclerostin in humans. The secreted glycoprotein consists of 213 amino acids. (47)

Sclerostin is expressed during fetal development as well as SOST mRNA in a few tissues postnatal and in adults (36, 37, 48, 49). Several studies tested for expression of SOST mRNA and sclerostin protein in different human and murine tissues. (36, 37, 49) SOST mRNA was found in different human tissues – colon, skin, placenta, aorta, heart etc. – with highest expression in kidney, liver, cartilage, and bone - but no detection of protein. (36, 37, 50) (Figure 17) However, in mineralizing matrix imbedded cells can postnatally express sclerostin, for example osteocytes, cementocytes, and chondrocytes, when hypertrophic and terminally differentiated. (41, 51-54)

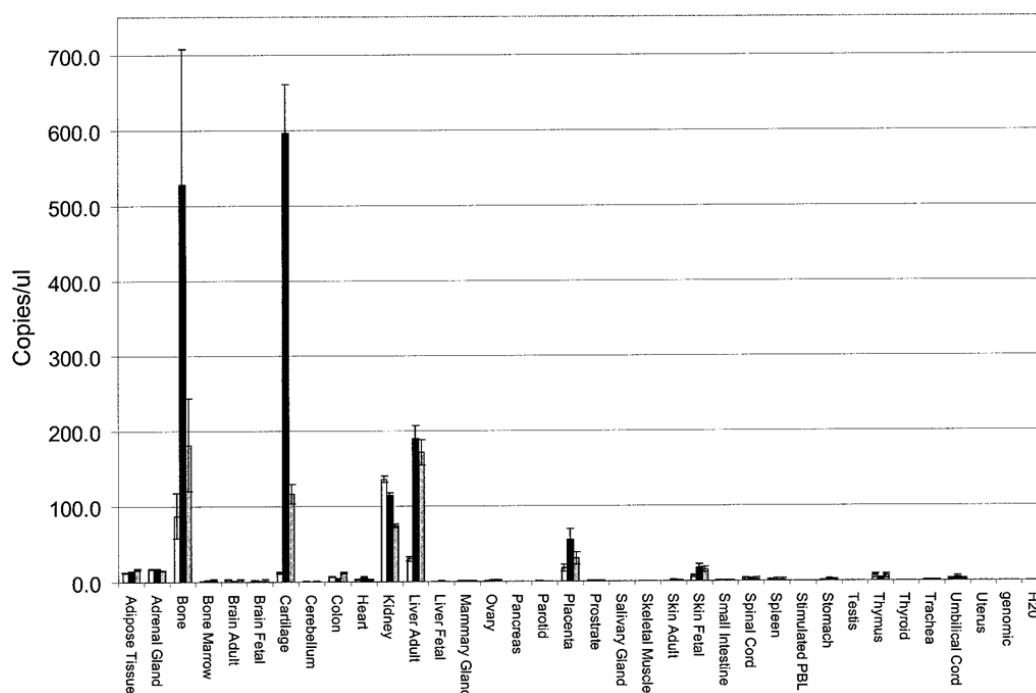


Figure 17: SOST mRNA expression in human tissues. Highest expression of SOST mRNA was found in bone, cartilage, kidney, and liver. Copies/μl are shown as raw mean SOST values (open bars), normalized to PPI (solid bars), and normalized to DAD1 (gray bars). (37)

1.3.3 Wnt-signaling path way

Sclerostin is secreted by osteocytes and transported to the bone surface via canaliculi to the circumferencing osteoblasts. (51) Sclerostin hinders osteoblastogenesis and promotes apoptosis of osteoblasts. Therefore the formation of new bone is lowered. (55) Wnt signalling can be influenced through different means; e.g reduction of sclerostin expression via mechanical loading. (56)

In canonical Wnt signaling, Wnt forms a complex through binding to LRP 5/6 and frizzled, a transmembrane receptor. GSK3 β is inhibited, β -catenin accumulates and migrates into the nucleus where it activates TCF/LCF. Subsequently transcription of target genes starts. Sclerostin – also Dkk and Sfrp - inhibit the differentiation of osteoblasts through blocking of the Wnt-signaling pathway. The protein binds the receptors LRP 5/6 so Wnt cannot bind and no complex is formed. A degradation of β -catenin follows through phosphorylation and proteolysis by GSK3 β . TCF/LEF are not activated and transcription of a target gene is not possible. (57, 58)

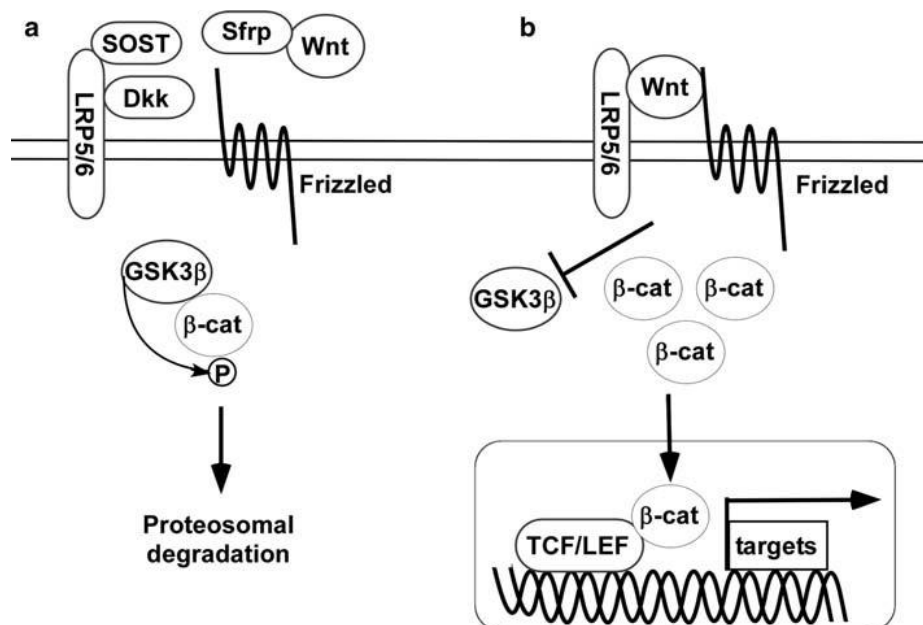


Figure 18: Wnt-signaling pathway. (a) SOST, Dkk and Sfrp are inhibitors that block binding of Wnt to LRP 5/6 and Frizzled. Without Wnt GSK3 β causes degradation of β -catenin. (b) If Wnt is present β -catenin migrates into the nucleus, activates TCF/LEF and target gene transcription starts. (57)

Recently LRP 4 has been described to bind sclerostin and Dkk in vitro. Choi et al. concluded that the receptor is involved in the growth and turnover of bone. (59) Further results suggest that sclerostin and Dkk regulate LRP 5 induced Wnt signaling independently. (60)

1.3.4 Current studies of sclerostin

The physiological importance of sclerostin is observed in experiments with genetically modified mice. These observations lead to the conclusion that sclerostin inhibits the formation of new bone. (58, 61, 62) For example a sclerostin knock-out mouse has pathologically increased bone formation. (61, 63)

1.3.4.1 α -hSOST

The increase of new bone formation is of therapeutical interest for treatment with sclerostin neutralizing antibodies. In the future, this could be a possible medication for the treatment of osteoporosis as well as for the enhancement of bone regeneration, e.g. after fractures. (64, 65) Several studies showed the potential of α SOST in animal models – mouse, rat and monkeys. (61, 64-69)

As mentioned before, physiological manifestations that are observed in SOST^(-/-) knock-out mice are increase in BMD, bone mass, formation, and strength. (61, 65) Studies for fracture healing - closed fracture of the femur - in rats showed that α SOST promotes at the fracture site strength and mass of bone. Further trabecular and cortical bone of healthy bones had enhanced strength, formation and mass. (68) The same results were observed by neutralization of sclerostin with α SOST in healthy male rats. (67) Another study demonstrated the increase in bone formation with α SOST through screws implanted at metaphyseal sites. A 50% increase of the pull-out force, in comparison to a control, was achieved. (66)

Ominsky et al. investigated the effect of α SOST on fracture healing in male cynomolgus monkeys with a fibular osteotomy. (68) The effect of humanized α SOST was studied in females cynomolgus monkeys with intact gonads. (69) In the male osteotomy model the fracture gap was thinner compared to controls and in both models bone strength, formation, and mass was increased. (68, 69)

In 2011 Padhi et al. - for Amgen® - published data of the first clinical phase I study with a monoclonal antibody for sclerostin, AMG 758, in humans. It was reported that the antibody, used in single dose, showed rise in markers for bone formation and BMD, as well as a dose-related decline in markers of bone resorption. The study medication was classified as generally safe and well tolerated. (70) The results encouraged further studies on this sclerostin neutralizing antibody and are now in phase II of clinical studies. (71)



Figure 19: $SOST^{-/-}$ mouse in comparison to a normal wild-type (WT). The knock-out (KO) mouse shows a higher BMD than the wild-type. (61)

