A clinically translatable concept for periodontal ligament engineering around dental implants

The characterization of patient-friendly materials with optimal biomechanical properties

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Citation for published version (APA):

de Jong, T. (2017). *A clinically translatable concept for periodontal ligament engineering around dental implants: The characterization of patient-friendly materials with optimal biomechanical properties*. [Thesis, fully internal, Universiteit van Amsterdam].

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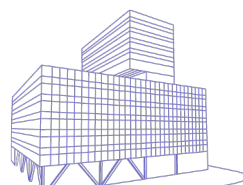
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A clinically translatable concept for periodontal ligament engineering around dental implants

The characterization of patient-friendly materials
with optimal biomechanical properties

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. ir. K.I.J. Maex
ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Aula der Universiteit
op vrijdag 17 februari 2017, te 11:00 uur

door Thijs de Jong
geboren te Nijmegen

PROMOTIECOMMISSIE

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Faculteit der Tandheelkunde

Voor mijn meisjes

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Chapter I

General introduction and overview of thesis chapters

GENERAL INTRODUCTION

When teeth are lost, it is becoming increasingly popular to replace them with dental implants. In 2002, approximately 700,000 dental implants were placed in the United States.[1] This number has risen to an estimated 1,260,000 dental implants placed in 2014, and is expected to double in 7 years.[2] The increasing popularity of dental implants may be explained by the high initial success rate of implant procedures, which was reported to be higher than 97% for single tooth replacements.[1]

However, after implantation, implant related complications are highly prevalent. For example, 80% of patients that receive dental implants develop bacterial peri-implant mucositis, an infection of the gingiva surrounding the implant. [3] Although mucositis is curable, in 28-52% of patients it progresses to peri-implantitis, a more severe condition that is accompanied by irreversible loss of alveolar bone.[3,4] Peri-implantitis-related bone loss may result in implant loosening and eventually in loss of the implant.[5] In a systematic review of 51 studies, Berglundh et. al showed that within 5 years of implantation already 6.5% of implants are lost as a result of peri-implantitis.[6] The majority of these implants are lost due to bone reduction.[6,7] Moreover, more than 10% of implants are lost within 5 years when bone augmentation is necessary prior to implant placement.[6,8]

Peri-implantitis is mainly caused by bacterial invasion of the alveolar bone that surrounds the implant. Peri-implantitis progresses rapidly, and has a high recurrence rate, even after successful treatment.[9–11] The reason for this is that dental implants are placed directly into the alveolar bone, where they need to osseointegrate to provide stability. To improve osseointegration, the implant surface roughness is highly increased.[12] Unfortunately, increased surface roughness also improves biofilm attachment, and aids in bacterial colonization. [13] Furthermore, loads on the implant are directly transmitted to the bone, which can result in micro-fractures of the alveolar bone. This increases bone loss and allows further bacterial invasion.[14]

Dental implants differ from natural teeth in many aspects. One of them is that teeth are protected and supported by a group of specialized tissues called the periodontium. The periodontium consists of the gingiva, alveolar bone, root cementum, and the periodontal ligament (PDL) (figure 1). The gingiva functions as a protective barrier against pathogens from the oral cavity. The alveolar bone, root cementum and PDL facilitate the attachment of the teeth to the maxillary and mandibular bones.[15] The PDL consists of an aligned connective tissue, that is interposed between, and anchored to, the mineralized surfaces of the cementum and alveolar bone. The PDL provides mechanical stability,

and functions as a shock absorber that protects the tooth and alveolar bone from the high forces of mastication.[15] Moreover, the PDL provides sensory information to the masticatory system, and forms a physical barrier against pathogens.[15]
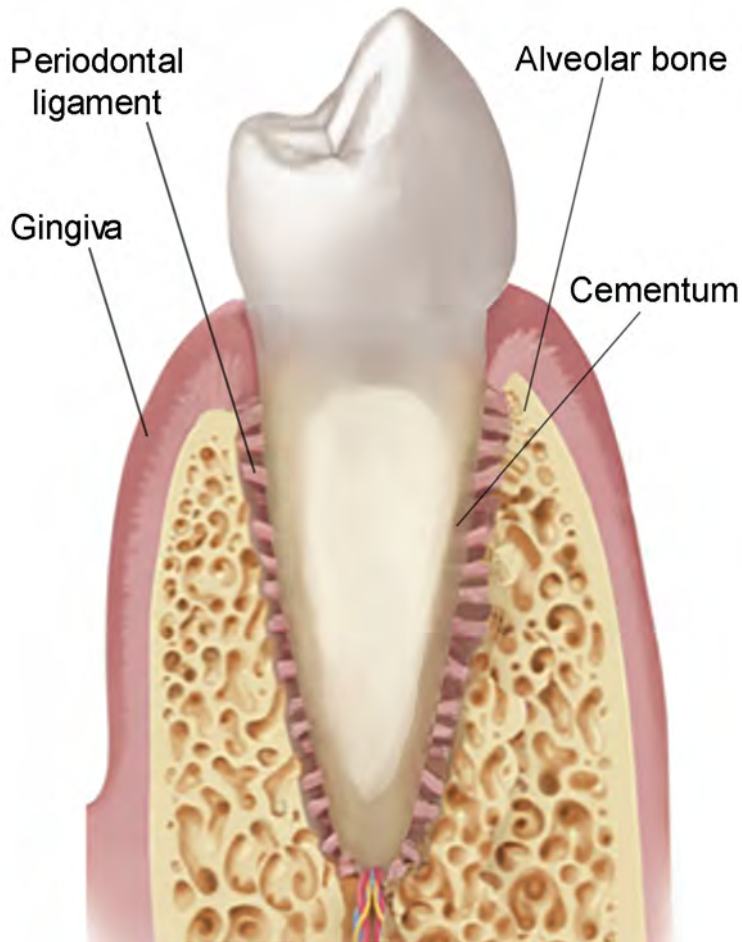
Figure 1: Schematic overview of the periodontium

CONCEPT DESIGN
As stated above, the way dental implants attach to the jaw bone greatly differs from natural teeth. With only the alveolar bone and gingiva present, dental implants do not have a complete periodontium. However, they would most likely benefit from a structure such as the PDL, as the main reasons for their problems are caused by unbuffered loading and bacterial progression along their rough surface (figure 2).
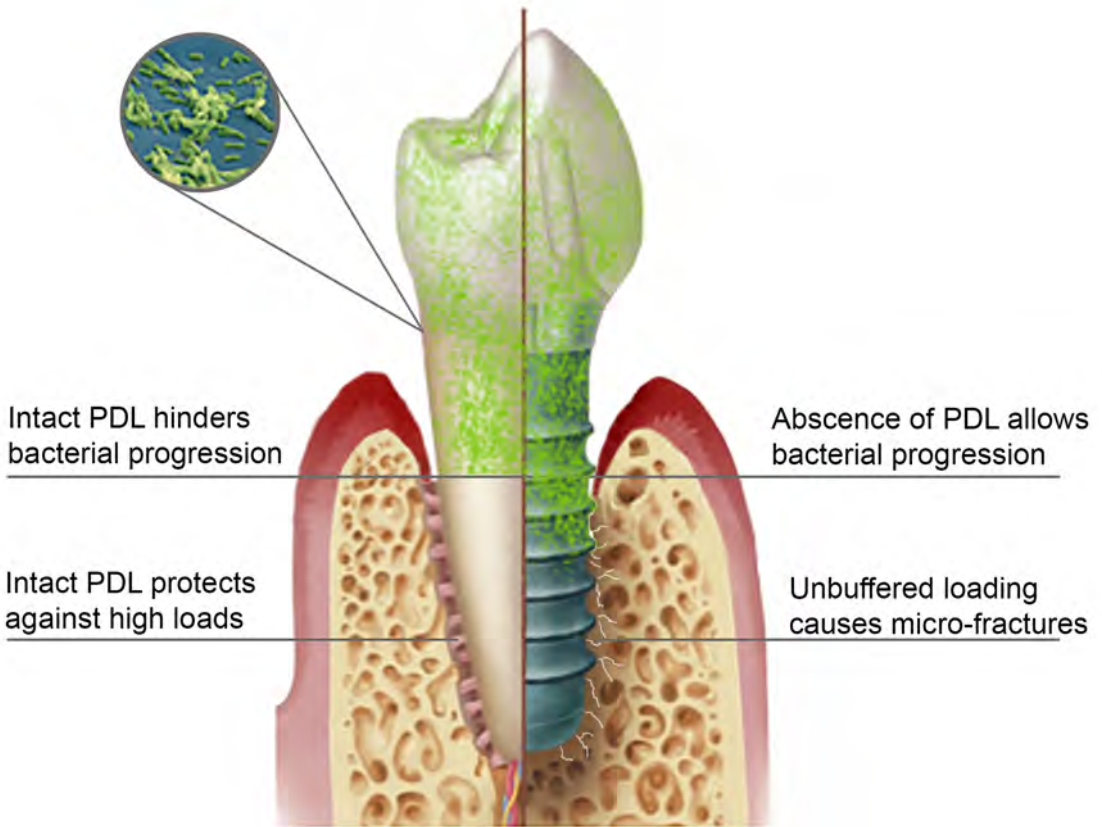
Figure 2: Schematic overview of the differences between natural teeth and dental implants. The healthy periodontium protects the tooth, and buffers loading from mastication. The surface of dental implants does not prevent bacterial infiltration, and unbuffered loading may lead to micro-fractures of the alveolar bone.

This thesis is centered around PDL tissue engineering, and presents a concept for the tissue engineering of a PDL around dental implants. The premise of this concept is to ultimately translate these implants to the clinic. Such premise has a great impact on the choice of biomaterial and stem cell type, and may create restrictions that would not exist if clinical translation was not aspired. To speed up clinical translation, cells and materials are not only required to perform their specific task for PDL generation, but need also be patient-specific, or approved for clinical use. Furthermore, the cells and materials should be clinically relevant, which means that their suitability for clinical tissue engineering greatly depends on availability, accessibility, and possible donor-site morbidity.

In chapter 2 the natural development of the PDL is reviewed, which provides a solid background for PDL tissue engineering. This knowledge formed the basis for the design of the concept for PDL tissue engineering around dental implants (figure 3). This concept is based on three distinct processes that are key for the formation of the PDL.

CONCEPT DESIGN
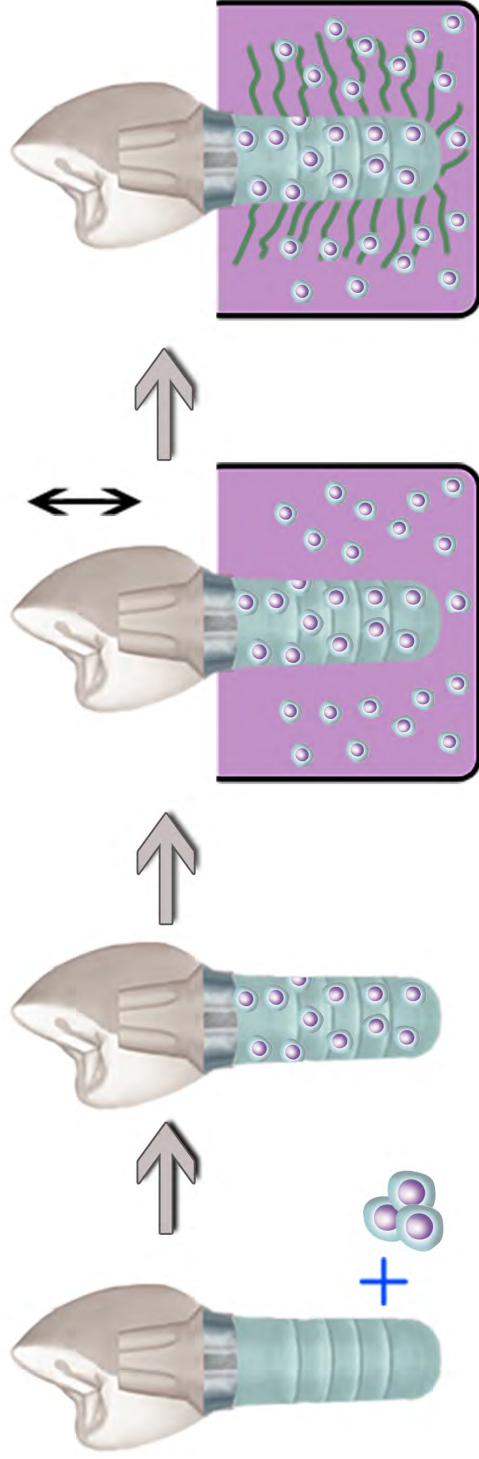
The Implant surface is seeded with stem cells from the intentended implant recipient.

The seeded implant is cultured in a gel containing stem cells from the same donor. Biomechanical stimuli are applied in order to stimulate the cells to secrete and align a ligamentous matrix.

Figure 3: In-vitro concept for PDL tissue engineering around dental implants

The first is that during PDL development, initial fiber formation starts on the root surface.[16] In the concept, this is mimicked by seeding stem cells from the intended implant recipient on the implant surface. The second process is the growth of fibers into the PDL proper, which constitutes the non-mineralized central part of the PDL. During development, the PDL proper consists of a randomly orientated collagenous mass that contains PDL-precursor cells.[17] In the concept, this mass is mimicked by a hydrogel that houses the same stem cells that were seeded on the implant surface. Thirdly, forces resulting from dental loading determine the alignment of PDL fibers, and stimulate PDL fibroblasts to produce collagen to accordingly increase PDL strength.[18] In the concept, this is mimicked by applying strain on the stem cells in the hydrogel.

Because the presented concept is intended for clinical translation, we adopted a backwards approach in its realization. Thus, first the hydrogel and stem cell type were chosen and characterized. The cells adopted for this concept are human adipose-derived stem cells (hASCs). These cells are suitable candidates for clinical tissue engineering because of their easy accessibility, rapid proliferation, and multi-lineage differentiation potential (figure 4 and appendix 1).[19–25]

The hydrogel adopted to realize the concept is fibrin, which is the body's own scaffold material after vascular damage or injury. Fibrin was chosen because it is a temporary matrix that is inductive for angiogenesis and tissue repair by regenerative cells.[26,27] Furthermore, fibrin was chosen because it is created from two blood-plasma proteins, thrombin and fibrinogen, which can be isolated from autologous blood. However, these components can also be obtained off-the-shelf in forms approved for clinical use.[28] The combination of hASCs and fibrin matrices is very suitable for rapid clinical translation, as it allows the creation of patient-specific constructs for tissue engineering. Moreover, these constructs have the great advantage that they can be created and implanted in a single surgical procedure.[29]

The research presented in this thesis focusses on the cells' direct mechanical and chemical environment. It focusses in particular on the chemical and mechanical properties of the scaffold materials, and how to optimize them for clinical PDL tissue engineering. Furthermore, the role of mechanical straining is explored. The goal of this thesis is to:

Take the first steps towards the generation of a periodontal ligament around dental implants, with patient-friendly materials and cells, specifically adipose-derived stem cells, fibrin and gelatin.

OVERVIEW OF THESIS CHAPTERS

A protocol was developed to create hASC-seeded fibrin matrices. <u>Chapter 3</u> describes how fibrin matrix stiffness affects incorporated hASCs while they remodel the fibrin to a collagenous matrix. This chapter also explores the effects of stiffness on the differentiation of the hASCs.

Chapter 3 yielded an optimal formulation for hASC-seeded fibrin matrices, which was used for the rest of the studies in this thesis. In this specific composition, hASCs extensively produce collagen, however the collagen fibers are deposited in a random orientation. A random collagen orientation is biomechanically inferior to an aligned collagenous tissue such as the PDL. Therefore in <u>chapter 4</u> is described how mechanical strain can be applied to align hASC-seeded fibrin matrices without compromising these cell's regenerative potential.
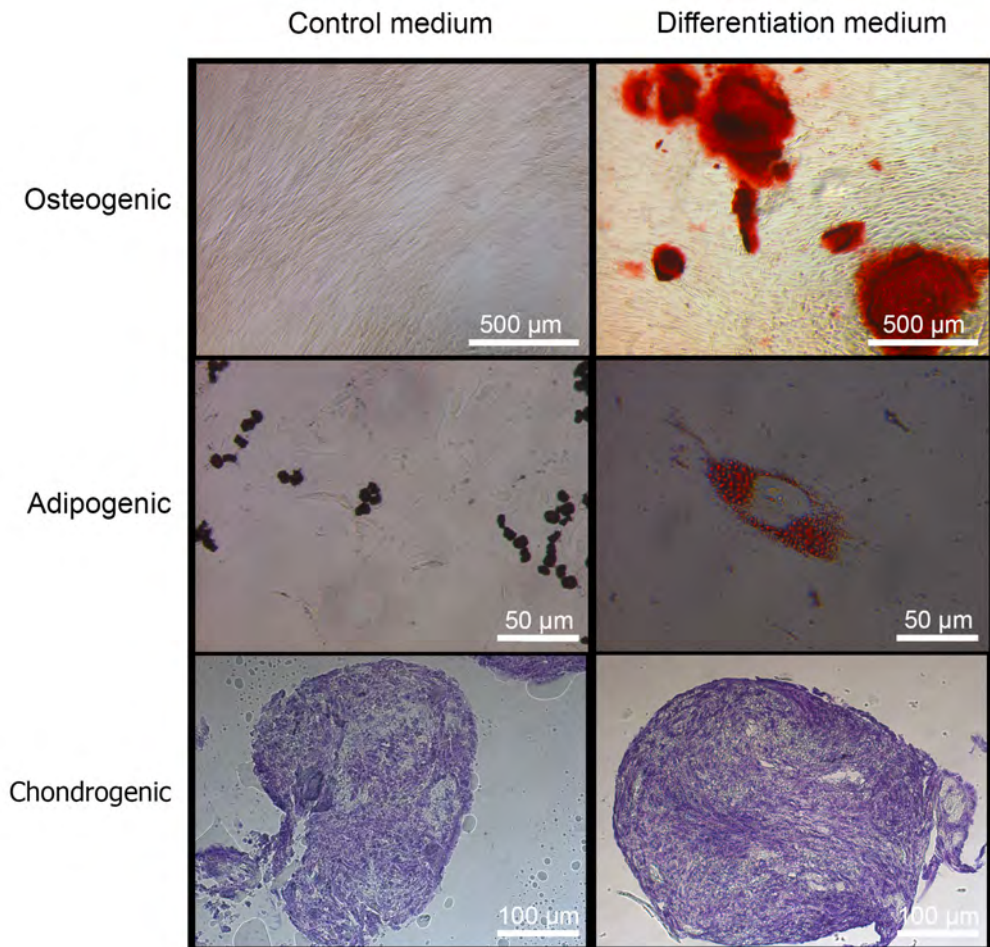
Figure 4: Light microscopy photographs of the multi-lineage differentiation potential of the adipose-derived stem cells that were used in all experiments in this thesis (see also appendix 1)

The second part of this thesis revolves around the implant surface. During PDL development, the PDL fibers are attached to a soft collagenous matrix, which is subsequently mineralized (see chapter 2). To attach PDL fibers to the titanium implant surface, such a soft matrix is created by electrospun gelatin, that is subsequently crosslinked. Chapter 5 describes the electrospinning and crosslinking processes, and how crosslinked electrospun gelatin meshes affect hASC survival and metabolism. Furthermore, this chapter describes the function and differentiation of hASCs seeded on the gelatin's surface.