1.3.4.2 Regulatory factors

The regulation of the expression of sclerostin is the aim of active research. At present it could be shown that functional loading (63, 72) but also oxygen tension are involved in the regulation of sclerostin expression. (73) Analysis of the effect of hormones, like PTH are also available. Although these studies are mostly limited to bone cells, especially osteocytes. (74-77)

1.3.4.3 Sclerostin in dentistry

In recent studies the expression of sclerostin in the periodontium has been observed. Sclerostin is expressed by cementocytes and therefore expressed in the periodontium. (52, 78) Also fibroblasts, isolated from the periodontium, express sclerostin as well as odontoblasts. (78, 79) These observations are not really surprising since cementocytes and also osteocytes – later are the main producers of sclerostin (51-54, 58, 62) – are imbedded in a mineralized matrix. (52, 78) Sclerostin, and also the Wnt-signaling pathway, are involved in the control of embryonic development of the tooth and its surrounding tissues. (48, 79) The Wnt-signaling pathway is not only active in embryonic periodontal development but also in PDLF of adult mice. (80)

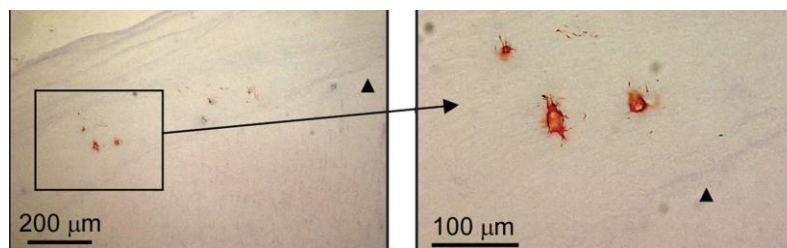


Figure 20: Expression of sclerostin by cementocytes (triangle) in a wisdom tooth (3rd molar) (52)

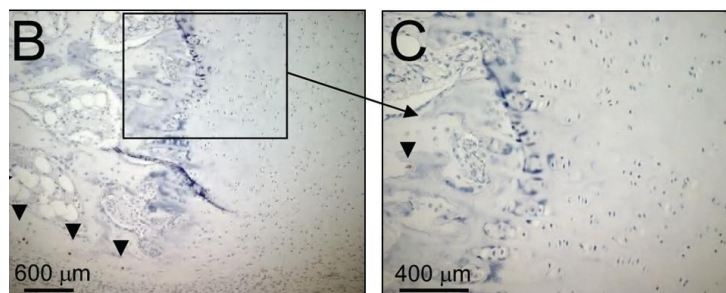


Figure 21: Expression of sclerostin by osteocytes (triangle) in cortical bone. (52)

At present there are no clinical studies concerning sclerostin as a possible target for dental treatment – in inflammation or dental defects. As mentioned before clinical studies with sclerostin neutralizing antibodies are showing promising results. Yet, these studies concentrate on the postcranial skeleton, so an impact on the periodontium may be possible and cannot be ruled out. Of interest for clinical use of such antibodies in dentistry could be healing of implants into the alveolar bone or minimalizing the risk of tooth loosening and loss in risk groups, e.g. patients with osteoporosis. (65, 81)

1.4 Problem

At present there are no studies about the regulation of sclerostin in the periodontium. These studies are of importance to understand the cellular processes of periodontal regeneration as well as pathological changes. Theoretically, a deficiency of sclerostin leads to a hypercementosis and thus to a narrowing of the periodontal gap. The constriction of the gap can lead to an ankylose, a boney anchoring of the tooth within the bone without PDL. This could maybe explain the difficulties of tooth extraction by people with sclerosteosis. (45)

In contrast, the overproduction of sclerostin may lead to the loss of mineralized periodontal structures – cementum and alveolar bone. So the regulation of the expression of sclerostin in the periodontium may have a physiological function, and a pathological role can also not be ruled out. Therefore it is important to identify the factors - expressed in the periodontium – that are involved in the regulation of sclerostin expression.

1.5 Aim

Investigations on how sclerostin is regulated in the periodontium may shed light on the role of sclerostin in the periodontium. Therefore, the aim of this study was to reveal effects of signaling molecules on the expression of sclerostin in periodontal fibroblasts.

1.6 Research questions

1. Do signaling molecules have an effect on the expression of sclerostin in periodontal fibroblasts?
2. Does sclerostin effect cell functions through an autocrine mechanism?

2 Materials and Methods

2.1 Materials

2.1.1 Media

- Serum-containing (SC-medium): α -MEM (PAA E15-832), 10% fetal calf serum (FCS; PAA 15-151) and 1% antibiotics (P/S/A; PAA).
- Serum-free medium (SF-medium): α -MEM and 1% antibiotics (P/S).
- Osteoblast differentiation medium (OD-medium): α -MEM, 20% FCS, 1% P/S/A, 10mM glycerophosphate (GP), 10^{-7} M dexamethasone (DEXA) and 50 μ M ascorbic acid-di-phosphate (AA-2-P)

2.1.2 Signaling molecules

- h platelet released supernatant (hPRS) (82)
- rh basic fibroblast growth factor (rhbFGF; R&D Systems 233-FB)
- rh bone morphogenic protein-6 (rhBMP-6; R&D Systems 507-BP)
- L-mimosine (L-Mimo; Sigma-Aldrich M0253)
- rh parathormone (rhPTH; Bachem 1026840)
- rh platelet-derived growth factor (rhPDGF-BB; R&D Systems 220-BB)
- rh SOST/sclerostin (rhsclerostin; R&D Systems 1406-ST-025)
- rh transforming growth factor beta-1 (rhTGF- β 1; R&D Systems 240-B-002)

2.1.3 Antibodies

- Anti-human goat monoclonal SOST (α -hSOST; R&D Systems 1406-AF)
- Anti-human goat polyclonal β -actin (α -h β Actin; Santa Cruz I-19 sc-1616-R)
- Polyclonal goat anti-rabbit Ig with HRP (α -rabbit HRP Dako P0448)

2.1.4 Viability, proliferation and protein synthesis assay

- ³[H]leucine (Hartmann Analytic MT 672E; Leucine, L-[4,5-H₃])
- ³[H]thymidine (Hartmann Analytic MT 6035; Thymidine, [methyl-³H]-)
- Dimethyl sulfoxide (DMSO; Merck 1.02952.1000)
- Scintillate cocktail (NEN Perkin Elmer 601 3611 Microscint 0)
- Top Seal A 96 well (NEN/Perkin Elmer 6005185)

2.1.5 Western Blot

- Acrylamid bis solution 40% (AA-bis; Merck/VWR 1.00640.1000)
- Blotting paper (Amersham HyphorTM)
- LandMarkTM Broad-Range Protein marker (Mbiotech MBI-20050)
- Microscopy Hemacolor® staining kit (Merck 1-11674.0001)
- Nitrocellulose membrane (VWR GHERPN203D)
- Western Blotting Detection System (AmershamTM RPN 2132)
- X-ray film (Amersham 28102)

2.1.6 ELISA

- Sclerostin ELISA-kit (Biomedica 100503; BI-20492 Sclerostin)

2.1.7 PCR

- DNase I Amplification Grade (Invitrogen 18068-015)
- Loading Dye (Fermentas #R0611)
- MassRulerTM DNA Ladder, Low Range (Fermentas #SM0383)
- Micro Amp Fast 96-well reaction plate 0,1 ml (Applied Biosystems 4346907)
- Primer pairs (GenXpress)

SOST

f 5'-ccaccctttgagaccaaagacgt-3'

r 5'-ggcccatcggtcacgtacg3'

β-actin (83)

f 5'-gcatcccccaaagttcaca-3'

r 5'-aggactgggccattctcctt3'

- RNeasy Mini Kit (Qiagen 74104)
- Sealing (IBL)
- Super ScriptTMIII Platinum® SYBR® Green one step qRT-PCR Kit with ROX (Invitrogen/Fisher Scientific 11746-100)
- Water – molecular biology grade (AppliChem 7732-18-5)

2.1.8 Equipment

- Beta counter (Packard Top Count NXTTM)
- Boyden Chamber (NeuroProbe #5956)
- Photometer (Molecular Devices SPECTRAmax® PLUS³⁸⁴)
- Radioactive cassette (Amersham Hypercassette)
- Western Blot apparatus (Bio-Rad Mini Protean® Tetra Cell)
- PCR-machine (Applied Biosystems, StepOnePlus Real-Time PCR System)

2.1.9 Additional materials

- 6 well-culture-plate (Costar 3506)
- 24 well-culture-plate (Costar 3527)
- 96 well-culture-plate clear (TRP® 92096)
- 96 well-culture-plate white (VWR/Nunc 13602)
- Counting chamber (Digital Bio DHC-N01)
- Methanol (Sigma-Aldrich 24229)
- Migration polycarbonate membrane 8µm pores (NewoProbe PFB8 302814)
- Plastic tubes (Sterillin® 128A)
- Reaction tube (Eppendorfer®)
- Syringe filter 0,2 µm pores (Corning 431229)
- Tissue culture dish (Sarstedt 83.1802)
- Tissue culture flask (Sarstedt 83.1812)

2.2 Methods

2.2.1 Cells

For the project human periodontal fibroblasts from extracted retained human third molars were used - fibroblasts of the gingiva (GF) and the periodontal ligament (PDLF). The GF cells were taken from gingival remains, the PDLF were scraped from the last third of the roots and put into tissue culture dishes. The cells were cultivated until the culture dish was covered. Then transferred to tissue culture flasks with SC-medium or stored in frozen nitrogen until further use. For the cells' sustainment the medium was replaced every 2-3 days. The cell culture flasks were changed after four passages and stored in an incubator at 37°C, 95% humidity and 5% CO₂.

Splitting

When the density of cells was high enough, the wall of the cell culture flask covered with nearly no free room in between the cells, they were harvested. The medium was aspirated, the cells washed with PBS and given TE to dispatch the cells from the tissue culture flask. SC-medium was added to stop the reaction and the cells mechanically flushed from the cell culture flask's wall and put into a plastic tube. Then they were centrifuged, resuspended in new SC-medium and returned into the cell culture flasks in a lower concentration. With each splitting the cells aged one passage; the cells were used till passage eight.

Harvesting

For the experiments the cells were split and the number of cells determined with a counting chamber. The cell concentration was adjusted with the dilution factor. The used concentrations were 1.7×10^5 c/ml (equals 50,000 c/cm²) and 1×10^6 c/ml (equals 300,000 c/cm²) depending on the experiment.

2.2.2 Alkaline phosphatase staining

ALP is an enzyme produced by mature osteoblasts. In histochemical staining alkaline phosphatase is used as a differentiation marker to determine the differentiation activity of osteoblasts. The protocol used was taken from D'Ippolito et al. (84) In this protocol the staining caused a yellow coloring in precursor cells and blue staining in differentiating cells.

Cells were plated on 96 well-culture-plates - 1.7×10^5 c/ml in SC-medium. After 24 h the cells were stimulated with SF-medium in presence and absence of rhTGF- β 1 (10 ng/ml), rhsclerostin (100 ng/ml), rhPTH (100 ng/ml) and α -hSOST (1:500), alone and in combination. After 72 h of incubation by 37°C, the cells were washed with PBS and affixed with neutral-buffered formalin. Subsequently washed with PBS, incubated with staining solution (pH 9), washed with a.d. Lastly the turnover rate of the substrate was photometrically measured at 550 nm and photos taken.

2.2.3 Viability assay, proliferation assay, protein synthesis assay

Cells were plated on 96 well-culture-plates - 1.7×10^5 c/ml in SC-medium. After 24 h the cells were stimulated in SF-medium with rhPDGF-BB (30ng/ml), PRS (1:5) and rhbFGF (10 ng/ml) alone and in combination with rhsclerostin (100ng/ml, 10 ng/ml, 1 ng/ml). After incubation for 24 h the analyses were done with corresponding assays.

Viability assay

MTT was added to the stimulated cells. After incubation for 2 h the MTT-media mix was removed, DMSO put into the wells and the plate tapped lightly until the formazan crystals dissolved. The OD was measured by 550 nm in the photometer. The assay analyses the impact of signaling molecules on cell viability, e.g. cytotoxicity. Yellow MTT substrate is reduced to violet formazan crystals by reducing enzymes, produced only in living cells. In a photometer, the

OD of the transformed substrate is measured and shows the effect of signaling molecules on the viability of cells in comparison to an unstimulated control.

Proliferation assay

To the stimulated cells $^3\text{[H]}$ thymidine was added, incubated for 6 h, washed in PBS and dried overnight. Subsequently a scintillate cocktail was added, a sealing put on top and the plate put into the beta-counter. The assay analyses the impact of signaling molecules on cell proliferation. The added $^3\text{[H]}$ thymidine is integrated into the DNA of proliferating cells. The incorporated $^3\text{[H]}$ thymidine sends out scintillating light signals which are detected by the beta-counter. The counted signals per minute (cpm) correspond with the proliferation rate. This shows the effect of signaling molecules on the proliferation of cells, either inhibiting or increasing, compared to an unstimulated control.

Protein synthesis assay

To the stimulated cells $^3\text{[H]}$ leucine was added, incubated for 6 h, washed in PBS and dried overnight. Next a scintillate cocktail was added, a sealing put on top and the plate put into the beta-counter. The assay analyses the impact of signaling molecules on cell protein synthesis rate. The added $^3\text{[H]}$ leucine is integrated into produced proteins. The incorporated $^3\text{[H]}$ leucine sends out scintillating light signals which are detected by the beta counter. The cpm correspond with the rate of protein synthesis. This shows the effect of signaling molecules on the protein synthesis rate of cells, either inhibiting or increasing, compared to an unstimulated control.

2.2.4 Boyden-Chamber-Assay

The migration assay analyses the chemotactical effect of signaling molecules on cells. An agent is chemotactical when it induces cells to move in its direction. The Boyden-chamber (85) consists of two chamber parts, a lower and an upper, with a thin permeable membrane in-between, that catches migrating cells.

The lower chamber was loaded with cells - 1×10^6 c/ml in SF-medium – a thin membrane put on it and the upper chamber added on top. The upper part was filled with different stimulations, PRS (1:5), Sclersotin (100 ng/ml), PTH (100 ng/ml), in SF-medium - alone and in combination. After incubation for 3 h at 37°C the membrane was washed and dried. Subsequently the cells were affixed with methanol, put into a red colour solution followed by a blue colour solution for staining, washed under water and dried. Cells that migrated through the membrane were stained violet; compared to an unstimulated control it showed if signalling molecules had a chemotactical impact. (Figure 22)

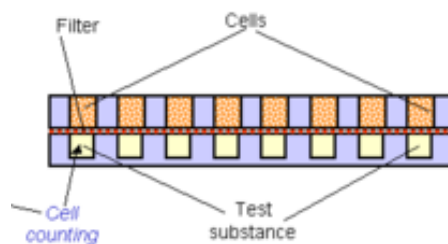


Figure 22: Standard Boyden-Chamber-Assay. (86)

2.2.5 Western Blot

The Western Blot, or protein immunoblot, is used for the search of specific proteins from samples. This is achieved through separation of proteins by size in gel electrophoresis, followed by transfer of the proteins onto a membrane and binding of antibodies to the wanted protein.

Sample preparation

Cells were seeded in 6-well culture plates – 1.7×10^5 c/ml in SC-medium - and left for 24 h to settle down. Subsequently the cells were stimulated in OD-medium with signaling molecules for 24 h, except for one row of samples which were incubated for 3 h, 6 h, 24 h, 48 h and 72 h. After the incubation period medium was removed, cells washed with PBS and mixed with SDS-sample buffer. Following cells were scraped from the well bottom using a police rubber man, put into reaction tubes, sonicated, thermocycled at -95°C , centrifuged, transferred to new tubes and stored at -20°C until further use.

Signaling molecules used were - alone and in combination – rhPDGF-BB (30 ng/ml), TGF- β 1 (10 ng/ml, 1 ng/ml, 1 pg/ml, 10 pg/ml, 1 pg/ml), PTH (100ng/ml), rhBMP-6 (30 ng/ml), PRS (1:5) and L-Mimo (1 nM). The primary antibodies (1:1000) with their corresponding secondary antibody (1:2000) used were α -h β Actin with α -goat HRP and α -hSOST with α -goat HRP.

Procedure

A polyacrylamide gel was prepared, consisting of a separating gel and on top a stacking gel with slots for the samples. The samples were loaded, the gel plates put into the electrophoresis apparatus, immersed in running buffer and run by 200 V for 45 minutes. Depending on the concentration of the acrylamid gel proteins were divided by size; smaller proteins wandered faster and further through the gel than bigger ones.

Next the segregated proteins were transferred from the polyacrylamide gel on a nitrocellulose membrane. Therefore a “sandwich” was built, sponge – filter paper – gel – membrane – filter paper – sponge. Put into the apparatus with transfer buffer and a cool aid for 45 minutes by 350 mA. Afterwards the

membrane was washed in TBS, incubated with blocking buffer (5% milk powder in TBS-T) for 1 h and again washed with TBS-T to remove excess blocking buffer. Then primary antibody (1:1000 in 5% BSA-TBS-T) specific to the target protein was added. After incubation over night by 4°C the membrane was washed in TBS-T, specific second antibody (1:2000 in 5% milk powder in TBS-T) added for 1 h and washed again in TBS-T. The blocking buffer bound unspecific to protein binding sites and later the specific binding antibodies replaced the bound milk proteins on the antigens. Subsequently the membrane was covered with detection solution and put in a radioactive cassette. In the darkroom an x-ray film was put on the membrane and developed. Chemo-luminescence was used for detection. The detection solution bound to the HRP on the secondary antibodies and blackened the x-ray film in the places where the proteins were on the membrane. The size and intensity of the blackening site showed the quantitative amount of specific bound protein. (Figure 23)