Finally, chapter 6 offers a glance at the completed concept, where an electrospun-gelatin-coated dental implant is submersed in a hASC-seeded fibrin matrix. Furthermore, chapter 6 answers if the hASCs should be seeded on the crosslinked electrospun gelatin mesh, in the fibrin matrix, or on both materials. Chapter 6 also shows how this cell seeding affects the hASCs' production of extracellular matrix on the interface between the two materials.

In chapter 7, the main conclusions of this thesis are summarized. Chapter 8 places the conclusions in a broader perspective, and adresses the limitations of this thesis, and future perspectives for the concept.

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Chapter II

The developing periodontal ligament

lessons for periodontal tissue engineering

Thijs de Jong, Astrid D. Bakker, Theodoor H. Smit

ABSTRACT

Context
The periodontal ligament (PDL) connects the tooth root and alveolar bone. It is an aligned fibrous network interposed between, and anchored to both mineralized surfaces. Periodontal disease is common and deteriorates the PDL's capacities as shock absorber, barrier for pathogens, and sensor of mastication. Although disease progression can be stopped, current therapies do not primarily focus on tissue regeneration.

Goal
Functional PDL regeneration may be achieved with innovative techniques such as tissue engineering. However, the PDL's small dimensions and complex architecture, combined with the burden to withstand high forces, make PDL tissue engineering very challenging. This challenge may be met by studying PDL development. Understanding PDL development provides clues as to which specific events need to be mimicked for the formation of this intricate tissue.

Method
We provide an overview of PDL development during distinct stages of odontogenesis, with specific focus on the formation of PDL fibers and their attachment to bone and cementum. We consider the spatial arrangement of the fibers during development, and the ability of mechanical forces to modulate this arrangement.

Concluding remarks
We suggest that the proper mechanical environment may initiate self-organization of biomaterials and regenerative cells, leading to creation of a functional PDL architecture.

INTRODUCTION

The periodontal ligament (PDL) constitutes the connection between tooth and jaw. The PDL is an aligned fibrous network that is anchored firmly to the alveolar bone on the one side and the root cementum on the other side. It provides mechanical stability to the tooth and acts as a shock absorber to protect the tooth and alveolar bone from damage created by the high forces associated with mastication. In addition to this, together with the gingiva, the PDL forms a protective barrier against pathogens from the oral cavity.[1] Finally, the neural network in the PDL plays an important role in the sensory input of the mastication system.[2]

These capacities deteriorate if the PDL is damaged by periodontitis, a commonly occurring and progressive disease that leads to loss of the PDL and the supporting alveolar bone. If left untreated, periodontitis eventually results in tooth loss.[3] Therapies for periodontitis exist, but primarily aim at controlling symptoms and stopping disease progression rather than at regeneration of lost tissues.[4] Novel therapeutic measures that aim at tissue regeneration are urgently required, considering that periodontitis has a high recurrence rate, and because the number of patients with periodontal disease is expected to increase with the ageing population.[5,6] Such therapeutic measures may be derived from innovative techniques such as tissue engineering and regenerative medicine.[7] However, the PDL's small dimensions and complex architecture, combined with its ability to functionally cope with high forces, make PDL tissue engineering an enormous challenge. This challenge may be met by studying PDL development and by learning from the specific events that lead to the formation of this intricate tissue.

Here we describe the development of the PDL during distinct stages of odontogenesis. We focus on the formation and alignment of the PDL fibers, and their attachment to bone and cementum, as these aspects are most important for PDL function. This paper covers general principles of PDL development, although specific events in the developing PDL differ between incisors, premolars and molars, and whether or not permanent teeth have a deciduous predecessor.[8] The molecular mechanisms that regulate PDL development, function, and remodeling have extensively been reviewed elsewhere and will not be addressed here.[9–11] By carefully describing the morphological changes occurring with the development of the PDL we aim to offer possible solutions for problems encountered when engineering the PDL.

COMPOSITION, STRUCTURE, AND ORGANIZATION OF THE MATURE PERIODONTAL LIGAMENT

The PDL spans 100-400 µm between the alveolar bone and root cementum.[1] It is a heterogeneous tissue with an extensive blood supply, neural network, and a diversity of cell populations.[12] These include bone cells, cementum

cells, epithelial rests of Mallasez, endothelial cells and neural cells.[1] However, the predominant cell type is the PDL fibroblast, which is responsible for the production and maintenance of the PDL fibers, which constitute the largest functional component of the PDL.[13]

The structural strength of the PDL is provided by fibers that are mainly composed of type I collagen, with minor contribution of collagen type III. These collagens form cross-banded fibrils that measure 54-59 nm in diameter.[1] Several minor collagens are also present in the PDL including types V, VI and XII. However, of the minor collagens, mainly type V collagen is associated with the PDL fibrils. It is found either in the core of the fibrils or in the space between fibril bundles, where it appears to regulate fiber assembly.[14,15] The majority of collagen fibrils in the PDL proper, the non-mineralized central part of the PDL, are arranged in well-defined fiber bundles called principal fibers. The principal fibers are categorized by their location and orientation along the root.[12]

By principal fiber organization, the PDL can be subdivided into the dentinogingival and alveolodental ligament (figure 1). The dentinogingival fibers extend in oblique-coronal direction from the cementum to the interproximal gingiva; transseptal fibers extend from the cementum and cross the alveolar crest to the cementum of the adjacent tooth. The alveolodental ligament represents the bulk of the PDL volume and contains alveolar crest fibers, horizontal fibers,
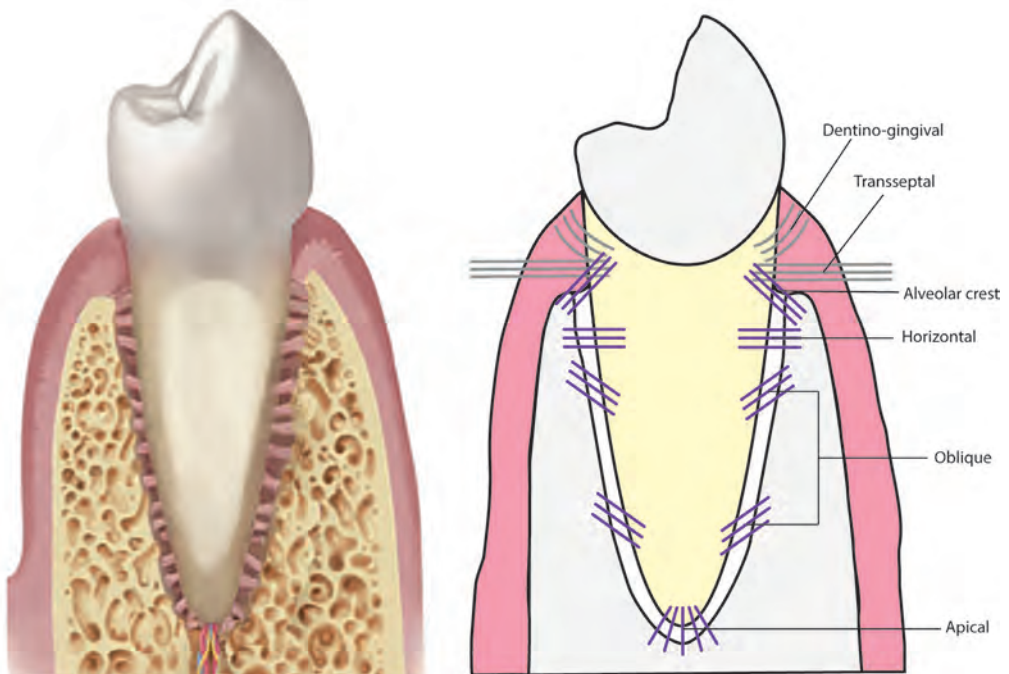

Figure 1: Schematic overview of the periodontal ligament's principal fibers.

oblique fibers, apical fibers, and in the case of multi-rooted tooth, interradicular fibers. The alveolar crest fibers run from the cervical part of the root to the alveolar bone crest in oblique-apical direction. Apical from the alveolar crest fibers, the horizontal fibers run perpendicular from the cementum to the alveolar crest. The oblique fibers are the most numerous fiber in the PDL, and run from the cementum in oblique-coronal direction to the alveolar bone. The apical fibers run radiating from the apex of the root to the base of the alveolus. Interradicular fibers are only found in multi-rooted tooth and run radiating from the radicular cementum to the interradicular alveolar bone (not shown). In addition to the collagenous network, elastic Oxytalan fibers run parallel to the cementum surface and form a branching network that surrounds the root. The function of Oxytalan fibers is not fully understood, but they have been associated with the neural and vascular network of the PDL, and are thought to regulate vascular flow.[12]

The complex spatial orientation of PDL principal fibers is essential for PDL function, as it allows three-dimensional support and protection against the multi-directional forces from mastication and other oral movements.[16] On the other hand, this complex fiber arrangement, combined with the PDL's small dimensions, greatly complicate PDL tissue engineering.

Maintenance of periodontal function
PDL tissue integrity is maintained by an extremely high collagen turnover, with a half-life of about one day for PDL collagen fibers.[17] This collagen turnover is mainly accounted for by the PDL fibroblasts.[13,18] However, because the bone-bound and cementum-bound fibers are also subject to continuous remodeling, osteoclasts, osteoblasts and cementoblasts are essential to maintain PDL function, as they facilitate its attachment to the mineralized surfaces.[1,19–21] It was found that collagen on the cementum side is more mature than on the bone side, which indicates that PDL remodeling occurs faster on the bone side of the ligament.[22] This higher remodeling rate at the bone-side may be the result of the high rate of remodeling of the alveolar bone itself, while the cementum has a much slower turnover.[20,23]

Because the PDL is a highly dynamic tissue that is continuously remodeling, using pre-formed constructs for PDL regeneration will probably prove unsustainable over time. Likely, a more durable way to engineer the PDL will be with a self-organizing system of cells and fibers that utilizes the natural turnover of alveolar bone and cementum. The requirements for such a system are discussed later in this paper.

ORIGIN AND DEVELOPMENT OF PERIODONTAL LIGAMENT FIBERS
All periodontal structures: cementum, PDL, and alveolar bone derive from the dental follicle, the superficial mesenchymal layer enveloping the developing tooth epithelium and mesenchymal papilla.[9] When the crown is completed

and the root starts forming, cells in the dental follicle differentiate into PDL fibroblasts, osteoblasts and cementoblasts.[24] These cells remodel the dental follicle to the PDL, which starts at the cemento-enamel junction and proceeds in apical direction.[8] Remodeling of the fibers proceeds from the mineralized surfaces toward the PDL proper.

Formation of Sharpey's fibers
The anchorage of the PDL to the mineralized surfaces of the alveolar bone and the root is most essential for the PDL's function. The proper anchorage can therefore be defined as one of the major challenges for regeneration of the PDL. Interestingly, the formation of Sharpey's fibers, which facilitate this anchorage, is the first step in PDL development.[1] Sharpey's fiber formation starts on the tooth side first and begins with the formation of cementum on the developing root dentin.[8] Here, only acellular extrinsic fiber cementum (AEFC) is discussed, because it is the predominant tissue that connects the tooth root to the PDL.[20] Events in the development of AEFC and other cementum types show many similarities, which are reviewed by Bosshardt and Schroeder.[20]
AEFC is formed after odontoblasts from the dental papilla deposit non-mineralized root dentin. The AEFC matrix is produced by fibroblast-like cells that are situated immediately coronally of Hertwig's epithelial root sheet. These fibroblast-like cells project numerous cytoplasmic processes towards the non-mineralized dentin-matrix, and produce the initial collagenous cementum-matrix. This initial matrix is attached to the non-mineralized dentin, which results in a dense collagenous fiber fringe orientated perpendicular to the root surface.[20] This fiber fringe is invaded by cementoblasts that originate from the dental follicle. They start producing additional cementum matrix that is subsequently mineralized.[25] Mineralization of the cementum matrix advances from the dentin surface towards the PDL space. However, importantly, the mineralizing front stops before the fiber fringe is completely mineralized, leaving approximately 10-20 µm of collagenous fiber protruding in the PDL space (figure 2a).[20] It is at these sites that the fibers of the early PDL are able to attach, and so form a connection between the root and PDL (figures 2b and 2c).[26]
Sharpey's fibers on the bone side form in a different manner. When the root and PDL start to develop, the alveolar bone is already mineralized, but remains subject to extensive remodeling.[9,23] It is at the sites of remodeling that the Sharpey's fibers are attached to - and embedded in - the bone matrix.[27] Bone-bound Sharpey's fibers can be formed in several ways. The first is at areas where osteoblasts deposit osteoid, the non-mineralized bone matrix. Both newly formed and existing fibers from the PDL can attach to the osteoid directly, and become entrapped when the bone matrix is calcified.[19] Similarly, PDL fibers can attach to areas where bone was resorbed by osteoclasts that leave a strand-like ground-substrate in the resorption pit.[28] Also these fibers are entrapped

Figure 2: Light micrographs illustrating the change in orientation of human periodontal ligament fibers from prefunctional (A,B) to functional (C) in regions where acellular extrinsic fiber cementum (AEFC) prevails on the root surface. Reproduced with permission from Bosshardt and Schroeder (1996).[20]

A) The 5-6 µm thick AEFC layer has approximately developed over 2 years. The AEFC matrix fibers, i.e. fringe fibers (FF), continue for 15-20 µm into the PDL space (PL) and abut to a fibro-cellular meshwork running broadly parallel to the root surface.

B) The 14 µm thick AEFC layer has developed over 5 years. Most FFs continue for a short distance, i.e. 15 µm, into the periodontal space, whereas a few FFs appear slightly elongated. The orientation of the fibro-cellular meshwork occupying the periodontal space is less strictly parallel to the root surface than that in (A).

C) The 14 µm thick AEFC layer has developed over the same period of time than that in (B), but is located more cervically. The FFs are continuous with the PL fibers, which run now obliquely to the root surface.

in the calcified matrix after osteoblasts orchestrate matrix mineralization. Finally, PDL fibers can attach to the bone at areas where fibers from the osteoid 'escape' mineralization and are left protruding in the PDL space after newly formed bone has been mineralized completely (figures 3a and 3b).[27] Similar to events at the tooth side of the PDL, fibers from the PDL space are able to attach to the Sharpey's fibers by lateral and longitudinal apposition of fibrils. [20,26]

The Sharpey's fibers from the bone and those from cementum differ in size and number, which might be due to their specific method of development. The dimensions of fibers emanating from the bone are determined by the size of the osteoid patch or the resorption pit they are attached to.[21] As fibers on the cementum-side must attach to the fiber fringe, they are thinner and more numerous (figures 2 and 3).
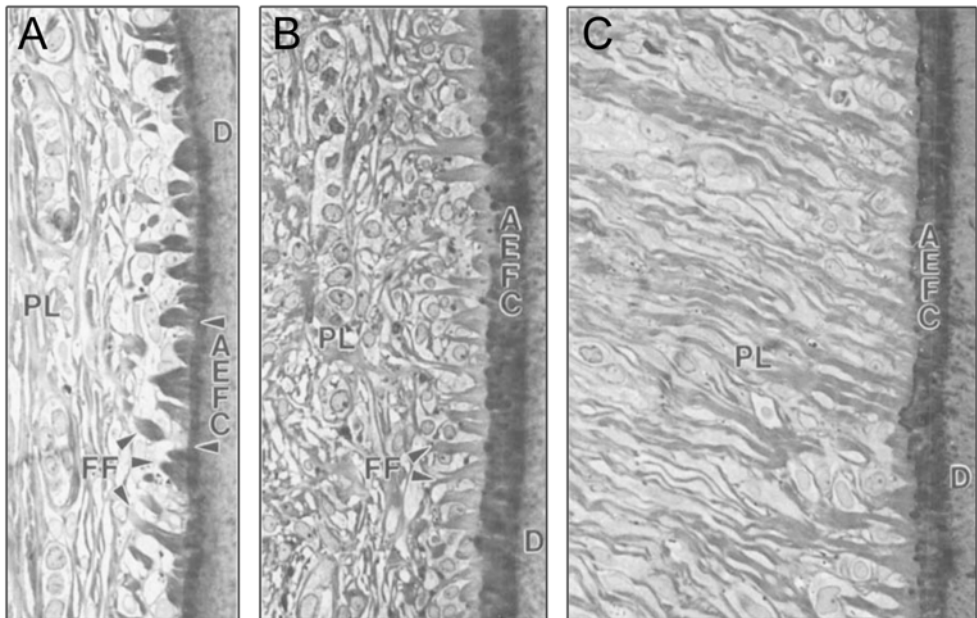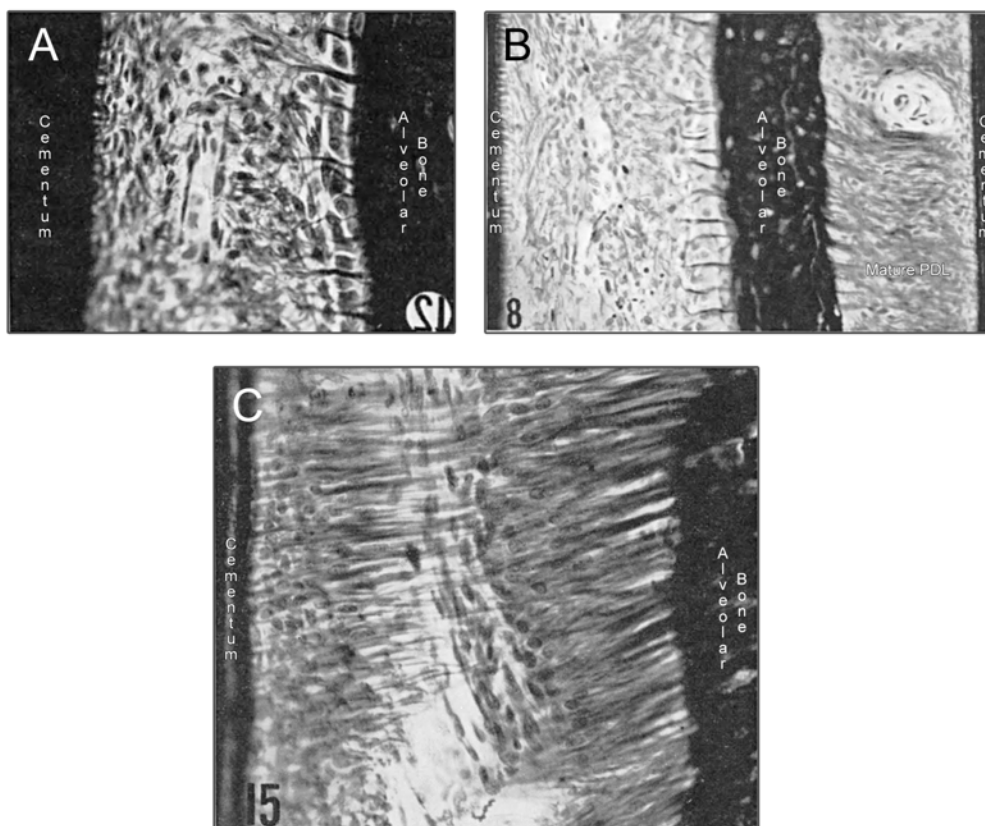
Figure 3: Light micrographs illustrating the change in orientation of squirrel monkey periodontal ligament fibers from prefunctional (A,B) to functional (C) near the alveolar bone. Reproduced with permission from Grant & Bernick (1972).[29]

A) At mid root, the ligament is less developed. Thick fibers project from bone and extend between osteoblasts into the periodontal ligament. These fibers splay outward, as if to unravel. The broad central zone of the ligament is occupied by loose collagenous elements. At the cemental surface, short, closely spaced fibers present a brushlike appearance.

B) The classically organized and apparently continuous principal fibers of the functioning first molar (on the right) contrast with the still developing fibers of the third premolar (on the left). On the premolar, fine, short, brush-like fibers line the cementum surface and are separated from the longer, more widely spaced bony fibers by a broad zone of loosely structured collagenous elements.

C) A higher magnification of the principal fibers of the functioning tooth shows thick fibers that appear to pass from bone to tooth.

Fiber growth, from Sharpey's fiber to continuous periodontal ligament

During Sharpey's fiber formation, the central part of the PDL space is composed of a loosely arranged collagenous mass.[29] This mass contains numerous mesenchymal precursor cells, which, among other cell types, can differentiate into PDL fibroblasts.[13] During PDL development, these fibroblasts effectively shape the PDL into its functional configuration by producing new extracellular matrix and by functionally remodeling the existing structures.[13] Matrix
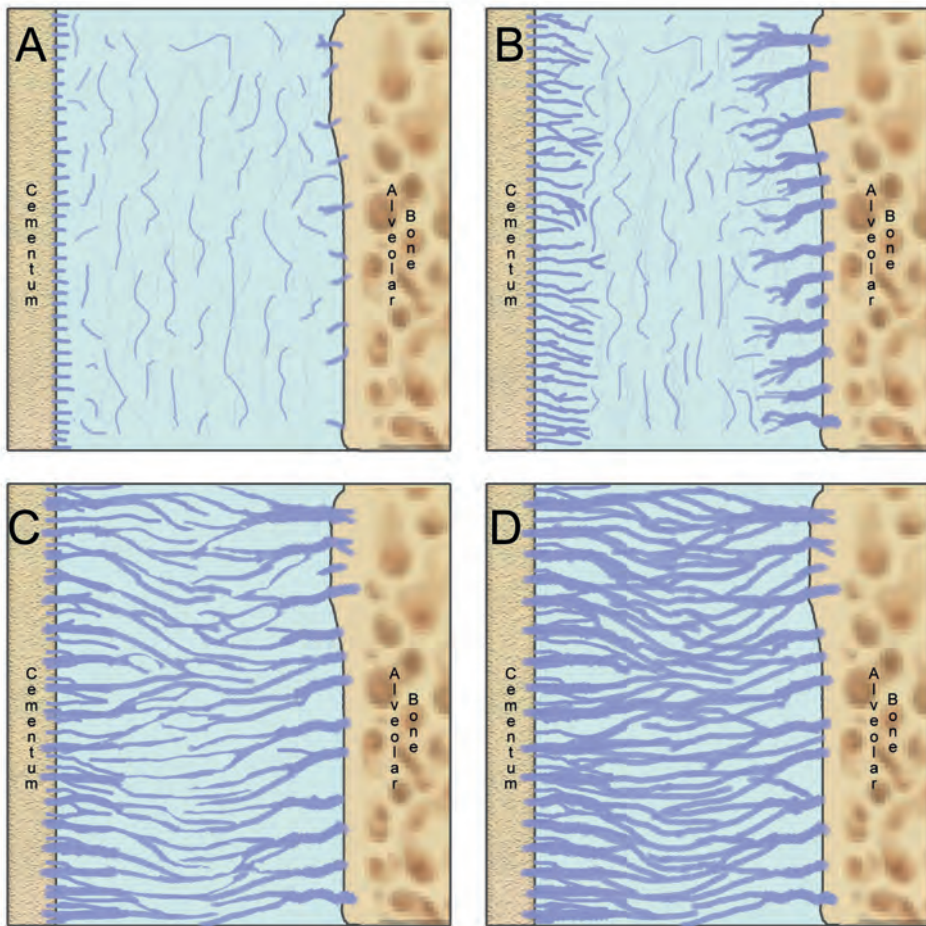
Figure 4: Schematic overview of fiber growth during PDL development.
A) Pre-emergence stage: closely spaced fibers emerge from the cementum that are aligned perpendicular to the long axis of the root. Fibers emerging from the bone can only occasionally be observed.
B) Pre-occlusional stage: fibers from cementum and bone protrude in the PDL space, they are not continuous and are separated by loosely structured collagenous elements.
C) First occlusal contact: Fibers that extend from the alveolar bone are thicker than fibers extending from the cementum. The osseos fibers appear to unravel toward the central part of the PDL, where they intertwine with the finer cemental fibers.
D) Full occlusal function: Although the Sharpey's fibers on the tooth side remain thinner and more numerous than on the bone side, overall the fiber bundles thicken and form a network that passes from cementum and alveolar bone without a distinguishable central zone.

remodeling starts almost simultaneously on the bone and tooth side, and begins at the ends of Sharpey's fibers that protrude as collagenous stumps into the PDL space (figure 4a).[20] From these stumps, PDL fibers grow into the PDL space by lateral and longitudinal apposition of fibrils (figure 4b).[26,30] The growing collagen bundles spread in the central part of the periodontal space.

There they and branch out to form an anastomosing network of intertwining fibers from the bone side to those on the tooth side (figures 3c and 4c).[30,31] Subsequently these fibers are crosslinked, and span the PDL space without distinguishable central zone (figure 4d).[30–32]

The way Sharpey's fibers are formed, and how PDL fibers grow toward each other from the mineralized surfaces further underscore the need of a self-regulating construct for PDL regeneration rather than a pre-formed implant. Such a construct should focus on the generation of suitable boundary conditions on the surfaces of cementum and alveolar bone, while retaining plasticity in the central part of the PDL space to allow extensive tissue remodeling.

PRINCIPAL FIBER FORMATION AND ORIENTATION DURING DISTINCT STAGES OF ODONTOGENESIS

Pre-emergence stage:

When approximately one-third of the root has formed, the developing PDL consists of loosely structured collagenous elements. Near the cemento-enamel junction, fibers arise from the cementum, and follow the outline of the crown (figure 5a).[29] In the midroot region, closely spaced fibers emerge from the cementum that are aligned perpendicular to the long axis of the root (figure 4a). In this region, fibers emerging from the bone can only occasionally be observed.[21] In the periapical region, fibers near the cementum and alveolar bone are densely packed, but are more loose in the central third of the PDL space.[8] These fibers pass in occlusal direction, parallel to the long axis of the root.

Emergence in the oral cavity (pre-occlusional stage):

In the pre-occlusional stage, organized fibers can readily be observed in the coronal and cervical areas (figure 5b). The dentogingival fibers show their mature occlusional orientation and follow the enamel surface from the cementum to the interproximal gingiva.[29] When an adjacent tooth has not yet erupted, developing transseptal fibers extend over the alveolar crest in the oblique-apical direction toward the cemento-enamel junction of the non-erupted adjacent tooth.[8] However, when the adjacent tooth has erupted, the mechanical field around the erupting tooth changes.[16] This results in a re-orientation of the transseptal fibers in superior-oblique direction towards the erupted tooth.[29]

Further apically, the developing PDL is not so well organized. From the middle third of the root towards the apex, closely spaced brush-like fibers emerge from the cementum, while fibers from the bone are more widely spaced.[20] Although both fibers from cementum and bone protrude in the PDL space, they are not continuous and are separated by loosely structured collagenous elements (figure 4b).[29]
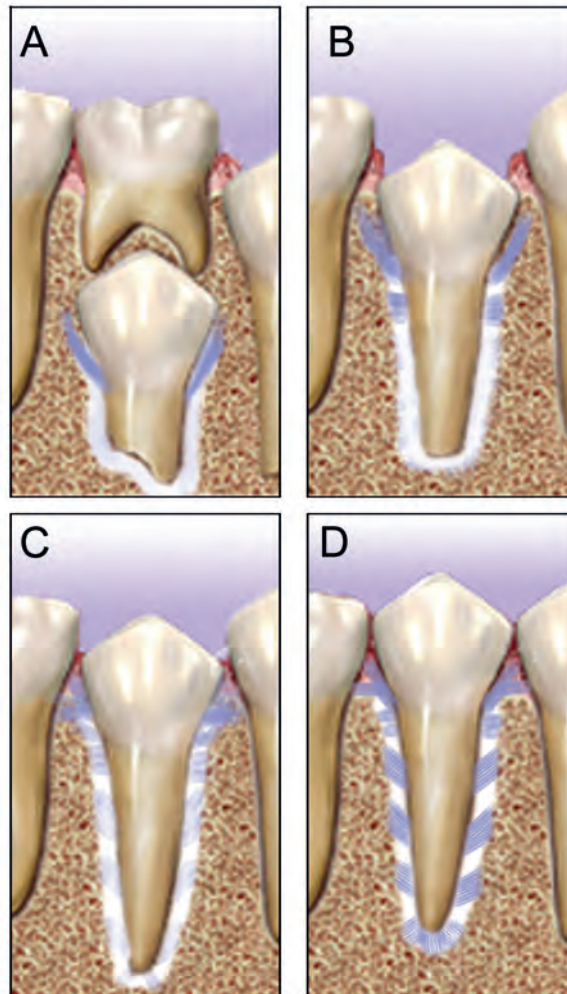
Figure 5: Schematic overview of principal fiber development. Note that the PDL is a continuous tissue that covers the entire root, the gaps are added only to emphasize principal fiber orientation.
A) Pre-emergence stage: Near the cemento-enamel junction, fibers arise from the cementum, and follow the outline of the crown.
B) Pre-occlusional stage: organized fibers can readily be observed in the coronal and cervical areas. Further apically the developing PDL is not so well organized.
C) First occlusal contact: PDL fiber organization is more advanced. The dentogingival and transseptal fibers are well developed and show their typical orientation. From the midroot level and further apically, thick arborizing fibers from the alveolar bone extend into the PDL space, and thin brush-like fibers extend from the cementum. However, these fibers do not intertwine and are separated by loose collagenous elements.
D) Full occlusal function: all principal fiber groups in the ligament are present. Overall the fiber bundles thicken and form a network that passes from cementum and alveolar bone without a distinguishable central zone.

First occlusal contact:
When the tooth reaches first occlusal contact, PDL fiber organization is more advanced (figure 5c). At this stage, the dentogingival and transseptal fibers are well developed. In the cervical third of the root, closely spaced fibers emerge from the cementum in an oblique downward direction toward the alveolar bone. Near the bone these fibers join with more widely spaced Sharpey's fibers. Fibers that extend from the alveolar bone are thicker than fibers extending from the cementum.[20] The osseos fibers appear to unravel toward the central part of the PDL, where they intertwine with the finer cemental fibers. From the midroot level and further apically, thick arborizing fibers from the alveolar bone extend into the PDL space, and thin brush-like fibers extend from the cementum. However, these fibers do not yet intertwine and are separated by loose collagenous elements (figure 4c).[29]

Mature ligament (full occlusal function):
At this stage, all principal fiber groups in the ligament are present (figure 5d). [12] The Sharpey's fibers on the tooth side remain thinner and more numerous than on the bone side. As a result of dental loading, overall the fiber bundles thicken and form a network that passes from cementum and alveolar bone without a distinguishable central zone (figure 4d).[8,16]

LEADS FOR TISSUE ENGINEERING OF THE PERIODONTAL LIGAMENT
PDL tissue engineering present several specific challenges. First, the very narrow PDL space, which spans approximately 150-400 micrometer from alveolar bone to tooth, limits the possibilities for the placements of off-the-shelf constructs. [8] The second challenge consists of the engineering of a soft tissue between two mineralized surfaces, and subsequently anchoring it to them. Though this is a challenge in itself, it is further enhanced by the fact that maintaining a boundary between these structures, and preventing advancement of the mineralization front is critical.[33] Thirdly, PDL engineering constructs need to be able to functionally cope with high forces, and should contain a self-repair mechanism to maintain their integrity, as damage resulting from these high forces is almost inevitable.[13] Finally, to cope with these high forces, natural PDL fibers are aligned according to the magnitude and direction of loading, which increases their mechanical strength in this direction. Several techniques exist for the alignment of tissue engineering constructs.[34] However, the challenge in functional alignment of engineered fibers lies with the PDL's three-dimensional structure around the root, and its corresponding three-dimensional loading pattern.[35]

Biomaterials for Periodontal ligament engineering
Numerous biomaterials have already been proposed for PDL engineering. Among them are natural and synthetic polymers, glasses, ceramics, and

hydrogels.[36–39] Furthermore, a great many of methods have been proposed to improve these materials, for example by altering their structural properties or by pre-loading them with growth or differentiation factors.[7,40,41] As stated above, the choice of material depends on the specific tissue engineering goal. It is likely that glass or ceramic constructs may are most suitable to engineer the alveolar bone or cementum surface. But when taking the limitations of the PDL space into account, soft injectable materials, such as polymers and hydrogels are likely the most suitable to engineer the PDL proper. Because injectable polymers and hydrogels generally have a high plasticity, they may aid in the creation of an optimal environment for PDL development.

Immediately after the injection of a hydrogel, an interface is created between the hydrogel and existing structures. Similar interfaces exist in many instances during PDL development. For example, when looking at the formation of cementum bound Sharpey's fibers, a pronounced interface exists between the relatively dense dentin matrix and the loose collagenous structure surrounding the developing root.[20,29] It is inevitable that this results in a difference in mechanical properties, in particular stiffness, between the dentin matrix and its surroundings.[42,43] The same principles apply to the other interfaces formed during PDL development, for example between the bone-bound Sharpey's fibers and ligament proper.[44] These differences in stiffness are of great importance when cells are incorporated in the hydrogel, as cells are known to respond to the mechanical properties of their substrate.[45] Moreover, mesenchymal stem cells differentiate to tissue-specific lineages when the elasticity of the target tissue is mimicked.[46] After hydrogel injection, it is very likely that the local stiffness in the center of the hydrogel dictates that the cells there retain a fibroblast-like phenotype. On the other hand, the cells at the interface between soft hydrogel and stiff substrate may differentiate towards an osteogenic phenotype, and have the ability to aid in the mineralization fibers.[46–48]

Another important feature of hydrogels is that they allow three-dimensional PDL engineering. Their initial lack of structural alignment allows the material to self-organize according to the local straining conditions. Mechanical and geometrical signals are an important mediators of tissue organization. The interposition of a soft hydrogel between two hard mineralized surfaces may trigger a self-regulating alignment-response perpendicular to the surfaces. [49] Next to this intrinsic alignment, also cytoskeletal contraction and external mechanical loads can exert an alignment response.[50–54]

Mechanical loading
External mechanical loading is an important feature of normal PDL physiology, and should not be overlooked when designing constructs for PDL tissue engineering.[1] Although loading causes micro-damage to the PDL fibers, it is essential for normal PDL development and function.[16] Dental loading strains the fibroblasts, and stimulates their production of collagen.[55,56]

Furthermore, the magnitude and direction of the strain direct the PDL fibroblasts to accordingly remodel the PDL fibers.[57,58] A clear example of this is the thickening of principal fiber bundles as soon as the tooth reaches functional occlusion.[8] Additionally, loading of the PDL is essential to prevent calcification of the ligament fibers, and thus to maintain the PDL space.[59] This is even the case for transplanted teeth, as loading prevents ankylosis and improves periodontal healing.[59] Furthermore, we showed that a strain regime that mimics dental loading reduces the calcification potential of adipose-derived stem cells embedded in a fibrin hydrogel.(unpublished results)

Though loading is of essential importance for PDL regeneration, as stated above, hydrogels do not initially possess the mechanical strength to support a tooth. This initial support may come from existing structures, or may be provided by external fixtures, for example orthodontic retainers.

Regenerative cells for Periodontal ligament engineering
Several cell types have been proposed for PDL engineering.[60] Among them are PDL progenitor cells, mature PDL fibroblasts, gingiva fibroblasts, and mesenchymal stromal cells from bone marrow or adipose tissue.[61–63] These different cell types have their distinct advantages and disadvantages. However, to be successful for PDL regeneration, the cell type of choice should at least possess the following characteristics: 1) they should have the ability to produce and remodel collagen, 2) be mechano-responsive and have the ability to functionally cope with the high forces associated with PDL loading, and 3) remain proliferative to account for cell death. Importantly, the cell type of choice must be amenable to clinical application. This means that accessibility, availability, donor-site morbidity and patient discomfort should also be taken into account when choosing a suitable cell for PDL tissue engineering.

Finally, the natural PDL is an innervated tissue, and is highly vascularized.[1] A vascular system is essential for the survival and maintenance of function of PDL tissue engineering constructs, while nervous innervation is essential for proprioception. This must be considered when choosing the appropriate hydrogel, as it must permit the invasion from endothelial cells and nerve endings in order to functionally incorporate the tissue engineering construct into the body. A final caveat is that virtually all knowledge on the developing PDL is derived from animal studies. Although, the structure of the PDL in animals very much resembles that of humans, translation of animal data to the human situation should be done with care.[64]

CONCLUSIVE REMARKS
The PDL is a highly organized tissue between two mineralized surfaces, which is capable of coping with extremely high forces. The complex arrangement of this tissue makes PDL tissue engineering an enormous challenge. Because the

limited PDL space impedes the placement of pre-formed constructs, we suggest the use of a construct that combines a self-organizing hydrogel with relevant regenerative cells. Such constructs, placed in the appropriate local mechanical environment, allow to recreate the events that lead to PDL development, and so will aid in the functional regeneration of lost periodontal tissues.

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Chapter III

Matrix remodeling and osteogenic differentiation
of human adipose-derived stem cells increases
with higher fibrin matrix stiffness

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ABSTRACT

Introduction
Fibrin-matrices of different stiffness can be used for tissue engineering. The differentiation and extracellular matrix (ECM) remodeling properties of mesenchymal stem cells can be influenced by matrix stiffness. We hypothesized that stiffer fibrin matrices slow matrix degradation and favor the osteogenic differentiation of human adipose-derived stem cells (hASCs).