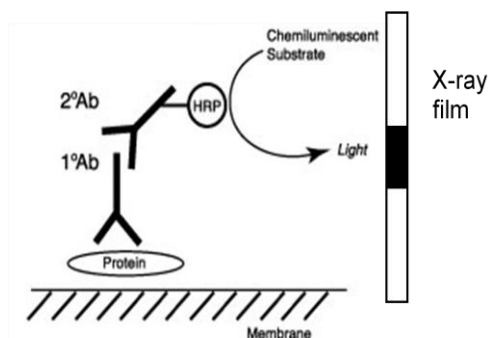


Figure 23: HRP Western Blot radioactive detection system. (87)

2.2.6 ELISA

The method is used for detection of a specific protein; based on the accumulation of antibody-antigen-antibody-enzyme complexes. The specific protein's antigens bind to the antibodies on the plate, the second enzyme-linked antibody binds on the protein. The substrate reacts with the linked enzyme and causes a color change. (Figure 24)

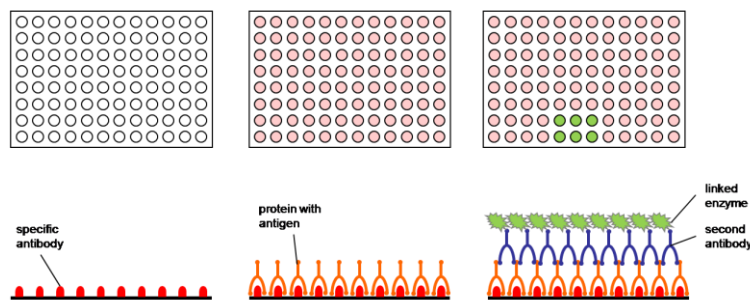


Figure 24: Standard ELISA

Sample preparation

Cells were seeded in 24 well-culture-plates – 1.7×10^5 c/ml in SC-medium. After 24 h the cells were stimulated in OD-medium with TGF- β 1 (10 ng/ml) for 72 h. Afterwards the supernatant was transferred to reaction tubes. The cells were washed with PBS, which was replaced by Triton (0,2%), sonicated, centrifuged, put into new tubes and stored at -20°C until further use.

Procedure

Samples were put into cell plates, coated with antibodies specific for a protein. Then a second specific enzyme-linked antibody was added, which bound to the protein. After incubation for ~24 h the cell plates were washed, conjugate buffer added, incubated for 1 h, washed and substrate added. After 30 minutes the procedure was stopped and the OD measured at 450 nm and 630 nm for sclerostin. A standard curve was created and the protein concentrations estimated for sample data.

2.2.7 PCR

In the PCR, a strand of mRNA is used as template, primers - with specific sequence - bind to the mRNA and build the complementary DNA sequence. Through an increase in temperature, the complementary strands dissolve. By lowering the temperature new primers can bind to the DNA strands and a new cycle is started. The constant repeat of cycles amplifies the specific DNA sequences exponentially and allows an assessment of the amount of mRNA contained in the original sample. (Figure 25)

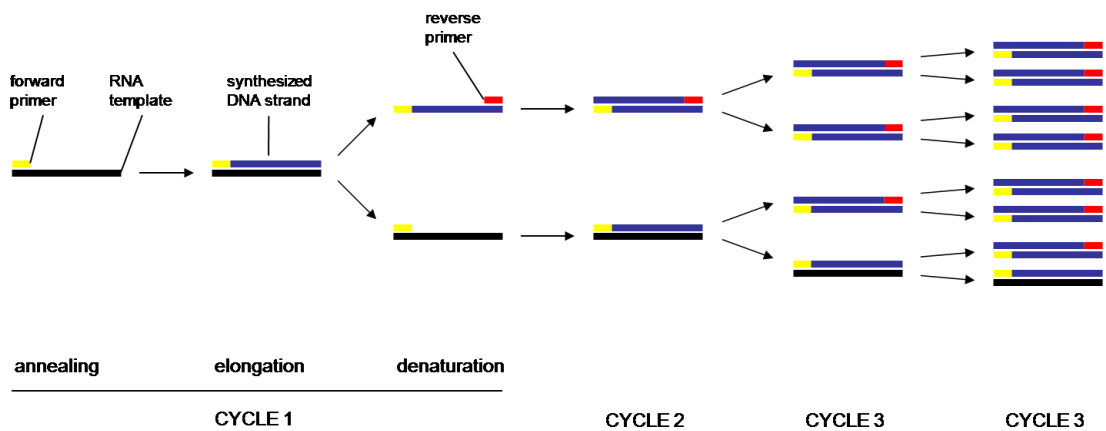


Figure 25: Standard PCR

Sample and RNA preparation

Cells were seeded in 6-well culture plates – 1.7×10^5 c/ml in SC-medium - and left for 24 h to settle down. Subsequently the cells were stimulated in OD-medium with TGF- β 1 (10 ng/ml) for 12 h and 24 h. After incubation, the cells were washed with PBS, which was replaced by lysis buffer containing beta-mercaptoethanole (β -ME). Following cells were scraped from the well bottom using a police rubber man, put into reaction tubes, homogenized with sonicator and 70%-ethanol added. In the next step the RNA was purified with the RNAeasy kit in several washing and elution steps following the protocol and in a.d. resuspended. The samples were stored at - 80°C until further use.

RNA quantification and DNase digestion

For verification of RNA presence and quality the samples were loaded on an agarose gel in TAE-buffer and checked - via built in β -ME – under UV light and photographed. For RNA quantification the samples' OD was measured and the concentration calculated for 1 μ g RNA per sample per experiment. Before the samples could be used for an experiment the DNase had to be digested, otherwise the DNases would degenerate the, during PCR generated, DNA. For the DNase digestion a DNase I-kit was used. The dilution of the RNA concentration of 1 μ g and the DNase digestion were done separately for each experiment with the required amount of RNA needed for the experiment.

Procedure

Master Mix was made of buffer and enzyme from the SYBR® Green one step qRT-PCR Kit mixed with RNase-free/DNase-free/purified water, divided into separate reaction tubes and the respective primers added. Digested and diluted RNA was put into the wells of the PCR plates first then the MM according to the loading scheme was added and the plate and a seal put on top. Directly after the plate was put into the PCR machine. The standard program consisted of 40 cycles and a followed melting curve. The analysis of the qRT-PCR was done with Microsoft® Excel 2007 and the $2^{-\Delta\Delta CT}$ method after Livak and Schmittgen. (88, 89) The stimulated samples were normalized to an external control gene, the house keeping gene β -actin. β -actin was chosen since it is expressed in different tissues and cells at a similar level.

3 Results

3.1 Question 1

Do signaling molecules have an effect on the expression of sclerostin in periodontal fibroblasts?

3.1.1 Screening

To answer objective 1, GF and PDLF were screened with different signaling molecules - PTH, TGF- β , BMP-6, PDGF-BB - and the effect on protein expression looked at via Western Blot. To ensure that α -hSOST binds sclerostin and not another of similar size a sample with rhsclerostin (30 ng/ml) was used for verification.

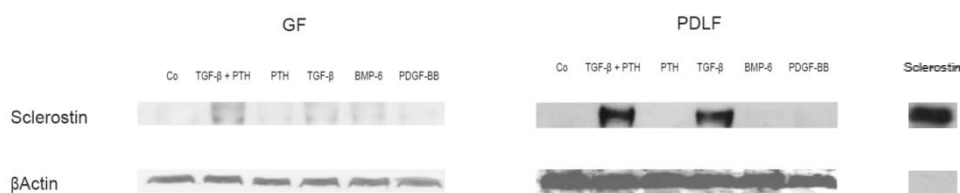


Figure 26: Screening with signaling molecules – Western Blot. GF and PDLF showed an increase in expression of sclerostin by stimulation with TGF- β compared to the unstimulated control. For verification of sample functionality β -actin was used; rhsclerostin for the α -hSOST specificity.

The Western Blot showed an increase in the expression of sclerostin by TGF- β and TGF- β + PTH in comparison to the unstimulated control. PTH alone, BMP-6, and PDGF-BB displayed no difference to the control. The band for rhsclerostin was the same height as the other bands. This supported the conclusion that the bound protein was sclerostin and that TGF- β increased the expression of sclerostin.

3.1.2 Reproducibility

To see if the TGF- β -induced effect was reproducible, GF and PDLF from different donors were incubated with TGF- β . The change in expression was observed on mRNA level with PCR and on protein level with Western Blot and ELISA.

3.1.2.1 PCR

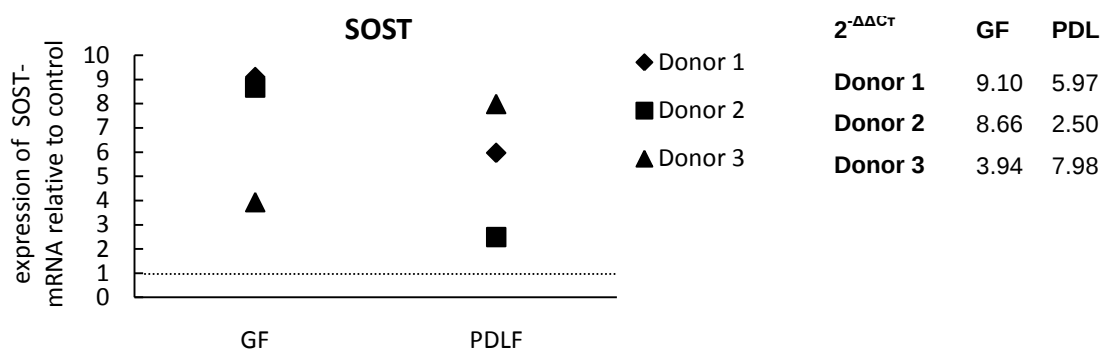


Figure 27: Reproducibility – PCR. The expression of SOST-mRNA is in all three donors of GF and PDLF higher than the control (dotted line). The fold-change differs between the donors.