Materials & Methods
hASCs were incorporated at different densities into soft and stiff fibrin matrices composed of 2mg/ml fibrinogen and 0.1 or 1.0 IU/ml thrombin. The Young's moduli of the matrices were determined by nano-indentation. Fibrin degradation was determined during a 14 day culture period by ELISA. qPCR and histology were used to assess ECM remodeling and osteogenic differentiation.

Results
Fibrin matrices polymerized with 1.0 IU/ml thrombin were 69% stiffer than those polymerized with 0.1 IU/ml. Stiffer matrices degraded more than soft matrices. Higher cell seeding densities increased matrix degradation. Cells in stiffer matrices produced more Alkaline Phosphatase and ECM than cells in softer matrices. RUNX-2 expression was almost ten times higher in stiff matrices than in soft matrices.

Discussion
Only stiff fibrin matrices induced osteogenic differentiation of hASCs. Unexpectedly, this was accompanied by enhanced cell-mediated matrix remodeling. These results suggest that a mechanical threshold for differentiation and ECM-remodeling was reached for cells embedded in the stiff matrices.

INTRODUCTION

It is expected that the growing and ageing population will cause an increase in the demand for repair or replacement of connective tissues such as ligaments, bone and dental tissues.[1,2] This demand could be met by innovative methods, such as tissue engineering and regenerative medicine. However, despite the extensive research that is being done in these fields, 'bench-to-bedside' translation is only rarely seen. One reason for this is that many tissue-engineered products require a delicate, complex and time-consuming interaction process between materials, soluble factors and cells. To overcome these challenges, we aim to create easy-to-use tissue-engineering products that are made with clinically relevant cells and autologous or clinically approved materials that are available as off-the-shelf products.

Human adipose-derived stem cells (hASCs) are suitable candidates for clinical tissue engineering because of their accessibility, rapid proliferation, and multi-lineage differentiation potential in-vitro as well as in-vivo.[3–10] A candidate material for the scaffold is fibrin, which is the body's own scaffold material after vascular damage or injury. After injury, thrombin catalyzes the polymerization of soluble fibrinogen monomers to an insoluble fibrin network.[11,12] This network forms a hemostatic clot that provides an inductive matrix for angiogenesis and tissue repair by regenerative cells.[13–17] Fibrinogen (physiological concentration: 2-4 mg/ml) and thrombin (0.5-1.1 IU/ml) are blood-plasma proteins and can be isolated from autologous blood.[9,11,18] However, they are also available off-the-shelf in forms approved for clinical use. Fibrin scaffolds are suitable for clinical tissue engineering, because they are very user-friendly. Fibrin scaffolds can either be pre-formed, or polymerized in-situ by injection with a dual syringe. This permits defects, however complex, to be filled completely, with the fibrin matrix integrating seamlessly with the surrounding tissues in the defect. Furthermore, when cells are added to the fibrinogen or thrombin components, they will be distributed homogenously when the fibrin matrix polymerizes.[19]

The composition of a polymerized fibrin matrix is determined by the concentrations and ratio of fibrinogen and thrombin, and by the presence and concentrations of stabilizing factors such as protease-inhibitors and cross-linking agents.[20] By varying these parameters, the number of fibrin fibers, the fiber thickness and the number of branch points between fibers will change, which influences the stiffness of the fibrin matrix. [11,12,21,22] Matrix stiffness is important, especially when cells are incorporated. Cells sense mechanical signals, and the mechanical properties of the extracellular environment can influence their behavior.[23] One property of cell behavior that is greatly influenced by matrix stiffness is the cells' ability to remodel their environment.[14,24] For many remodeling processes, the first step before new extracellular matrix (ECM) can be deposited is the removal of existing ECM or tissue engineering scaffold material by enzymatic degradation.

[25,26] Although the mechanism and rate of degradation of biomaterials are material-specific, they are influenced by environmental factors such as pH, temperature, and the material's structural and mechanical properties.[27–29] This also applies to fibrin matrices, e.g. when their stiffness increases, they become more difficult to degrade.[29–31] It is of vital importance to know the degradation characteristics of materials that are intended for implantation, because their clinical success depends greatly on the balance between material degradation and new tissue formation.[32]

Another aspect of cell behavior that is strongly influenced by matrix stiffness is cell differentiation. Engler et al. showed that mesenchymal stem cells differentiate to tissue-specific lineages when the elasticity of the target tissue is mimicked.[33] These experiments have been repeated in several studies, whose results indicate that relatively stiff matrices steer stem cells towards osteogenic lineages.[33–37] Most of these studies however, describe effects of matrix stiffness on cells seeded on top of substrates in monolayer. Although these two-dimensional models offer tremendous amounts of information, we strongly believe that the solution to most tissue-engineering problems requires a three-dimensional approach.

In this study, we used three-dimensional cell culture of hASCs seeded in fibrin matrices of different stiffness. We studied the effects of fibrin matrix stiffness on matrix degradation, and on the differentiation of hASCs, because these factors are of vital importance for the clinical success of tissue engineered constructs after implantation.[32] We hypothesized that matrix degradation is slower in stiffer fibrin matrices, and that stiffer matrices are favorable to the osteogenic differentiation of hASCs.

MATERIALS AND METHODS
Unless specified otherwise, materials were obtained from Life Technologies, Bleiswijk, the Netherlands, and the manufacturer's instructions were followed.

Human adipose stem cells (hASCs)
Subcutaneous adipose tissue was harvested from the abdominal wall of 5 healthy women (age = 44.8 ± 8.35 years; range 33-54 years) undergoing elective abdominal wall correction at the Tergooi ziekenhuizen Hilversum, The Netherlands. All participating donors provided their written informed consent. The written informed consent and study protocol were reviewed and approved by the Ethics Review Board of the VU University Medical Center, Amsterdam, The Netherlands.[38] The vascular stromal fraction, which contains the hASCs, was obtained as described previously.[38] The cells were pooled and the plastic adherent fraction was cultured up to passage 3 in minimal essential medium alpha (αMEM) with 2% human platelet lysate (PL), 10 IU/ml heparin (Leo Pharma, Amsterdam, The Netherlands), 100 IU/ml penicillin (P), 100 IU/ml

streptomycin (S) and 0.25 IU/ml fungizone (F).[39] The PL was created from 5 transfusion bags (180-350 ml) containing clinical-grade random-donor-pooled platelets (Sanquin, Amsterdam, the Netherlands), and was constant for all experiments.

Fibrin matrix formation and culture
hASCs were detached with phosphate-buffered saline (PBS) containing 0.5 mM EDTA/0.05% trypsin and suspended at 200,000 or 400,000 cells/ml in gel medium: M199 medium with 1% P/S. Unfractionated, plasminogen-depleted human fibrinogen (330 kDa) (STAGO BNL, Leiden, the Netherlands) was dissolved at 4 mg/ml in gel medium. The dissolved fibrinogen was mixed with gel medium –with or without cells– in a 1:1 ratio, resulting in a final fibrinogen concentration of 2mg/ml and cell concentrations of 0, 100,000 or 200,000 cells/ml. Fibrin matrices were polymerized by adding 4.5 µl bovine thrombin (STAGO BNL), at 10 or 100 IU/ml, to 450 µl of the fibrinogen/cell mixture, to reach final concentrations of 0.1 IU/ml or 1.0 IU/ml of thrombin. Per matrix, 400 µl of fibrin was polymerized one hour at room-temperature. During polymerization, no additional fluid or medium came in contact with the fibrin matrices, to prevent unbound fibrinogen from washing away. After polymerization, 400µl of culture medium was added, which consisted of αMEM with 5% PL, 10 IU/ml heparin, and 1% P/S/F. The fibrin matrices were cultured for fourteen days in a humidified incubator at 37˚ C and 5% CO2. Culture medium was refreshed three times per week.

Mechanical characterization of the fibrin matrices
The Young's moduli of non-cell-seeded fibrin matrices were determined with the Piuma Nano-indenter (Optics 11, Amsterdam, the Netherlands).[40,41] To assure full fibrin matrix polymerization, measurements were taken two hours after addition of thrombin to fibrinogen.[42] Nano-indentation was performed at 4 locations per matrix. The indenter-probe had a tip-diameter of 81 µm, and stiffness of 0.27 N/m. The indentation depth was 20 µm, with 1 second indentation time. Indentation speeds varied from 4-40 µm/s.

Fibrin degradation ELISA
The amount of fibrin degradation products was determined by ELISA as described previously.[43]

Histology and immuno-histology
On the fourteenth day of culture, live/dead staining was performed on whole fibrin matrices with the LIVE/DEAD® Viability/Cytotoxicity Kit. After culture, fibrin matrices were fixed in PBS containing 4% formaldehyde and embedded in paraffin. 7 µm sections were cut. To observe cell morphology and ECM production, sections were deparaffinized and stained with Mayers' Hematoxilin

& Eosin or Goldner's trichrome staining. Von Kossa staining was used to observe matrix calcification: after deparafinization sections were incubated in 1% Silver-Nitrate for 30 minutes under strong UV light. Unreacted silver was removed with 5% Sodium Thiosulfate. Sections were counterstained with nuclear fast red (Vector labs, Brunschwig Chemie, Amsterdam, the Netherlands). To observe alkaline phosphatase (ALP) enzyme activity an adapted protocol for staining fresh samples was used: sections were incubated in Tris-buffered saline (pH 7.5) (Sigma Aldrich, Zwijndrecht, the Netherlands) and in Tris-buffer (pH 9.1): 0.1 M Tris HCl, 50 mM NaCl, 1 nM ZnCl and 5 mM MgCl (Sigma aldrich). The sections were stained overnight with NBT/BCIP substrate in Tris-buffer. Immuno-staining for Runt related transcription factor 2 (RUNX-2) was performed on matrices containing 200,000 cells/ml. After deparaffinization, peroxidases were blocked with 3% H_2O_2 (Sigma Aldrich), and antigen retrieval was performed in boiling 10 mM citrate buffer (pH 6.0) (Sigma Aldrich). After blocking with 10 % goat serum (Vector labs Vectastain ABC elite kit, Brunschwig Chemie, Amsterdam, the Netherlands), sections were incubated overnight at 4°C with 2.25 µg/ml of a custom made RUNX-2 antibody that was described previously.[44] Immuno-localization was visualized with a goat-anti-rabbit ABC peroxidase kit (Vector laboratories) and with DAB+ substrate (DAKO, Heverlee, Belgium). Images were captured with the Leica DRMA HC-F14 microscope and Qwin software (Leica).

RNA-isolation and cDNA-synthesis
RNA-samples were obtained from fibrin matrices containing 200,000 cells/ml. The fibrin matrices were minced with a scalpel blade, lyzed in 700 µl Trizol reagent, and phase separated with 140 µl chloroform (Sigma Aldrich). The water phase was used for RNA-isolation with RNeasy mini spin columns (Qiagen, Venlo, the Netherlands). cDNA was synthesized from 250ng RNA with the superscript VILO cDNA synthesis kit.

Quantitative polymerase chain-reaction (qPCR)
qPCR was performed as described previously, to determine the RNA-expression of markers for proliferation, ECM remodeling, osteogenesis, chondrogenesis, and adipogenesis.[45] The primer-sequences are listed in appendix 2. All genes were expressed relative to a normalized housekeeping (HK) gene, which was calculated from the genes UBC and YWHAZ with the formula HK = $\sqrt{(UBC*YWHAZ)}$.

Statistical analysis
Prism 5 (GraphPad) was used for statistical analysis. For the ELISA and qPCR data, two-way ANOVA with Bonferonni multiple comparisons post-hoc test was used. For the nano-indentation and histology experiments, the student's T test was used. Each n represents an independent experiment, P-values <0.05 were considered significant.
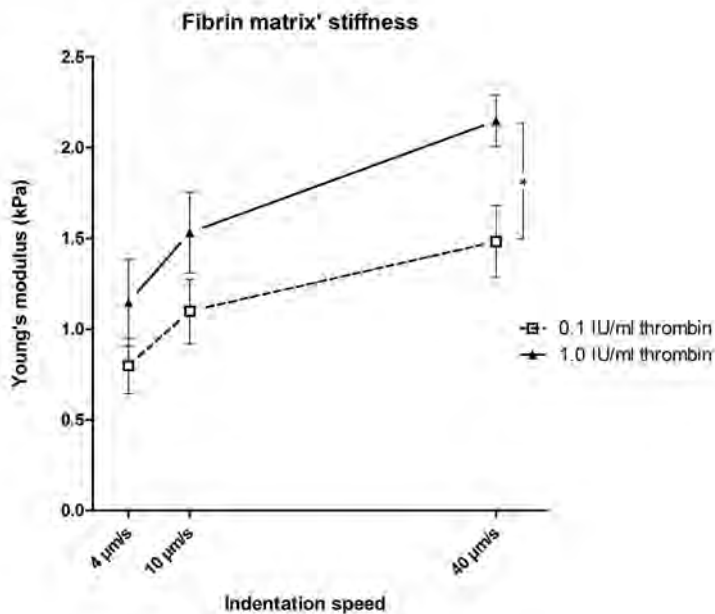
Figure 1: Mechanical properties of the fibrin matrices, as determined by nano indentation. The matrices polymerized with 1.0 IU/ml and 0.1 IU/ml thrombin show strain stiffening at higher indentation speeds. Mean ± S.E.M.; N=4; *:P<0.05

RESULTS

Fibrin matrix stiffness increases with a higher thrombin concentration

All fibrin matrices showed strain stiffening with increasing indentation speeds (figure 1). Indentation at 40 μm/s yielded a Young's modulus of 2.14 ± 0.40 kPa in the matrices formed with 1.0 IU/ml of thrombin. These matrices were significantly stiffer than the 0.1 IU/ml thrombin matrices, which had a modulus of 1.48 ± 0.49 kPa (P<0.05; n=4).

Cell-mediated fibrin matrix degradation is higher in stiffer matrices

On culture day fourteen, cell viability in the soft matrices was 97 ± 2 %, and 97 ± 3 % for 100,000 and 200,000 cells/ml respectively. Cell viability in the stiff matrices was 98 ± 2 %, and 94 ± 4 % for 100,000 and 200,000 cells respectively (supplemental Figure 1). After culture, empty areas (voids) were visible within all the cell-seeded matrices, but not within the acellular matrices. The number of voids appeared higher in stiff matrices. (Arrows in figure 2a). All cell-seeded matrices had a significantly reduced volume compared to acellular matrices. The stiff matrices showed the largest volume reduction. (Figure 2a; also see supplemental figure 2). The culture medium of acellular matrices contained negligible amounts of fibrin degradation products. Increasing amounts of fibrin degradation products were found in the culture medium of all cell-seeded

A Soft matrix (0.1 IU/ml thrombin)
No cells   200,000 cells/ml

Stiff matrix (1.0 IU/ml thrombin)
No cells   200,000 cells/ml

I   II   III   IV

200 µm   200 µm   200 µm   200 µm

B

Fibrin degradation

Cummulative fibrin degradation products
(ng/ml)

Stiff matrix
(1.0 IU/ml thrombin)
● No cells
▲ 100k/ml
□ 200k/ml

Soft matrix
(0.1 IU/ml thrombin)
● No cells
▲ 100k/ml
□ 200k/ml

Day   1   2   3   6   8   10   14

C   MMP-2

2-way ANOVA:
Ftime: P<0.001
Fthrombin: n.s.

Relative MMP-2 gene expression
(fold change)

Day   1   3   6   10   14

D   MMP-9

2-way ANOVA:
Ftime: P<0.001
Fthrombin: n.s.

Relative MMP-9 gene expression
(fold change)

Day   1   3   6   10   14

0.1 IU/ml thrombin
1.0 IU/ml thrombin

◄ Figure 2: Representative pictures for Hematoxilin & Eosin staining, and ELISA and qPCR data of fibrin matrix degradation.
A) Hematoxilin and eosin staining of the (I,II) soft and (III,IV) stiff matrices, (I,III) without cells, (II,IV) with cells (200,000 cells/ml). In both the soft and stiff matrices, a higher number of cells was found at the culture medium exposed side (green asterisk, see supplementary results) Although in both the stiff and soft matrices voids from local fibrin degradation can be seen (black arrows), they appear more abundant in the stiff matrices. Compared to the soft matrices, the stiff matrices reduced significantly more in volume (also see figure s2).
[Hematoxilin & Eosin staining: blue/black = cell nuclei, purple/pink = cytoplasm]
B) Results for the Fibrin degradation ELISA: In the culture medium of non-cell seeded matrices hardly any fibrin degradation products were found. Increasing amounts of degradation products were found in the culture medium of all cell seeded matrices over time, but no differences were observed between the soft and stiff matrices in the first ten days of culture. During this time, degradation was independent of the number of initially seeded cells. Between the tenth and fourteenth day of culture, the stiff matrices degraded significantly faster than the soft matrices. In the stiff matrices the number of degradation products was significantly higher at a higher cell density. Mean ± S.E.M.; N=5; *:$P<0.05$, **:$P<0.01$, ***:$P<0.001$
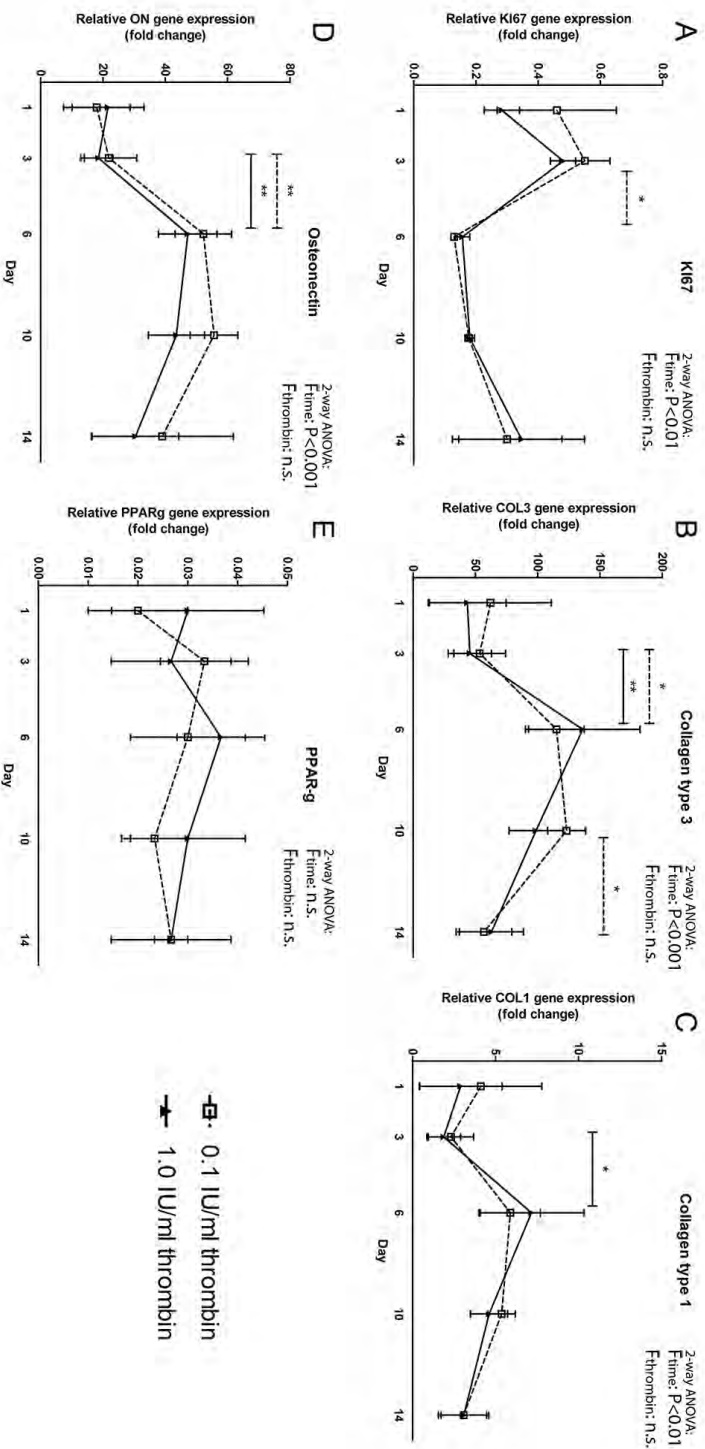C) qPCR data for RNA expression of the proteolytic enzymes MMP-2, and D) MMP-9. With two-way ANOVA, an effect of time (F_{time}) was observed for these proteolytic enzymes, thrombin concentration ($F_{thrombin}$) had no effect.

matrices during the 14 day culture period. No differences were observed between the soft and stiff matrices in the first ten days of culture (Figure 2b). Also, during this time period, fibrin degradation was independent of the number of initially seeded cells. Between the tenth and fourteenth day of culture, the stiff matrices degraded significantly more than the soft matrices (Soft vs. Stiff matrices: 100,000 cells/ml $P<0.01$; n=5| 200,000 cells/ml $P<0.001$; n=5) (Figure 2b). In the stiff matrices the number of degradation products was significantly higher at higher cell densities (Stiff matrices 100,000 vs. 200,000 cells/ml: $P<0.01$; n=5) (Figure 2b). Fibrin matrix degradation in both soft and stiff matrices was not affected by the addition of the antifibrinolytic molecule aprotinin (100 IU/ml) to the culture medium (data not show). RNA expression of markers associated with plasmin induced fibrinolysis decreased with increasing culture time (supplemental figure 3). The RNA expression of the proteases MMP-2 and MMP-9 significantly increased with culture time (Two-way ANOVA: F_{time} $P<0.05$), but were independent of matrix stiffness (Figures 2c and d).