In the PCR, all three TGF- β stimulated GF and PDLF donors showed an increase in the expression of SOST-mRNA compared to the control after normalization to β -actin. The increase of mRNA was 9.1-fold, 8.7-fold, 3.9-fold by the GF donors and 6-fold, 2.5-fold and 8-fold the PDLF donors.

3.1.2.2 Western Blot

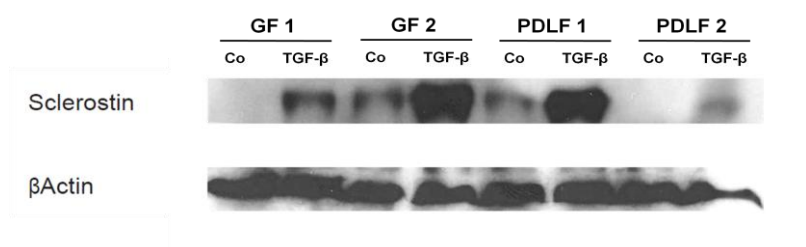


Figure 28: Reproducibility – Western Blot. GF 1/2 and PDLF 1 showed an increase in expression of sclerostin by stimulation with TGF- β compared the respective unstimulated control. For verification of sample functionality β -actin was used.

In the Western Blot, TGF- β increased the expression of sclerostin compared to the unstimulated control, but the level of the expression differed between donors. GF 2 and PDL 1 showed strong signal - more sclerostin protein - while GF 1 had a lighter and PDL 2 barely any signal compared to the respective unstimulated controls.

3.1.2.3 ELISA

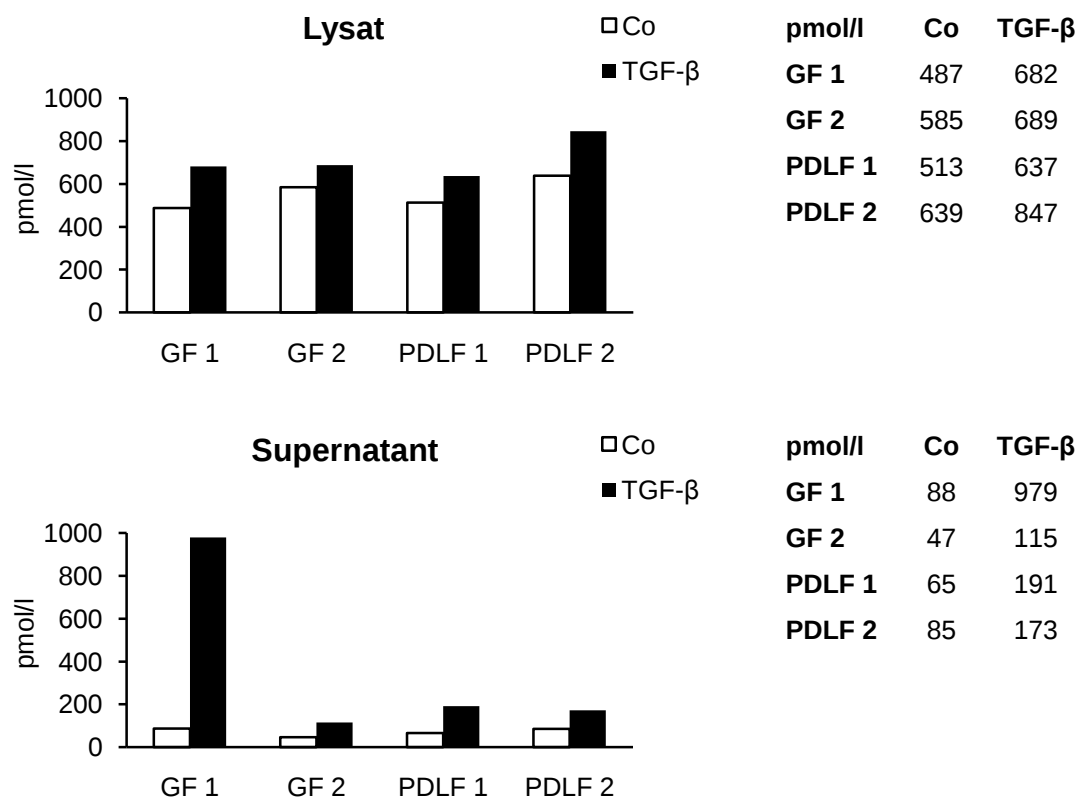


Figure 29: Reproducibility – ELISA. The expression of the protein sclerostin (in pmol/l) is shown for GF 1/2 and PDLF 1/2. All donors showed an increase by stimulation with TGF- β compared to respective unstimulated control – in lysat and supernatant.

The ELISA data also shows higher amount of sclerostin in TGF- β stimulated samples in comparison to the respective controls – lysat and supernatant. Although all donors produce more protein with stimulation, the amount differs between the donors as well as between origin of the cells.

Next it was of interest what kind of conditions were necessary to achieve the increased expression of sclerostin. Therefore, it was tested for time-dependency and dose-dependency.

3.1.3 Time

To see what time period was necessary for TGF- β to increase sclerostin, cells were incubated with TGF- β for different time points.

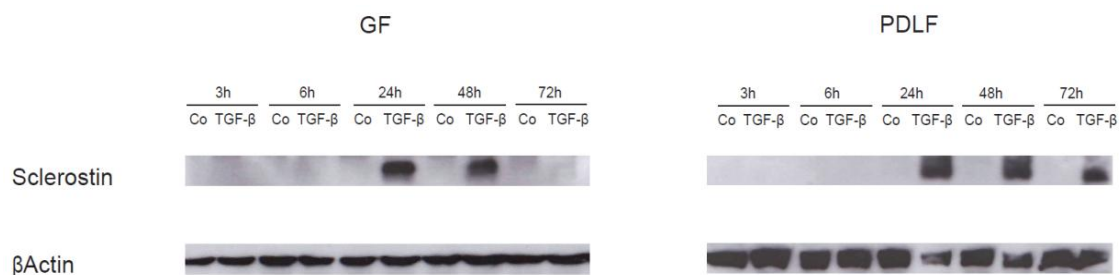


Figure 30: Time – Western Blot. GF and PDLF showed an increase in expression of sclerostin by stimulation with TGF- β after 24 h compared to the respective unstimulated control. For verification of sample functionality β -actin was used.

An increase of the expression of sclerostin was detected at 24 h on protein level via Western Blot in GF and PDLF. The Western Blot showed an increase of sclerostin between 24 h – 48 h in GF and 24 h – 72 h in PDLF. The increase was strongest after 24 h and decreased with continued time. Here, it took 24 h for TGF- β induced increase to take place.

3.1.4 Dose

To see what concentration of TGF- β was necessary to increase sclerostin, cells were incubated with TGF- β in different concentrations (dilution row 1:10).

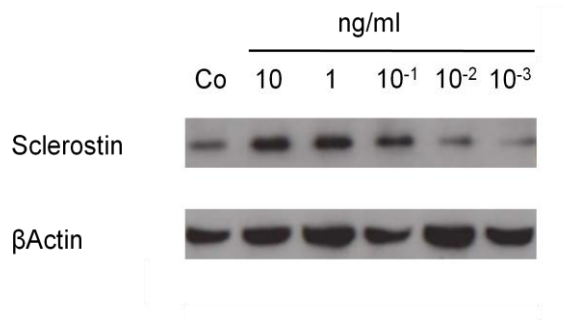


Figure 31: Dose – Western Blot. An increase in expression of sclerostin by stimulation with TGF- β 1 -10 ng/ml, 1 ng/ml, 0.1 ng/ml - compared to unstimulated control was shown. For verification of sample functionality β -actin was used.

The Western Blot showed that concentrations of TGF- β between 10 – 0.1 ng/ml increased the expression of sclerostin compared to the unstimulated control. 10 ng/ml and 1 ng/ml showed the same strength and 0.1 ng/ml a slightly weaker signal intensity.

3.1.5 Result 1

TGF- β increased the expression of sclerostin on mRNA and protein level. The effect is donor-, time-, and dose-dependent. This gives rise to the question what effect sclerostin has on cell functions.

3.2 Question 2

Does sclerostin effect cell functions through an autocrine mechanism?

To answer question 2 the effect of sclerostin (100 ng/ml, 10 ng/ml, 1 ng/ml) alone and in combination - with PDGF-BB, PRS, bFGF, PTH - was tested on the cell functions viability, proliferation, protein synthesis, and chemotaxis. It was also tested for a possible impact of sclerostin on fibroblastic differentiation.