The expression of genes for proliferation, ECM remodeling and differentiation change over time, but not with matrix stiffness
Gene expression of proliferation, differentiation and ECM remodeling markers varied over time on both soft and stiff matrices. After three days of culture the expression of the proliferation marker KI67 significantly decreased (Figure 3a). Simultaneously, the hASCs significantly increased the expression of the remodeling marker COL-3 (Figure 3b). The gene expression levels of differentiation markers, as quantified in this study, was generally not affected by matrix stiffness, except for the most abundant bone matrix protein collagen

Figure 3: qPCR data for RNA expression of markers for proliferation, ECM remodeling, and Osteogenic, Chondrogenic and Adipogenic differentiation. A) Proliferation marker KI 67, the ECM proteins B) Collagen type 3 and C) type 1, the osteogenic differentiation marker D) Osteonectin. With two-way ANOVA, an effect of time (Ftime) was observed for all these markers, thrombin concentration (Fthrombin) had no effect. After three days of culture we observed a shift from a proliferative phenotype to a phenotype that facilitates ECM remodeling and osteogenic differentiation. There was no differentiation towards other mesenchymal lineages; SOX 9, COL 2 and Adiponectin were detectable but not quantifiable, and E) PPAR-γ expression did not change with time or stiffness. Mean ± S.E.M.; N=3; * Depicts difference between consecutive time-points, *:P<0.05, **:P<0.01, ***:P<0.001

type 1. COL-1 expression was significantly increased by day 6 on stiff matrices, but not on soft matrices (Figure 3c). Osteonectin RNA-expression significantly increased between the third and sixth day of culture (Figure 3d). The expression of the adipogenic marker PPAR-γ did not change with time (Figure 3e). The expression of the adipose marker Adiponectin, and the chondrogenic markers SOX-9 and Col-2B was detectable but not quantifiable.

hASCs in stiff matrices produce more ECM and osteogenic differentiation markers
At the end of the culture period (14 days), we detected more ECM produced by cells in stiff matrices compared to those in soft matrices. Newly formed ECM was deposited mainly around voids in the fibrin matrices and consisted mainly of collagen. The Goldner's trichome staining and Von Kossa staining showed matrix calcification (figures 4a and c). All cells in the fibrin matrices were positive for ALP activity, but staining intensity appeared highest in the stiffer matrices (figure 4b). The percentage of RUNX-2 positive cells in stiff matrices was 46.3 ± 18 %, approximately tenfold higher than the percentage of positive cells in soft matrices, which was 4.8 ± 5.8 %. (P<0.05; n=3) (figures 4d and 5).

DISCUSSION
The aim of this study was to investigate hASC differentiation and fibrin degradation in fibrin matrices of different stiffness.. The specific reason for studying degradation was that not only the differentiation of cells, but also the rate of matrix remodeling determines the clinical success of a tissue-engineered construct.[32] We hypothesized that matrix degradation is slower in stiffer fibrin matrices, and that stiffer matrices are favorable to the osteogenic differentiation of hASCs. However, the ELISA data shows that this differentiation was accompanied by an increased cell-mediated degradation of the fibrin matrices.

Matrices polymerized with 1.0 IU/ml were significantly stiffer than matrices polymerized with 0.1 IU/ml thrombin. It was shown previously that mesenchymal stem cells incorporated into fibrin matrices differentiate according to matrix stiffness.[46,47] It is likely that the mechanical properties of the stiff, and not the soft, fibrin matrices triggered the hASCs to differentiate towards the osteogenic lineage, as demonstrated by increased ALP and RUNX-2 expression. With different stiffness, different intracellular signaling pathways are activated, which are able to affect gene and protein expression by these cells.[23,33,48] Examples of such pathways are Rho Kinase (ROCK) and non-muscular myosin 2 (NMM-2) which regulate cell differentiation, cell migration and contraction, and ECM remodeling by altering the cells' actin cytoskeleton.[33,49–51] Although we did not specifically study the cellular pathways involved, we assume that the stiff matrices, and not the soft matrices, surpassed a mechanical threshold
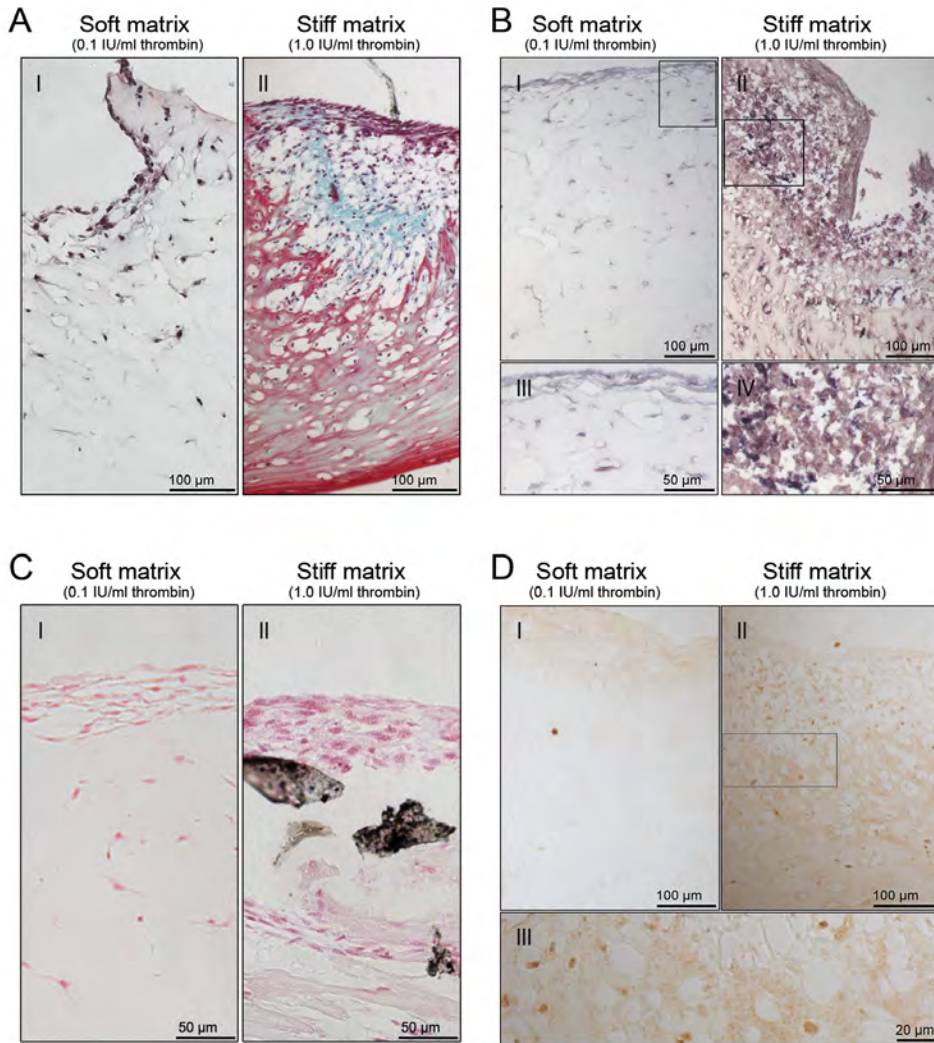
for activation of ROCK, NNM-2 or a similar pathway, causing only the cells in stiff matrices to undergo osteogenic differentiation. On the basis of the results for RNA-expression of the chondrogenic markers SOX-9 and COL-2 and the adipogenic markers Adiponectin and PPAR-γ, we conclude that our cells differentiate preferentially towards the osteogenic lineage. Besides matrix stiffness, the differentiation of hASCs may have been initiated by soluble factors. However, all matrices received the same culture medium, in which no factors were added that induce cell differentiation, and were polymerized with equal amounts of fibrinogen. This makes it unlikely that these sources influenced the differentiation of hASCs. The only difference between matrices was the amount of thrombin. However, because thrombin has an in-vivo half-life of minutes and is rapidly inactivated by platelet lysate in the culture medium, it is unlikely that

◄ Figure 4: Representative pictures of histological staining for Goldner's trichrome, ALP, Von Kossa and RUNX-2.

A) Goldner's trichrome staining of the (I) soft and the (II) stiff matrices (200,000 cells/ml). Compared to the cells in the soft matrices, the cells in the stiff matrices produce more ECM proteins, which are mainly deposited around the voids in the matrix. In the stiff matrices, early matrix calcification is visible. [Goldner's trichrome staining: blue/black = cell nuclei, red = collagen, green/blue = calcified tissue]

B) RNBI/BCIP staining for ALP enzyme activity in the cells in the (I,III) soft and (II,IV) stiff matrices (200,000 cells/ml). In (III) and (IV) higher magnifications for the boxed areas are shown. In both the soft and stiff matrices the cells are 100% positive for ALP activity. However, the staining intensity appears higher in the stiff matrices. [RNBI/BCIP staining: Purple/black = ALP enzyme activity]

C) Von Kossa staining for calcification in the (I) soft and (II) stiff matrices. Only the stiff matrices show matrix calcification. Displacement of the calcified tissue is visible, which likely is an artifact caused by sectioning the hard-soft tissue interface. [Von Kossa staining: Black = matrix calcification, pink/red = cell nuclei]

D) Rabbit anti human RUNX-2 staining in the (I) soft and (II,III): stiff matrices, in (III) a higher magnification of the boxed area is shown, immune localization is only visible in the cell nuclei (200,000 cells/ml). The expression of RUNX-2 is significantly higher in the stiff matrices (see figure 4). [Rabbit anti human RUNX-2 staining: Brown = RUNX-2 immuno-localization]

thrombin caused differentiation of the hASCs.[52–54] Fibrin matrix stiffness increases with higher thrombin concentrations because the number of fibrin fibers and branch-points increase whilst at the same time fiber diameter decreases.[22] Unfortunately, to date it is unclear to what extent differences in fibrin topology affects cell behavior compared to the pre-eminent effects of matrix stiffness.[23,33] We cannot exclude the possibility that matrix topology affected the behavior of the hASCs in our experiments. However, we assume that stiffness is the dominant factor, because the overall effect of topological changes in fiber structure is implied in changes in matrix stiffness.

It is probable that mechanical signals to the hASCs also caused enhanced matrix degradation and production of ECM proteins in our stiff matrices. Histology showed a marked volume-decrease of all cell-seeded fibrin matrices, but especially of the stiff matrices. This could be caused by matrix degradation, matrix contraction or both.[55] On the basis of the fibrin degradation ELISA data, and because we did not observe contraction, matrix degradation is the most obvious conclusion. On the basis of the qPCR data, it is most likely that the hASCs used proteases from the MMP family to facilitate matrix degradation. [56–58] Extracellular matrix degradation is usually a prerequisite for production of new ECM. Therefore, by qPCR we assessed collagen type 3, which is one of the first new matrix proteins produced during wound healing, and stained our matrices for collagen and mineralization.[25,26,59] Histology shows that the cells in our stiff matrices deposit more ECM, mainly around the voids in the matrices. Similarly, Catelas et. al found that bone marrow cells deposit ECM around 'holes' in the fibrin matrix, which were described by Kaijzel et. al as the
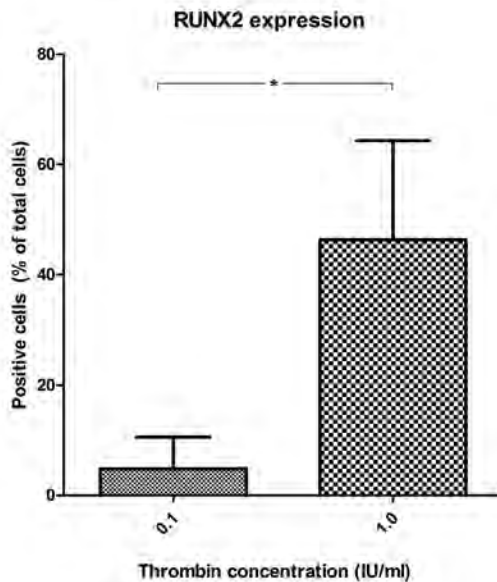
Figure 5: Quantification of RUNX-2 positive cells.
Compared to the cells in the soft matrices, significantly more cells are positive for RUNX-2 protein in the stiff matrices. Mean ± S.E.M.; N=3; *(P<0.05)

result of local degradation of the fibrin matrix.[46,60] Contrary to our results, others have shown that increased fibrin matrix stiffness is associated with decreased fibrin matrix degradation.[31] However, in most studies, the fibrin matrix stiffness is increased by using fibrinogen and thrombin concentrations that far exceed physiological levels, or by adding stabilizing agents such as the protease-blocker aprotinin or cross-linking agent factor XIII.[22,29,61] With clinical application for our matrices in mind, no stabilizing agents were used, because these have been associated with increased risk for stroke, renal failure and myocardial infarction.[62,63] Furthermore, fibrin polymerization time strongly reduces with increasing fibrinogen and thrombin concentrations, which makes clinical application of supraphysiological fibrin matrices difficult or even impossible.[21,42,64] We used fibrinogen and thrombin concentrations within the range that is normal in human plasma. Apart from being easy to handle, fibrin matrices similar in composition to ours have shown to promote angiogenesis, which is essential for clinical success after implantation.[5,37,54] Although the enhanced degradation of our stiff matrices was unexpected, we do not find it surprising that hASCs are able to degrade them. During wound healing, fibrin matrices similar in composition to ours are purposely degraded to facilitate new ECM production.[65]

From our data, it is unclear if ECM remodeling preceded osteogenic differentiation or the other way around. However, it is clear that these processes

are closely related to each other, and it is likely that the initial stiffness of the matrices initiated them. It seems that a threshold was reached by our stiff matrices, because the hASCs behaved vastly different after what appears to be only a small increase in matrix stiffness. Although this requires further study, it is likely that activation of the same pathways that caused the cells in the stiff matrices to differentiate were also responsible for enhanced ECM remodeling, as they are known to regulate this.[33,49–51] We measured the stiffness of acellular matrices in order to compare the effects with cell seeded matrices. Duong et. al showed that incorporated cells do not affect fibrin matrix stiffness directly after polymerization.[66] However, in our case, the mechanical properties of the matrices will have changed during culture, because of matrix degradation, possible matrix contraction, and ECM production. Unfortunately, the nano-indenter probe we used was not able to measure in turbid platelet lysate medium, which prevented us from monitoring the stiffness during culture. The fibrinogen and thrombin concentrations were chosen on the basis of previous experiments by Koolwijk et. al, in which angiogenesis was studied on fibrin matrices created with 2mg/ml fibrinogen and 0.1 IU/ml thrombin.[67] Although the scope of this research was not angiogenesis, we chose 2mg/ml fibrinogen and 0.1 IU/ml thrombin as a standard concentration. To ensure significant changes in the mechanical properties of the fibrin matrices, we changed the thrombin concentrations by steps of 10-fold to reach final thrombin concentrations ranging from 0.01 to 10 IU/ml. Although our initial experimental setup also included matrices polymerized with 0.01 IU/ml and 10 IU/ml of thrombin, these matrices were left out of the results. In our opinion they are not suitable for tissue engineering purposes. The 0.01 IU/ml fibrin matrices polymerized to slow, causing a monolayer of cells to form underneath the fibrin, and the matrices created 10 IU/ml of thrombin showed too extensive matrix contraction and degradation. The cell densities in this study are clinically relevant, as they are consistent with routinely obtained yields from clinical hASC isolations.[18] We based our cell concentrations on an ongoing clinical phase 1 trial by members of our group in which hASCs are implanted for maxillary sinus floor elevation.[68] The initial experimental setup included matrices with 20,000 cells/ml. In this group, we found a similar pattern of fibrin degradation as with 100,000 and 200,000 cells/ml, however less distinct (supplemental figure 3). Therefore, we decided to exclude this cell density from further experiments. For the same reason, we performed immuno-staining and qPCR only on matrices seeded with 200,000 cells/ml.[46]

In summary, we investigated hASC incorporation in fibrin matrices with a clinical application in mind. Although the in-vivo behavior of our matrices requires further investigation, we showed here that fibrin matrices of different initial stiffness have vastly different effects on fibrin matrix degradation and on the differentiation of hASCs. Careful selection of the ratio and concentrations

of fibrinogen and thrombin, as well as the number of seeded cells, will aid in the creation of optimal fibrin scaffolds for connective tissue regeneration, for example soft tissues and non-weight bearing bone defects.