3.2.1 Viability

To test for possible effects of the growth factors on the viability of GF and PDLF a viability assay was used.

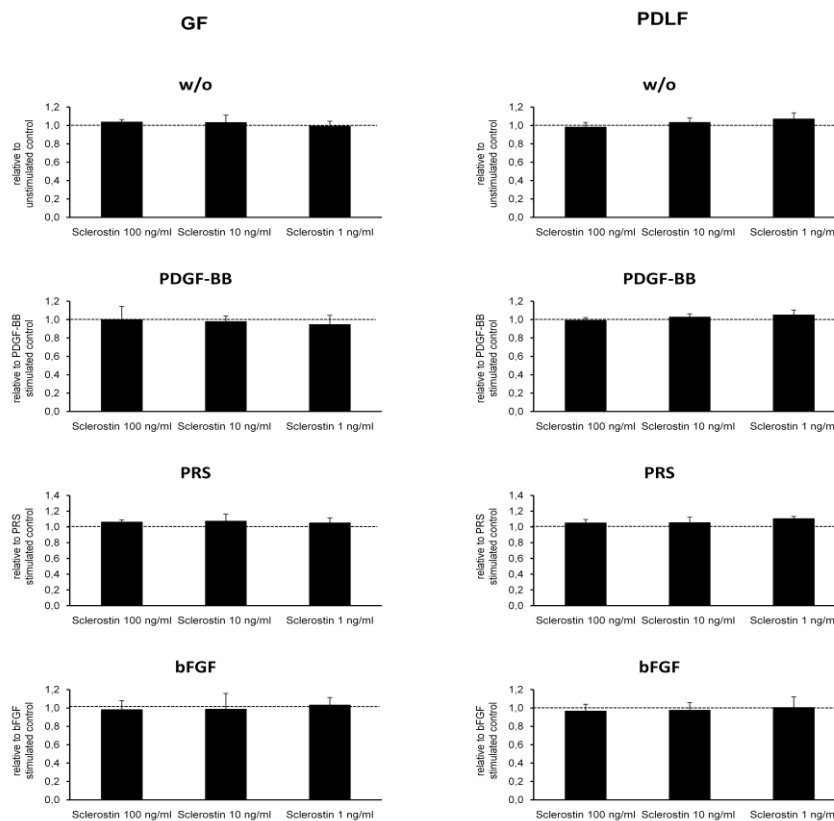


Figure 32: Viability. None of the growth factors used – w/o, PDGF-BB, PRS, and bFGF – showed an effect on viability in GF and PDLF compared to the respective unstimulated control (dotted line). Neither alone nor in combination with different concentrations of sclerostin.

Recombinant sclerostin alone showed no effect on the viability of GF and PDLF compared to control. Combination of sclerostin with PDGF-BB, PRS, and bFGF had no impact - in the used concentrations – compared to respective controls.

3.2.2 Proliferation

To test for possible effects of the growth factors on the proliferation of GF and PDLF a proliferation assay was used.

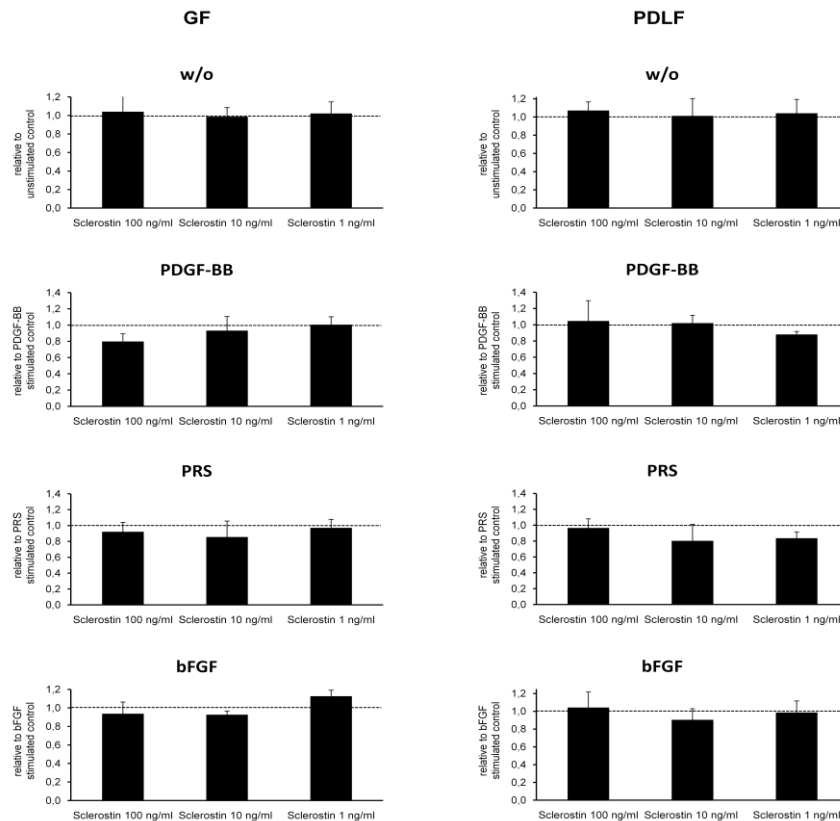


Figure 33: Proliferation. None of the growth factors used – w/o, PDGF-BB, PRS, and bFGF – showed an effect on proliferation in GF and PDLF compared to the respective unstimulated control (dotted line). Neither alone nor in combination with different concentrations of sclerostin.

Recombinant sclerostin – 100 ng/ml, 10 ng/ml, 1 ng/ml - alone showed no effect on the proliferation of GF and PDLF compared to the control. The combination of sclerostin with PDGF-BB, PRS, and bFGF had no impact - in the used concentrations - compared to the respective controls.

3.2.3 Protein synthesis

To test for possible effects of the growth factors on the protein synthesis rate of GF and PDLF a protein synthesis assay was used.

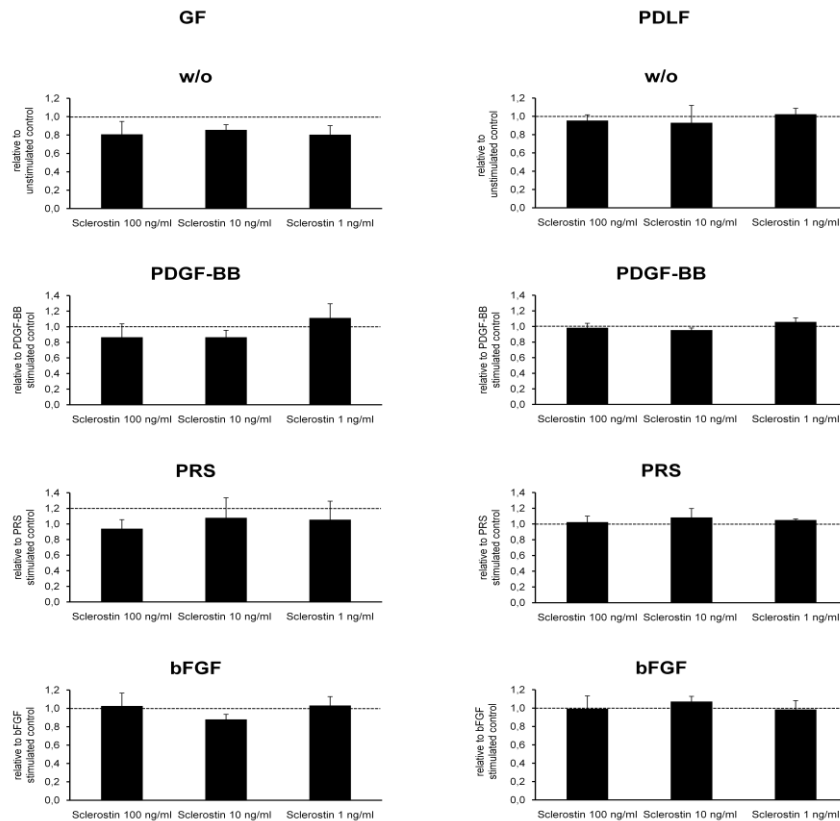


Figure 34: Protein synthesis. None of the growth factors used – w/o, PDGF-BB, PRS, and bFGF – showed an effect on proliferation in GF and PDLF compared to the respective unstimulated control (dotted line). Neither alone nor in combination with different concentrations of sclerostin.

Recombinant sclerostin – 100 ng/ml, 10 ng/ml, 1 ng/ml - alone showed no effect on the protein synthesis rate of GF and PDLF compared to the control. The combination of sclerostin with PDGF-BB, PRS, and bFGF had no impact - in the used concentrations - compared to the respective controls.

3.2.4 Chemotaxis

To test for possible effects of the signaling molecules on the chemotaxis of GF and PDLF a Boyden-Chamber-assay was used.