SUPPLEMENTARY FIGURES

Soft matrix (0.1 IU/ml thrombin)   Stiff matrix (1.0 IU/ml thrombin)
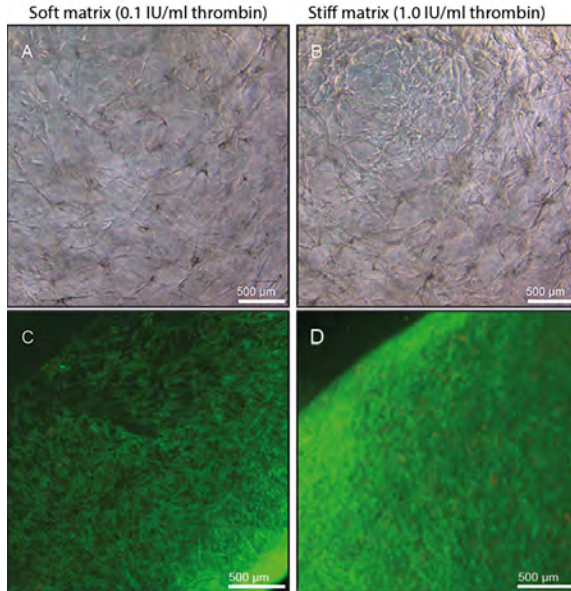
◄ Supplementary figure 1:
hASCs' characteristics inside fibrin matrices
Polarized light images of the A) soft and B) stiff matrices in culture on day 14 (200,000 cells/ml). The cells in both soft and stiff matrices show an elongated star-shaped morphology. The live/dead staining images of the C) soft and D) stiff matrix show predominantly live cells. [Live/Dead staining: green = live cells, red = dead cells]
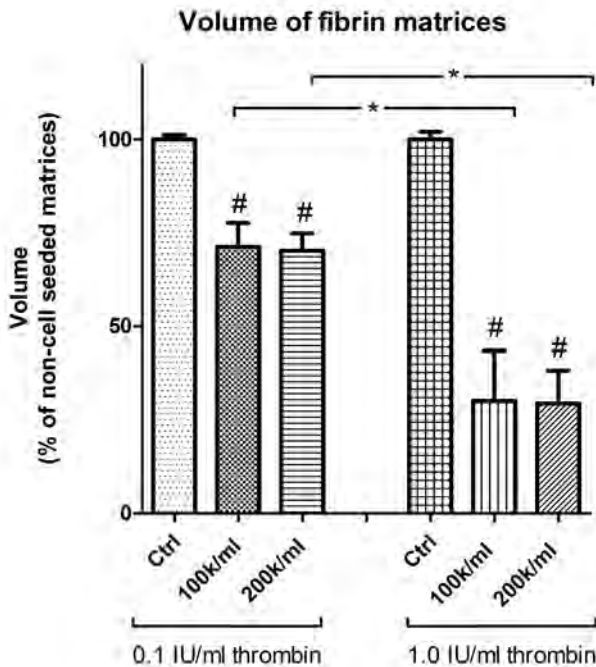
◄ Supplementary figure 2:
Volume of fibrin matrices
Compared to non-cell-seeded matrices, all cell-seeded matrices significantly reduced in volume. The stiff matrices showed a significantly higher volume reduction compared to the soft matrices. #: $P<0.05$ compared to control matrices; *: $P<0.05$
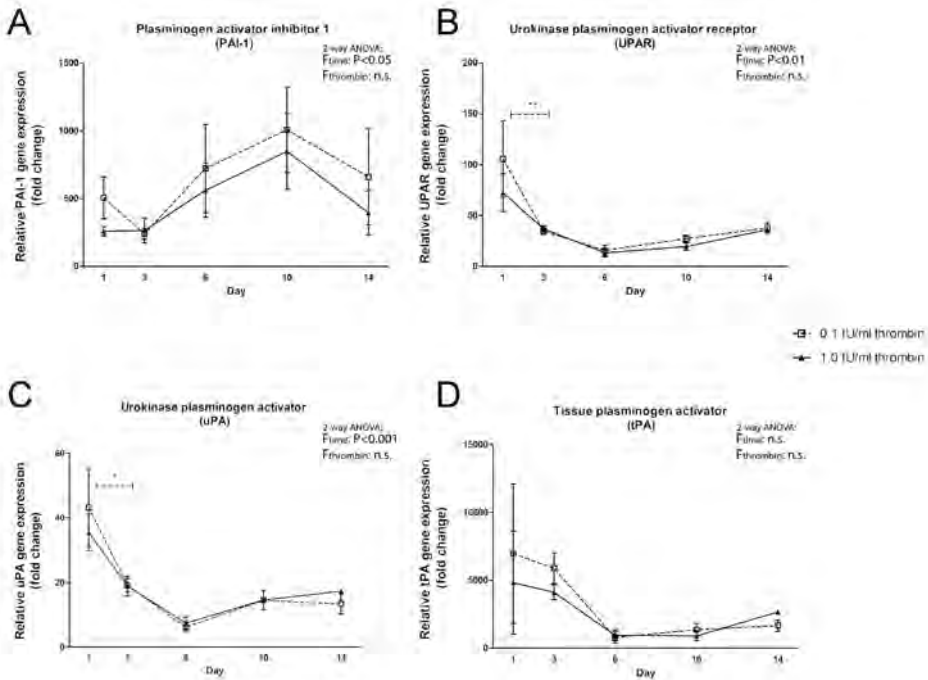
Supplementary figure 3: qPCR data for RNA expression of markers for plasmin activation.
A) PAI-1, B) UPAR, C) uPA, and D) tPA. An effect of time (Ftime) was observed for all these markers, thrombin concentration (Fthrombin) had no effect. After three days of culture we observed a decrease of activators of the plasmin system, and an increase of plasmin inhibitors. Mean ± S.E.M.; N=3; * Depicts difference between consecutive time-points, *:P<0.05, **:P<0.01
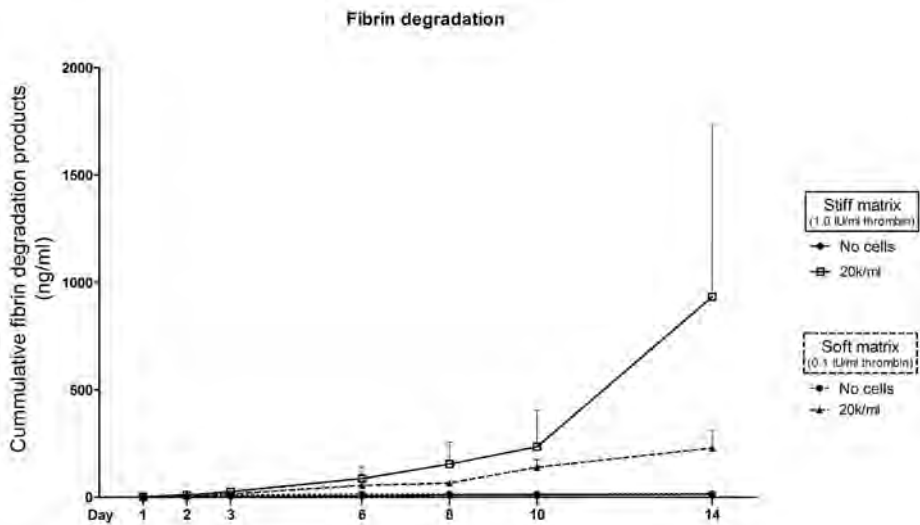
Supplementary figure 4: Degradation of fibrin matrices seeded with 20,000 cells/ml
Although less distinct, the degradation profiles of matrices seeded with 20,000 cells/ml is similar to that of matrices seeded with 100,000 and 200,000 cells/ml. After 10 days, matrix degradation appears more extensive in the stiff matrices. (n=3)

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Chapter IV

Adipose-derived stem cells for periodontal ligament engineering

The need for dynamic strain

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ABSTRACT

Introduction

The periodontal ligament (PDL) connects the tooth to the alveolar bone. Periodontal disease is common, but reliable methods for functional PDL regeneration do not exist. As a tool for PDL regeneration, we propose human adipose-derived stem cells (hASCs) embedded in fibrin. We showed previously that hASCs in fibrin extensively produce collagen, but in a non-functional, random orientation. Also, hASCs produce undesirable factors associated with matrix calcification. We hypothesize that with dynamic strain the matrix will align, and the calcification potential of hASCs reduces.

Materials & Methods

hASCs were seeded in fibrin matrices, which were strained for 3 days, either statically, or with a regime that mimics periodontal loading from mastication. Straining started at day 0 (immediate) or day 4 (delayed). Fiber and cell alignment, and ECM production were determined histologically. qPCR was performed for ECM proteins and calcification-associated genes.

Results

Cell and fibrin fiber orientation was random in non-strained fibrin matrices. Regardless of straining type and onset, cells and fibers aligned in the strain direction. In the immediately strained group, static strain decreased alkaline phosphatase and RUNX-2 expression, while dynamic strain decreased RUNX-2 and osteonectin expression compared to unloaded constructs. Continuous static strain also decreased expression of type III collagen and elastin, while dynamic strain did not. Delayed strain hardly affected gene expression.

Discussion

Provided that dynamic loading commences immediately, expression of calcification-associated genes by hASCs reduces, while matrix and cells align without compromising their regenerative capacity. These results combined confirm the potential for hASC-seeded fibrin matrices for funntional PDL regeneration.

INTRODUCTION

Teeth are connected to the alveolar bone by a soft connective tissue called the periodontal ligament (PDL). The PDL essentially is a network of collagenous fibers firmly anchored to the alveolar bone and root cementum via Sharpey's fibers.[1] The PDL provides mechanical support to the tooth, protection from pathogens and sensory input to the masticatory system.[1] These functions deteriorate if the PDL is damaged by periodontitis, a deep infection that leads to loss of the PDL and supporting alveolar bone.[2] Treatment for periodontitis exists, but current practice aims at minimizing disease progression rather than the regeneration of lost tissues.[3] Although extensive research is being conducted to regenerate periodontal tissues with constructs that combine biomaterials and cells with regenerative potential, the clinical translation of such constructs remains limited.[4] A reason may be that many of these constructs are composed of non-approved biomaterials, and that regenerative cells are either difficult to harvest or require extensive in-vitro expansion. We investigate a strategy to ease clinical translation by using clinically relevant autologous cells in combination with autologous or clinically approved biomaterials.[5] A candidate material for clinical tissue-engineering is fibrin, the body's own scaffold material after vascular damage or injury.[6] Fibrin is off-the-shelf available in forms approved for clinical use, but can also be easily processed from the patient's own blood.[7] Fibrin scaffolds are injectable and provide a temporary matrix that is inductive for angiogenesis and tissue repair by regenerative cells.[7] As a cell source, we propose human adipose-derived stem cells (hASCs), because these cells offer multi-lineage differentiation potential, show high proliferation, and are easily accessible in quantities that permit direct clinical application.[8] As both fibrin and hASCs can be harvested autologously, hASC-seeded fibrin matrices offer patient-specific tissue-engineering constructs. While these constructs remain to be tested in humans, preclinical data from Tobita et al. shows that adipose-derived stem cells, suspended in a carrier of platelet-rich-plasma, promote periodontal regeneration in rats and canines.[9,10] Previously, we showed that hASCs incorporated in fibrin matrices produce extensive amounts of collagen in-vitro.[5] This renders hASC-seeded fibrin matrices a promising candidate for human PDL regeneration. However, in its current form, the orientation of the collagen deposited in the fibrin matrices is random. In all healthy ligaments, cells and fibers align in the direction of principal strain, which increases the structural strength of the ligament in the direction of loading. [11] Another drawback of the reported fibrin matrices is that they increase the expression of the calcification-associated genes alkaline phosphatase (ALP) and Runt-related transcription-factor 2 (RUNX-2) by hASCs, stimulating cell-mediated matrix calcification.[5] Calcification is undesirable in PDL tissue-engineering, because it can result in ankylosis, a pathological condition in which the tooth fuses to the alveolar bone.[12] Thus, if hASC-seeded fibrin matrices are to be used for PDL tissue-engineering, it is necessary to induce cell and matrix alignment for

increased mechanical strength, and to reduce the expression of calcification-associated genes to prevent ankylosis.

For inspiration to achieve this, we looked at the native PDL. During PDL development, fibers are formed in random orientation around the erupting tooth root.[13] At the end stage of eruption, when occlusion occurs, forces of mastication start loading the teeth. This creates strain on the PDL fibers, which directs fiber and PDL fibroblast alignment.[1] Next to external strain from mastication, PDL fibroblasts exert intrinsic strain on the PDL fibers by cytoskeletal contraction.[14] Although straining causes micro-damage to the PDL, it is essential for maintaining PDL function.[1] Despite local damage, overall tissue integrity is maintained because of an extremely high turnover of matrix-proteins by PDL fibroblasts. These fibroblasts are directed by the magnitude and direction of local strain to accordingly remodel the PDL matrix. [13] Straining of the PDL is also essential to maintain PDL space and to prevent ankylosis.[15] The importance of mechanical stimuli becomes apparent when healing periodontal defects are deprived of occlusional loading. Without loading, ankylosis in these tissues is highly prevalent.[12,15] Thus, it seems that dynamic straining of the PDL reduces the calcification potential of the PDL fibroblasts.

hASC-seeded fibrin matrices have potential for PDL tissue-engineering. Here we examine if, with strain, we can align the fibrin matrix and hASCs, and simultaneously reduce the calcification potential of hASCs. With respect to these parameters, we furthermore asked ourselves if in a clinical situation straining should commence directly after application of the construct, or if the tissues should first be given some time to heal? To answer this question, we investigated whether it is best to apply strain immediately after fibrin polymerization, or if it is best to delay the straining onset by three days. For both immediate and delayed straining onset, we hypothesize that dynamic strain is necessary to achieve both alignment and reduced calcification, as is the case with the native PDL.

MATERIALS & METHODS
Unless specified otherwise, materials were obtained from Life Technologies, Bleiswijk, the Netherlands, and manufacturer's instructions were followed.

Human adipose-derived stem cells (hASCs)
Subcutaneous adipose tissue was harvested from the abdominal wall of five healthy women (average age 44.8 years, range 33-54) undergoing elective abdominal wall correction at Tergooi ziekenhuizen Hilversum, the Netherlands. All participating donors provided written informed consent. The written informed consent and study protocol had been reviewed and approved by the Ethics Review Board of the VU University Medical Center, Amsterdam, the Netherlands.

[16] The stromal vascular fraction (SVF), containing hASCs, was obtained as described previously.[16] The plastic adherent fractions of SVFs of the five donors were pooled, and cultured up to passage 4 as described previously.[5]

Fibrin matrix formation
hASCs-seeded fibrin matrices containing 200,000 cells/ml were created with 2mg/ml fibrinogen and 1.0 IU/ml of thrombin (STAGO BNL, Leiden, the Netherlands) as described previously.[5] Per matrix, 200 µl of fibrin was polymerized 1.5 hours at room-temperature in untreated 6-wells Flexcell Tissue Train culture plates on top of linear Trough Loaders (Flexcell international, Burlington – PH, United States of America).

Straining regime and culture
Directly after fibrin polymerization, 3 ml of culture medium was added, which consisted of αMEM with 5% human platelet lysate, 10 IU/ml heparin, and 1% penicillin/streptomycin/fungizone. Fibrin matrices were cultured up to 6 days in a humidified incubator at 37˚C and 5% CO_2. During culture, the cell-seeded fibrin matrices were uniaxially strained in the Flexcell FX4000 device (Flexcell international). Matrices were subjected to a dynamic straining regime that mimics dynamic external periodontal loading from mastication, as well as continuous intrinsic strain on the PDL fibers from PDL fibroblasts. This regime consisted of 8% continuous strain, with four dynamic loading periods per 24 hours; 6 hours between separate straining periods; straining frequency of 1 Hz; 90 cycles per straining period (90 sec); sinus displacement of 8 to 15%. [13,17–19] To distinguish the dynamic component of this regime from the static component, separate matrices were subjected only to 8% continuous static strain. Both dynamically strained matrices and statically strained matrices were compared to non-strained matrices that served as controls.
To determine the effects of onset of straining, two groups were created 1) Immediate straining group and 2) delayed straining group. In the immediate straining group, straining commenced immediately after fibrin polymerization. Matrices in this group were strained for 3 days during culture. In the delayed straining group, matrices were cultured without strain for 3 days. Thereafter the matrices were strained for 3 days during culture. Non-strained matrices were cultured for 3 or 6 days as controls for the immediate and delayed straining group respectively.

Histology and immunohistology
After culture, fibrin matrices were fixed in PBS containing 4% formaldehyde and embedded in paraffin. 10 µm sections were cut. To observe fibrin fiber alignment, sections were deparaffinized and stained for one hour with 1% picrosirius red (Sigma Aldrich). To observe cell morphology, sections were deparaffinized and stained with Mayers' Hematoxylin. To observe newly synthesized collagen,

sections were stained for dermatan sulfate, type I collagen and DAPI as described previously.[20] Images were captured with the either the Leica DRMA HC-F14 microscope or Leica DM600 B microscope and Leica application suite software (Leica). For cell alignment, images were analyzed with NIS elements software (Nikon). The strain direction was leveled with the horizon (equivalent to 0˚ and 180˚). Per fibrin matrix, cell alignment was determined on three distinct regions of interest (center and both ends). The values for these regions were averaged and categorized into subsets of 10˚.

RNA-isolation, cDNA-synthesis and qPCR

RNA and cDNA were obtained as described previously.[5] To assess the potential of hASC-seeded fibrin matrices for PDL regeneration, RNA from hASCs was compared to RNA from PDL fibroblasts. cDNA from PDL fibroblasts of six donors was provided by dr. ir. T.J. de Vries and mr. T. Schoenmaker, and was obtained as described previously.[21] Quantitative polymerase chain-reaction (qPCR) was performed as described previously.[5] Primer-sequences are listed in appendix 2. Genes were expressed relative to a normalized housekeeping (HK) gene, which was calculated from the genes UBC and YWHAZ with the formula HK = $\sqrt{(UBC*YWHAZ)}$.

Statistical analysis

Prism 5 (GraphPad) was used for statistical analysis. To analyze hASC alignment, the student's T-test was used. For qPCR data, data was normalized, strained matrices were compared to non-strained controls with student's T-test. Each n represents an independent experiment, P-values <0.05 were considered significant.

RESULTS
Fibrin fibers and hASCs aligned in the direction of strain

Fibrin fibers appear in random orientation in non-strained matrices. With both static and dynamic strain, fibrin fibers appear aligned in the direction of strain (figures 1A and 2A). hASCs in non-strained matrices are orientated randomly (figure 1B-C and 2B-C). With both static and dynamic strain, significantly more hASCs align in the loading direction (0-10˚ and 170-180˚), and significantly less hASCs align perpendicular to the loading direction (around 90˚). Alignment is independent on the onset of strain (figures 1B-C and 2B-C).