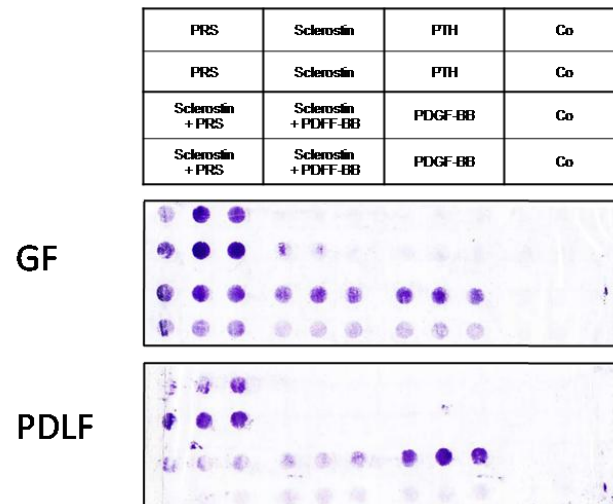


Figure 35: Boyden-Chamber-Assay. PRS and PDGF-BB – alone and in combination with sclerostin – induced cells to migrate. PTH, w/o, and sclerostin alone had no effect on cells. w/o verified that migratory had to be stimulant-dependent.

The cells in the unstimulated control did not migrate, meaning possible migration had to be induced by the signaling molecules. PRS and PDGF-BB alone and combined with sclerostin induced the cells to migrate. Neither sclerostin alone nor PTH provoked migration of cells. These data suggest that sclerostin shows no chemotactical effect on GF and PDLF in the used concentration.

3.2.5 Differentiation of fibroblasts

To determine, if signaling molecules have an effect on the differentiation of periodontal fibroblasts and therefore possible a regulatory effect on the expression of sclerostin, GF and PDLF were incubated with signaling molecules – sclerostin, TGF- β , PTH – and determined the differentiation activity via ALP staining.

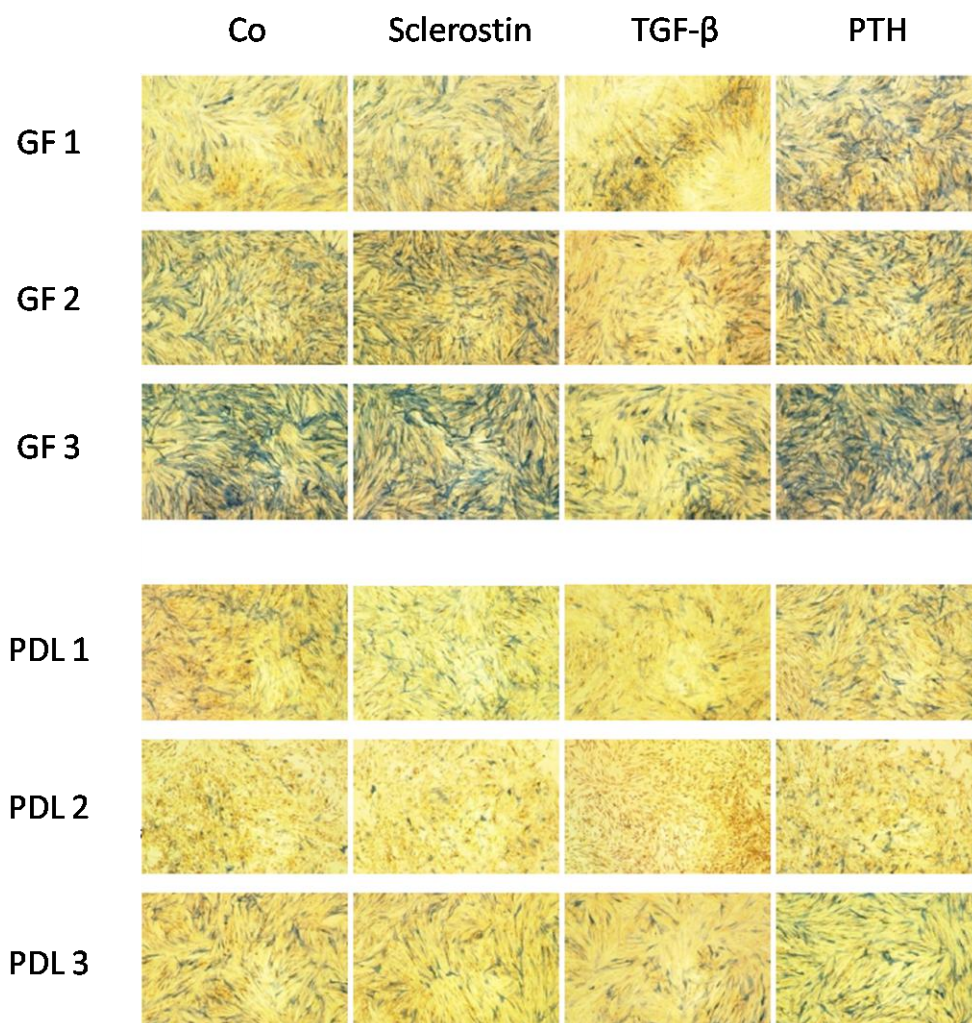


Figure 36: Differentiation - ALP staining. GF and PDLF are both positive for fibroblast differentiation GF stronger than PDLF. TGF- β showed an inhibitory effect, sclerostin and PTH no effect on the cells compared to the respective controls control.

GF and PDLF showed differentiation activity and were positive for blue staining, GF stronger than PDLF. GF 3 had the most intense staining followed by GF 1/2; PDLF 1/3 had the same amount of staining while PDLF 2 only showed single ALP positive cells. Sclerostin and PTH had no effect on the staining. TGF- β showed an inhibitory effect on differentiation in comparison to the respective unstimulated control.

3.2.6 Mediator of inhibiting effect

To see whether this inhibitory effect of TGF- β was mediated by sclerostin, mediated GF and PDLF were incubated with TGF- β and α -hSOST – alone and in combination – and tested for differentiation activity via photometry and ALP staining.

3.2.6.1 Photometry

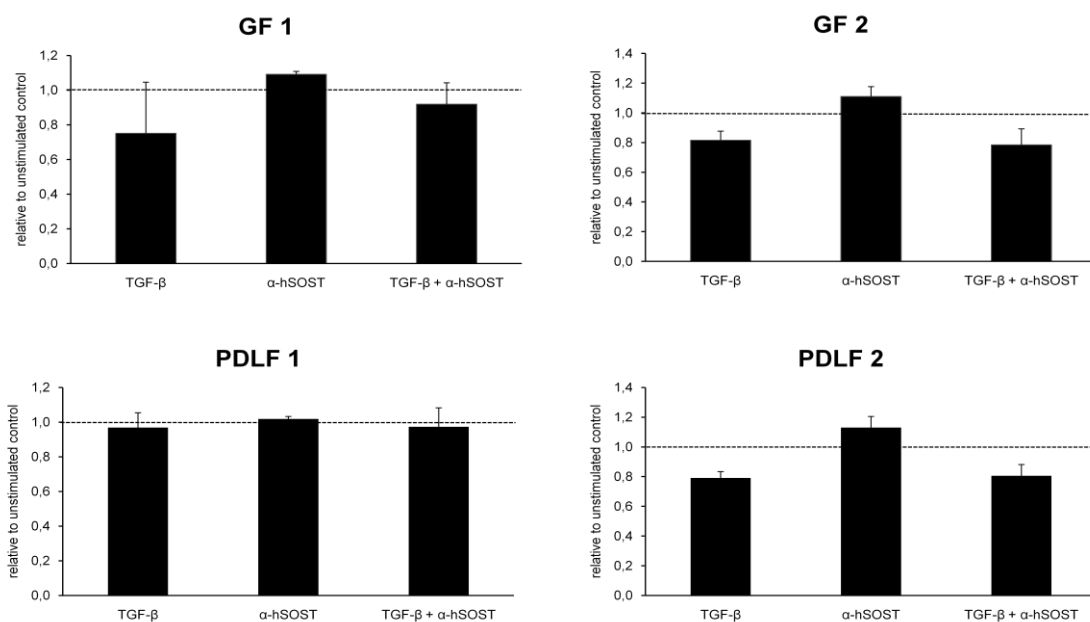


Figure 37: Mediation - Photometry. Compared to the respective unstimulated control (dotted line) TGF- β alone and in combination with α -hSOST showed an inhibitory effect, α -hSOST none. The effect was observed in GF 1/2 and PDLF 2; PDLF 1 had no reaction at all.

GF 1/2 as well as PDLF 2 showed a reaction to stimulation, only PDLF 1 had none. TGF- β and TGF- β + α -hSOST showed an inhibitory effect on ALP, α -hSOST alone had no impact, compared to the respective controls.