Static strain appears to reduce matrix production

Voids from local fibrin degradation can be observed in all matrices (asterisks in figures 1-4).[5] Because fibrin degradation is a prerequisite for new ECM production, matrices were double stained for dermatan sulfate (DS) and type I collagen (COL-1) Although the fibrin fibers produce a strong background signal, positive fluorescence from DS and COL-1 can be clearly distinguished (figures
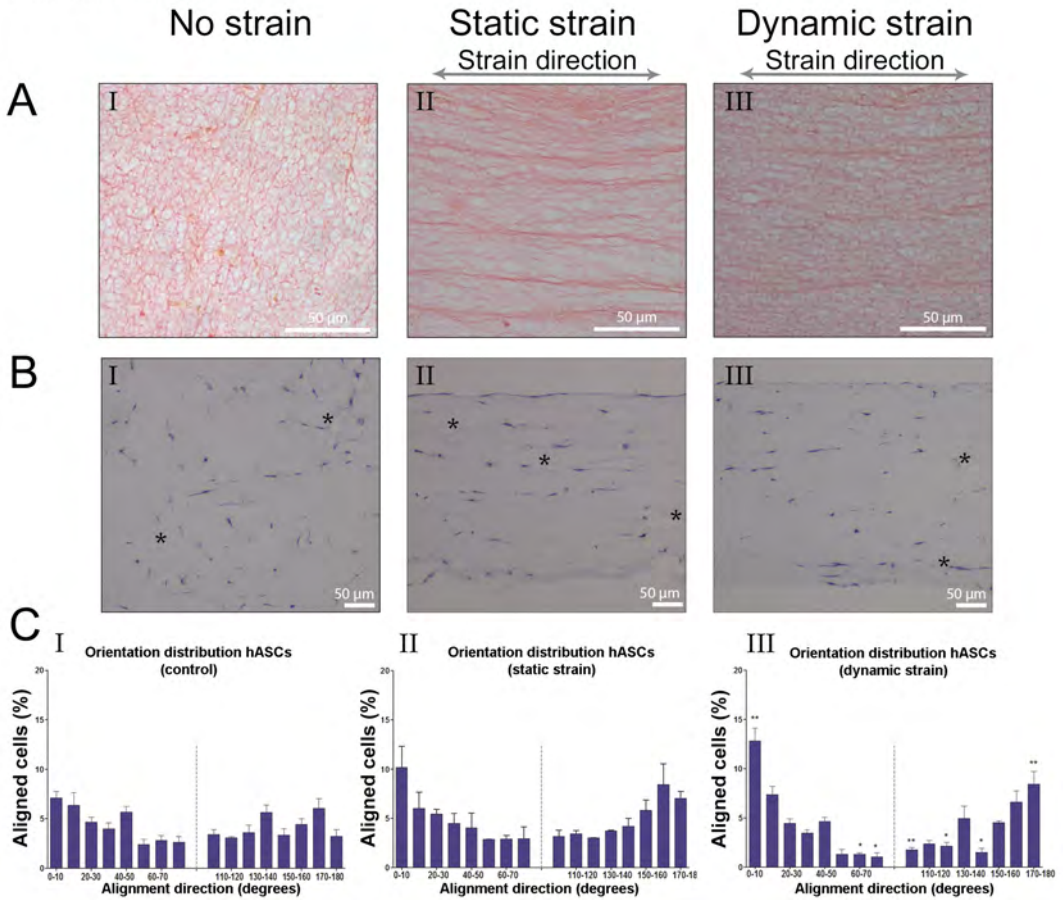
Figure 1: Fibrin fiber and cell alignment in immediately strained matrices. Representative images
A) Non-strained matrices show random fibrin fiber orientation. Statically and dynamically strained
matrices show fiber alignment in the strain direction. [Picrosirius red staining: red = fibrin fibers]
B) Non-strained matrices show random cell orientation. With static and dynamic strain, cells
align in the direction of strain. Local fibrin degradation is visible in all matrices (indicated by *).
[Hematoxylin staining: purple/blue: cell nuclei]
C) Compared to non-strained controls, significantly more cells align in the strain direction with
the dynamic regime. [controls n=5, static strain n=2, dynamic strain n=5] *: P<0.05, **: P<0.01

3 and 4). In the immediate strain group, DS is found in close vicinity of hASCs
in all straining regimes (figure 3). hASCs that received delayed static strain are
positive for DS, but staining for type I collagen seams weak. With delayed strain,
double staining for DS and COL-1 is clearly visible for both non-strained and
dynamically strained matrices (arrows in figure 4). DS and COL-1 are deposited
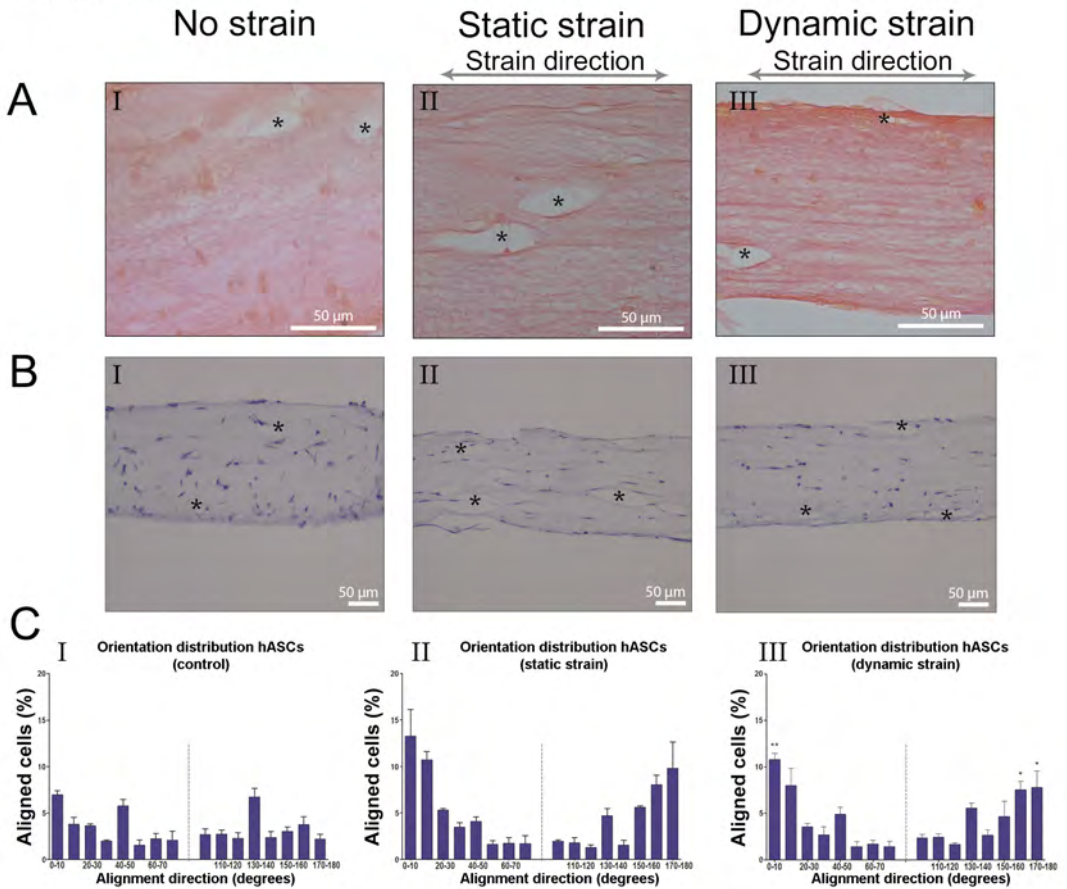in close vicinity of hASCs and areas of local fibrin degradation.

Figure 2: Fibrin fiber and cell alignment of matrices that received delayed strain. Representative images

A) Non-strained matrices show random fibrin fiber orientation. Statically and dynamically strained matrices show fiber alignment in the strain direction. [Picosirius red staining: red = fibrin fibers]

B) Non-strained matrices show random cell orientation. With static and dynamic strain, cells align in the direction of strain. Local fibrin degradation is visible in all matrices (indicated by *). [Hematoxylin staining: purple/blue: cell nuclei]

C) Compared to non-strained controls, significantly more cells align in the strain direction with the dynamic regime. [controls n=3, static strain n=2, dynamic strain n=3] *: P<0.05, **: P<0.01

Only static strain affects the expression of mRNA for proliferation and ECM proteins

In the immediate strain group, proliferation of hASCs appears not affected, as observed from expression of the proliferation gene KI67 (figure 5A). With immediate strain, hASCs in statically strained matrices express significantly lower amounts of mRNA for type III collagen, a marker for early tissue

Immediate strain

Figure 3: Immunolocalization of dermatan sulfate and type I collagen in immediately strained matrices. Representative images

Positive signal for dermatan sulfate is found in close vicinity of hASCs in all matrices. Local fibrin degradation is visible in all matrices (indicated by *). [Dapi staining: blue = cell nuclei, GD3A12/Alexa Fluor 488 staining: green = dermatan sulfate, anti- type I collagen/Alexa Fluor 594 staining: red = type I collagen]

regeneration (figure 5A).[22] Elastin-expression is significantly lowered by static strain (figure 5B). Static strain appears to reduce expression of type I and V collagen, the main proteins that make up the PDL matrix.[1] However, this effect is not significant. With dynamic strain, elastin gene-expression is lower, expression of type I, III and V collagen are similar to that of hASCs in control matrices.

In the delayed strain group, the proliferation-gene KI67 is upregulated in statically
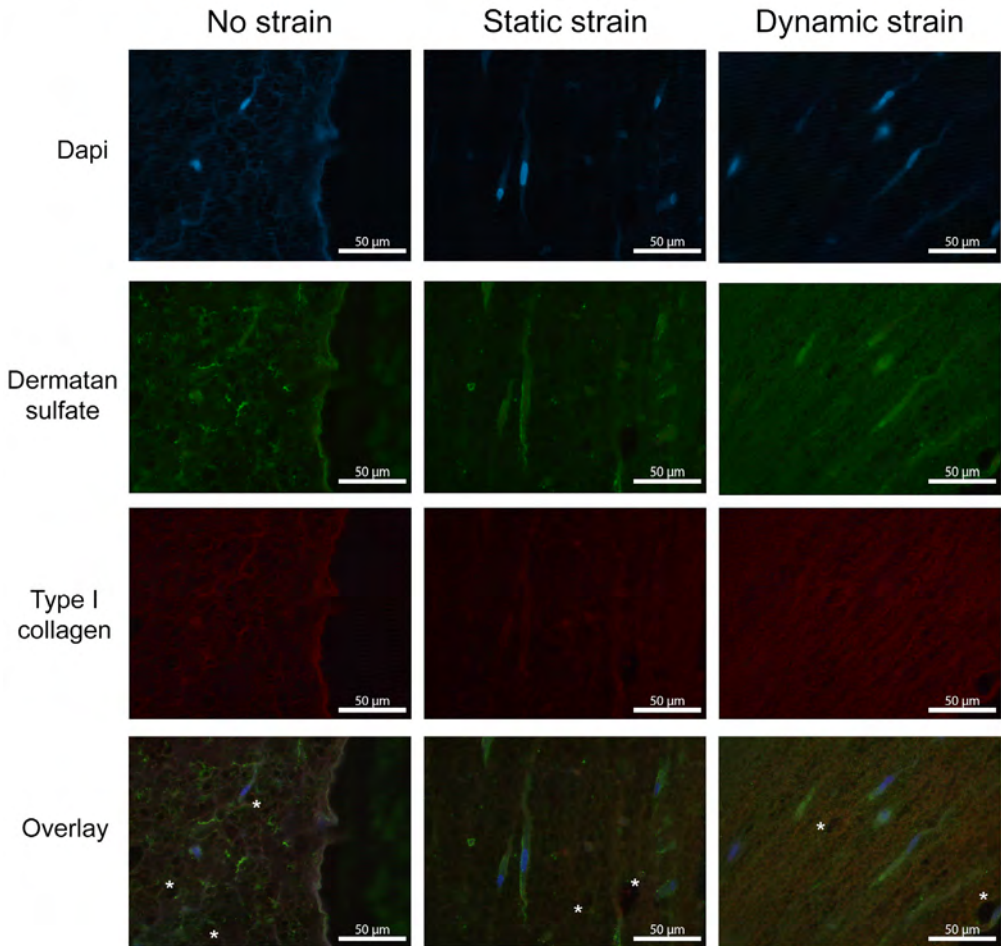
Delayed strain
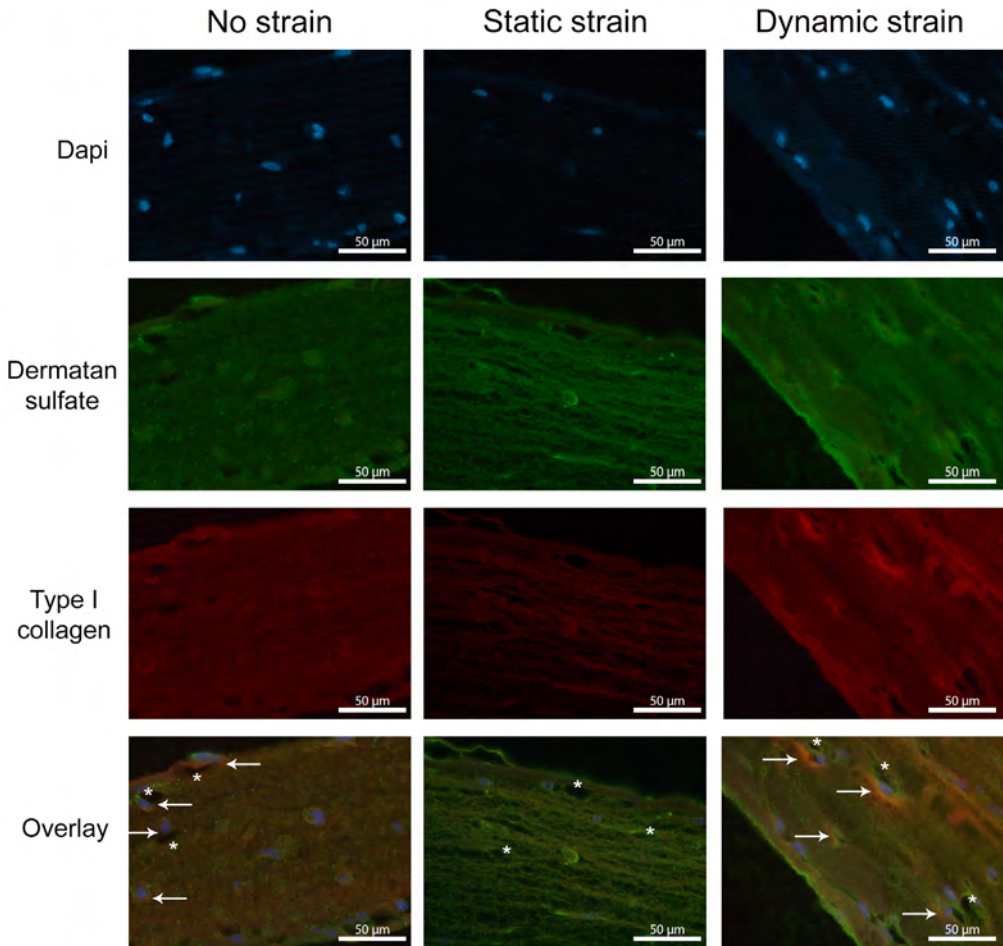
Figure 4: Immunolocalization of dermatan sulfate and type I collagen in matrices that received delayed strain. Representative images

Positive signal for dermatan sulfate is found in close vicinity of hASCs in all matrices. Double staining for type I collagen and dermatan sulfate is visible in non-strained matrices and dynamically strained matrices, but not in statically strained matrices. Double staining (indicated by arrows) is mainly visible in close vicinity of hASCs and local fibrin degradation (indicated by *). [Dapi staining: blue = cell nuclei, GD3A12/Alexa Fluor 488 staining: green = dermatan sulfate, anti- type I collagen/Alexa Fluor 594 staining: red = type I collagen]

strained matrices (supplementary figure 1). Except for the upregulation of type V collagen in statically strained matrices, delayed strain does not affect the expression of type III collagen and typical markers for PDL ECM (supplementary figure 1).

Compared to PDL fibroblasts, type III collagen mRNA expression by hASCs

was higher in immediately dynamically strained and control matrices. hASCs in statically strained matrices expressed levels of type III collagen similar to PDL fibroblasts (supplementary table 1). hASCs in all immediately strained matrices expressed less mRNA than PDL fibroblasts for type I and V collagen (supplementary table 1). In matrices that received delayed strain, hASCs expressed higher levels of type III collagen, and lower levels of type I and V collagen than PDL fibroblasts (supplementary table 1).

Immediate strain lowers early calcification-associated differentiation markers
Expression of mRNA for the early calcification-associated markers ALP, RUNX-2 and Osteonectin are reduced by immediately applied strain (figure 5C). The late marker Osteopontin was not affected by straining. Delayed strain, on the other hand, does not affect expression of early or late calcification-associated markers (supplementary figure 1). In all hASC-seeded fibrin matrices, the chondrogenic genes SOX-9 and COL-2, as well as the adipogenic genes Adiponectin and PPAR-γ were detectable, but not quantifiable.
Compared to PDL fibroblasts, expression of the early calcification-associated markers ALP and RUNX-2 was lower in hASCs for all immediately strained matrices (supplementary table 1). With delayed strain, ALP expression by hASCs was similar to that of PDL fibroblasts, while RUNX-2 expression was lower (supplementary table 1).

DISCUSSION
In this study, hASC-seeded fibrin matrices were subjected to static strain or dynamic strain that mimics periodontal movement from mastication. These regimes were used to investigate cell and matrix alignment and expression of differentiation markers associated with matrix calcification. Straining was commenced either immediately after fibrin polymerization, or after three days 'healing time'. Our results show that cells and matrix align with both static and dynamic strain, regardless of straining onset. However, only if strain is applied immediately, early markers associated with calcification are reduced. Both static and dynamic strain reduced these early markers. However, static strain simultaneously decreased expression of markers for ECM proteins, while dynamic strain did not.