3.2.6.2 ALP staining

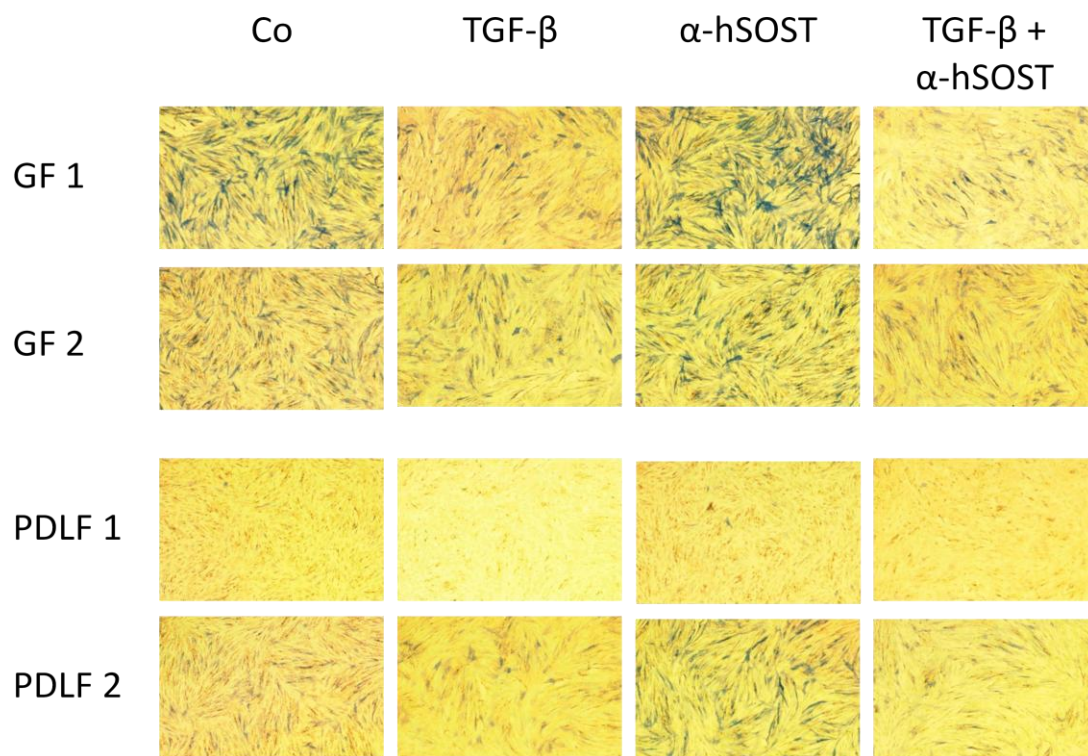


Figure 38: Mediation - ALP staining. GF and PDLF are both positive for fibroblast differentiation - GF stronger than PDLF. α -hSOST alone had no effect, TGF- β and TGF- β + α -hSOST showed reduced staining, compared to the respective controls. Therefore the observed inhibitory effect was TGF- β mediated.

GF and PDLF showed differentiation activity and were positive for blue staining, GF stronger than PDLF. GF 1 had the most intense staining followed by GF 2; PDLF 2 had a light staining while PDLF 1 had none. Compared to control α -hSOST alone had no effect. TGF- β and TGF- β + α -hSOST showed reduced staining compared to the respective controls.

3.2.7 Result 2

Recombinant sclerostin showed no effect on cell viability, proliferation, protein synthesis, and chemotaxis. Sclerostin had no effect on fibroblast differentiation – neither α -hSOST, recombinant protein nor sclerostin released in the presence of TGF- β . Therefore the observed inhibitory effect on differentiation was TGF- β mediated.

3.3 Summary

Objective 1

TGF- β increased the expression of sclerostin on mRNA and protein level. The effect is donor-, time-, and dose-dependent.

Objective 2

Sclerostin had no direct effect on cell viability, proliferation, protein synthesis, and chemotaxis. Fibroblast differentiation was not affected by sclerostin – neither α -hSOST, recombinant protein nor sclerostin released in the presence of TGF- β . Therefore the observed inhibitory effect was TGF- β mediated.

Summary

TGF- β 1 increased the expression of sclerostin in periodontal fibroblasts. Sclerostin itself had no effect on cell functions. However, an autocrine/paracrine mechanism cannot be ruled out.

4 Discussion

Our main finding is that TGF- β 1 up-regulates the expression of sclerostin in periodontal ligament fibroblasts and gingival fibroblasts. This observation is important since sclerostin, a key regulator of bone regeneration through inhibiting the Wnt-signaling pathway, is not exclusively expressed in osteocytes, but also in cells of the periodontium. (58, 78, 79) Sclerostin alone did not influence viability, proliferation, proteinsynthesis, chemotaxis, and osteogenic differentiation; an autocrine function could not be shown.

Regulation of sclerostin expression through TGF- β 1 in GF and PDLF has not yet been reported, thus being a novel finding. The effect of TGF- β 1 on the expression of sclerostin has only been looked at in one study by Sutherland et al. in osteoblasts. (90) However, compared to our study, the effect of TGF- β 1 on the expression of sclerostin was deemed negligible. (90) A study in neonatal mice showed enhanced gene expression of TGF- β receptors type 2/3 and sclerostin in the perichondrium of the temporomandibular joint. The functional role of sclerostin was defined as unclear; a connection between an increase of TGF- β and sclerostin was not drawn. (91) Gao et al. showed that the TGF- β receptors type 2/3 are expressed in the periodontium. (92) Also regulatory effects of BMPs on the expression of sclerostin have been published. Expression of sclerostin is induced by BMP-2/4/6 in osteogenic cells of human and mouse. (90) The knock-out of BMP receptor in osteoblasts caused decrease in sclerostin and enhancement of BMP signalling increased sclerostin. (93, 94) All together a regulation of sclerostin through TGF- β 1 is supported by earlier studies that showed the presence of TGF- β in the periodontium and a common increase in the expression of TGF- β and sclerostin.

Our finding that sclerostin is expressed in periodontal cells was also observed in other studies. Cementocytes in the cementum (52, 78) PDLF in the PDL (78), osteoblasts and osteocytes in bone (36, 51-55, 58, 62, 90) as well as in

periodontal cells in mineralizing matrices (52, 90). Also recently odontoblasts have been shown to be able to express sclerostin. (79) That GF produce sclerostin like PDLF is supported by the fact that sclerostin is also expressed in other soft tissues in adults (36, 37, 49, 50) and TGF- β 1 in gingival tissue (95). Further it was shown that GF and PDLF are similar to osteoblasts, with the potential to produce mineralizing structures like cementum or bone. (96) Summed up, the expression of sclerostin in GF and PDLF and the regulation by TGF- β 1 is supported by the presence of TGF- β 1 and/or sclerostin in PDL and gingiva.

The literature contradicted our data that showed no effect of rhSclerostin on proliferation, viability, and osteoblastogenesis. A study showed a significant decrease in proliferation and osteoblastogenesis, as well as a significant increase in apoptosis. (55) An inhibition of osteoblastic differentiation was also reported in hMSC. (53) The time-dependency of the expression of sclerostin – 0 h no sclerostin, 24 h sclerostin – in OD-medium has also been observed in PDLF. (78) The use of the same antibody for Western Blot as in our study supports that the found protein was sclerostin. (78) Our data on sclerostin's effect on cell functions differs from literature. Reasons therefore may be the use of different fibroblasts types and incubation times.

Of clinical relevance is the interaction of TGF- β 1 and sclerostin in dental defects and chronic inflammations. TGF- β is involved in wound healing (95); inhibition of sclerostin up-regulated healing in bone defects. (66). The study's data support a regulatory interaction between these two factors. Treatment with sclerostin neutralizing antibodies could improve and speed up the healing at defect sites or in chronic inflammation, enhance the healing of implants into the alveolar bone as well as be used in osteoporotic patients to lower the risk of tooth loss. (81)

Limiting factors of the study were, for example, that the effect of TGF- β 1 on sclerostin expression was only investigated in GF and PDLF. Other cell types -

cementocytes, osteoblasts, and osteocytes - express sclerostin as well (36, 51-55, 90) and therefore should also be tested. Bioassays to determine the consequences of increased sclerostin are missing. Experiments with supernatants without the presence of TGF- β 1 are a possibility for further research. The data for the impact of sclerostin on osteoblastogenesis differ from literature. It may be that the number of receptors was insufficient or a factor responsible for the expression of the receptors was missing. Further studies are of interest to determine the reasons. Due to the donors' anonymity, gender, age, health status, and life style - smoking, drinking, medications - were unknown. The data are general and not specific to gender, age, or health. Therefore, this background information may help to explain different reactions and results between donors. Another restriction of this study is the in vitro model. In this closed system only a limited number of cell types and signaling/transcription/differentiation factors are present that interact and react together.

Future studies should include in vivo animal or human models to show possible effects of TGF- β 1 induced sclerostin expression in the periodontium in an open system. Further, studies to decipher the mode of regulation for sclerostin and the signaling pathway between TGF- β 1 and sclerostin are needed.

Summarizing the findings suggest that in the periodontium the bone regulator sclerostin is produced, and up-regulated by TGF- β 1, a periodontal expressed growth factor. Thus, a functional role of sclerostin in the periodontium is possible. However, whether sclerostin has a physiological or a pathological function in periodontal tissues is still unknown.

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Figure 15: Reference (40)

Figure 16: Reference (47)

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Figure 18: Reference (57)

Figure 19: Reference (61)

Figure 20: Reference (52)

Figure 21: Reference (52)

Figure 22: Reference (86)

Figure 23: Reference (87) modified

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