Alignment is important if hASC-seeded fibrin matrices are used for PDL tissue-engineering, because it will strengthen the construct in the loading direction.[23] Fibrin fibers and hASCs aligned independent of the onset of strain and with both static and dynamic strain, but not in the unloaded controls. This implies that strain is required to align fibers and cells. It also shows the high plasticity of these matrices, which is favorable for their clinical application in PDL-regeneration. During application, matrices are injected in random orientation. Thereafter, the local strain will align and strengthen the construct
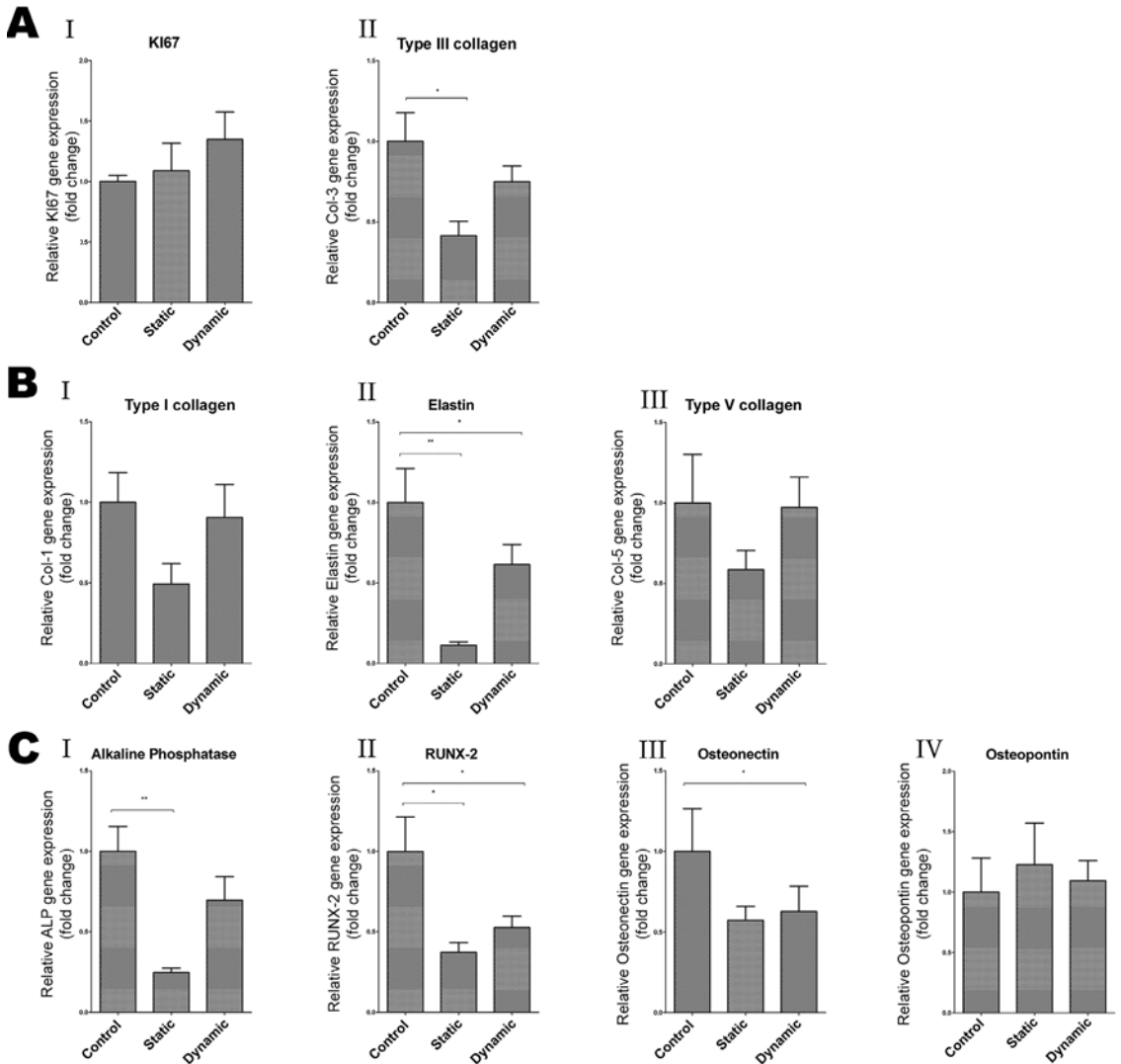
Figure 5: qPCR data for RNA expression of markers for proliferation, ECM remodeling, and matrix calcification in immediately strained matrices

A) Proliferation of hASCs appears not affected by straining, as can be observed from mRNA expression of the proliferation gene KI67. hASCs in statically strained matrices express significantly lower amounts of mRNA for type III collagen, an early marker for tissue regeneration.

B) Immediately applied strain does not affect type I and type V collagen expression, but lowers expression of elastin.

C) The early calcification-associated markers ALP and RUNX-2 are lowered by static strain. Dynamic strain lowers RUNX-2 and Osteonectin. The late calcification-associated marker Osteopontin is not affected by immediately applied strain.

[controls n=5, static strain n=4, dynamic strain n=5] *: P<0.05, **: P<0.01

appropriate to local conditions.[24] Several authors have described that static strain aligns cells, and that dynamic strain triggers a strain-avoidance response, in which cells align perpendicular to the strain direction.[25–27] In this study, no strain-avoidance was observed. The reason for this may be that the dynamic straining regime in this study contains both dynamic and static components. These components respectively mimic loading by mastication, and continuous strain on the PDL fibers that is exerted by PDL fibroblasts.[13,17–19] It is likely that cell and fiber alignment are not influenced by the dynamic component in our regime, because it composes only a small portion of the total strain. Nevertheless, the dynamic component mimics an important part of the in-vivo PDL physiology, and appears essential to maintain the hASCs ability to secrete ECM proteins. This becomes apparent from the mRNA expression of type III collagen and PDL ECM markers, and immunofluorescence of type I collagen. In matrices that received only static strain, these markers are consistently lowered, while dynamic strain was able to restore their expression to that of non-strained controls. In accordance to this, Lanyon and Rubin have reported that merely static strain is catabolic for many tissues, including bone and ligaments. [28] Our results suggest that short bouts of dynamic load, for example from mastication, are essential to maintain tissue remodeling capabilities in tissue engineering constructs for PDL regeneration.

If hASC-seeded fibrin matrices are used for PDL tissue-engineering, it is also important to reduce the calcification potential of hASCs at an early stage. Increased expression of calcification-associated differentiation markers can induce fibrin-matrix calcification, which may lead to ankylosis. In-vivo, matrix calcification is effectuated largely by mature osteoblasts, however also immature osteoprogenitor cells are capable of depositing calcified nodules. [29] In turn, these nodules act as a conductive scaffold, which causes a snowball effect on further matrix calcification.[30] By reducing the expression of early differentiation markers such as ALP, RUNX-2 and Osteonectin, it is likely that the differentiation cascade further downstream is disturbed. [30,31] An example of reduced calcification potential with impaired matrix calcification can be observed in fracture non-union, in which fracture ends do not consolidate but remain connected by a soft callus.[32] A major risk-factor that causes non-union is excessive movement of fracture ends, which strains the callus that contains bone marrow stromal cells.[31] Similarly, our results show that dynamic strain reduces early calcification-associated markers in hASCs, however only if straining commences immediately after fibrin polymerization. This suggests that, in respect to preventing ankylosis, it is best to start loading the construct immediately after application of hASC-seeded fibrin matrices for PDL regeneration.

hASC-seeded fibrin matrices are still far away from the mature PDL. This is

indicated by the low expression of markers for ECM proteins in hASCs when compared to healthy PDL fibroblasts. Nevertheless, from our results it is clear that the hASCs initiate ECM deposition similar to events in wound healing. This becomes apparent from the high expression of type III collagen, which is one of the first ECM proteins deposited during tissue regeneration.[22] Furthermore, after three days of culture, hASCs stained positive for dermatan sulfate, a glycosaminoglycan associated with collagen deposition.[20] After six days of culture, positive staining for type I collagen was observed. From our data it is not clear if newly deposited ECM aligns with strain. However, we speculate that alignment of newly deposited ECM will occur in strained hASC-seeded fibrin matrices, as several authors have shown that alignment of newly deposited ECM is coupled to strain-induced cell and fiber alignment.[24–27]

We chose the combination of fibrin and hASCs because of their potential for rapid clinical translation. Our in-vitro results show that the expression of calcification-associated genes by hASCs reduces when periodontal loading is commenced immediately after application, and that matrix and cells align in the loading direction without compromising their regenerative capacity. Although in-vivo experiments still have to confirm our results, here we demonstrate great potential for human PDL regeneration with hASC-seeded fibrin matrices.

SUPPLEMENTARY FIGURES

Delayed strain
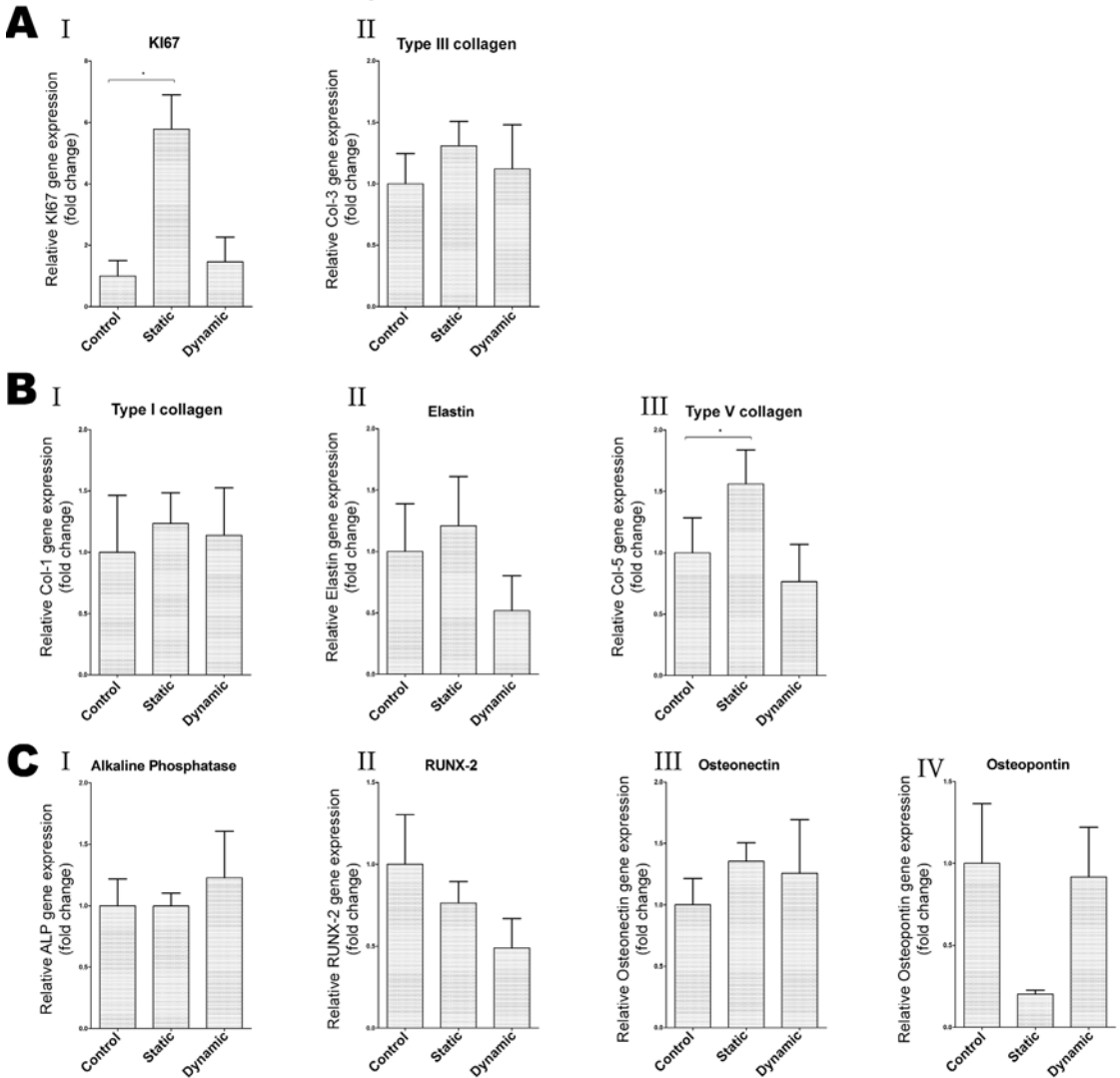
Supplementary figure 1: qPCR data for RNA expression of markers for proliferation, ECM remodeling, and matrix calcification in matrices that received delayed strain
A) Proliferation is increased in the statically strained matrices, as can be observed by increased KI67 mRNA expression. Delayed strain does not affect expression of type III collagen.
B, C) Except for upregulation of type V collagen mRNA in statically strained matrices, delayed strain does not affect typical markers for PDL ECM or matrix calcification.
[controls n=5, static strain n=4, dynamic strain n=5] *: P<0.05, **: P<0.01

Relative expression of hASC genes compared to healthy adult PDL fibroblasts

| | Immediate strain | | | | | | Delayed strain | | | | | |
| | Control | | Static | | Dynamic | | Control | | Static | | Dynamic | |
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
COL-3	319,94%	± 137,96%	132,99%	± 49,92%	239,85%	± 76,14%	513,33%	± 251,01%	672,05%	± 175,85%	576,25%	± 369,65%
COL-1	12,98%	± 5,83%	6,41%	± 2,83%	11,75%	± 6,51%	23,24%	± 21,52%	28,74%	± 10,12%	26,44%	± 17,98%
COL-5	3,23%	± 2,38%	1,88%	± 0,67%	3,14%	± 1,50%	14,06%	± 8,04%	21,96%	± 6,73%	10,73%	± 8,57%
ALP	63,78%	± 23,80%	15,85%	± 3,08%	44,37%	± 22,83%	98,43%	± 42,89%	98,25%	± 17,81%	121,05%	± 74,28%
RUNX-2	14,92%	± 7,85%	5,56%	± 1,59%	7,89%	± 2,56%	35,37%	± 21,44%	27,02%	± 7,83%	17,35%	± 12,52%

Percentage of marker expression compared to healthy PDL fibroblasts

XXX: P<0.05 compared to healty PDL fibroblasts

Supplementary table 1: Relative quantification of ECM and calcification-associated markers for hASCs and PDL fibroblasts
Compared to PDL fibroblasts, type III collagen mRNA expression by hASCs is higher in immediately strained dynamic and control matrices. hASCs in statically strained matrices express levels of type III collagen mRNA similar to PDL fibroblasts. hASCs in all immediately strained matrices express lower mRNA levels than PDL fibroblasts for the typical PDL ECM markers type I and V collagen. Expression of the early calcification-associated markers ALP and RUNX-2 is lower in hASCs than in PDL fibroblasts for all immediately strained matrices. Also in matrices that received delayed strain, hASCs express higher levels of type III collagen, and lower levels of type I and V collagen than PDL fibroblasts. ALP expression by hASCs is similar to that of PDL fibroblasts, while RUNX-2 expression is lower with delayed strain.
[controls n=5, static strain n=4, dynamic strain n=5, PDL fibroblasts n=6]

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Chapter V

Crosslinked electrospun gelatin for connective tissue engineering

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ABSTRACT

Introduction
Gelatin may be a biological alternative for synthetic meshes used in connective tissue repair. Gelatin meshes can be created by electrospinning. However, gelatin needs crosslinking for stabilization and to prevent it from dissolving rapidly. It is unknown whether crosslinked gelatin meshes are biocompatible. Here we studied the effect of crosslinking on the structure and biocompatibility of electrospun gelatin, and how crosslinked electrospun gelatin (cESG) affects cell migration and differentiation.

Materials and methods
Type B gelatin was dissolved in HFIP and electrospun to meshes of 55 µm thickness, which were crosslinked with EDC, and seeded with human adipose-derived stem cells (hASCs). SEM was used to study cESG structure. During 21 days of culture, hASC viability and metabolism, cell attachment, migration, morphology, cell differentiation and extracellular matrix production were assessed.

Results
Crosslinking did not alter the structure of cESG. During 21 days of culture, hASCs retained a star shaped fibroblast-like morphology and migrated through the mesh. hASCs proliferated and stayed metabolically active. Throughout culture, increasing amounts of type I collagen were produced by the hASCs. hASCs did not show differentiation towards osteogenic, chondrogenic, adipogenic or myofibroblast lineages.

Discussion
cESG is a non-instructive biomaterial with excellent biocompatibility. The combination of this material with hASCs is a promising candidate for connective tissue engineering.

INTRODUCTION

Synthetic meshes are widely used for the repair of connective tissues such as the abdominal wall, pelvic floor, and ligaments and tendons.[1] Although these synthetic materials initially show good clinical results in terms of restored stability, in the long term they may exhibit severe post-surgical complications such as breakage or exposure, tissue erosion, and pain related to scar formation. [2–4] This may be caused by the incompatibility of these materials with cells in terms of attachment to and migration through the material, cell survival, and the cell's inability to remodel these materials to an autologous matrix.[5,6] These problems may be overcome by the use of materials composed of natural extracellular matrix (ECM) components. These materials exhibit the same molecular structure and function as the tissues that they were derived from, and those that they need to replace.[7] Such a material is gelatin, an abundant and cheap by-product of the leather and meat industry that is created by controlled collagen hydrolysis.[8] Gelatin is composed of collagenous peptides of various size, is non-antigenic and degrades in fragments that stimulate cells to produce new extracellular matrix (ECM).[9] For these reasons, gelatin is extensively being used in the clinic, for example as a hemostatic agent, or as a bone filling material.[10,11] Gelatin can be processed to create several types of tissue-engineering scaffolds, such as hydrogels, nano-particles, and porous sponges. [12–14] However, a fibrous mesh structure is likely the most suitable scaffold type for connective tissue engineering, as this closely mimics the natural ECM. A technique to create such scaffolds is electrospinning. In electrospinning, a polymer in solution is held at a needle tip, and is placed in a high-voltage field. This creates a polymer jet that travels along the electric gradient, causing the solvent to evaporate, and the resulting polymer fiber to be stretched until it is captured on a collector.[15] Electrospinning has great potential for tissue engineering, because it offers great control over the size of fibers and their pores, and allows both synthetic and biological polymers to be used, including gelatin.[15,16]

By electrospinning it is thus possible to create defined porous fiber meshes of gelatin. However, gelatin dissolves in water within minutes, which makes untreated gelatin meshes unsuitable for connective tissue engineering. In order to make the meshes suitable for this application, it is necessary to stabilize them by crosslinking the gelatin fibers.[17] Gelatin can be crosslinked in various ways. However, not all crosslinking methods are suitable for biomedical application of the material, as many methods incorporate toxic crosslinking agents in the mesh.[17] This is not the case for N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC). EDC induces crosslinking of gelatin via amide bond formation by activating carboxylic groups to react with free amine residues, without altering the chemical composition of gelatin.[18] However, EDC crosslinking may alter the structural properties of the fibers and their pores, which could alter pre-determined properties of the material.[19] Furthermore,

EDC is toxic, and possible residues may compromise the survival and function of regenerative cells that are pre-seeded on the mesh or invade from surrounding stroma after implantation.[19] The survival of cells, their ability to proliferate and migrate, and their capacity to remodel the crosslinked electrospun gelatin (cESG) are essential for its success for connective tissue engineering.

Another important aspect that determines the success of cESG is the differentiation fate of regenerative cells on cESG, as biomaterials may be instructive to cell differentiation.[20,21] For connective tissue regeneration, it is imperative that the cells on the cESG retain a fibroblast-like phenotype. Differentiation to other mesenchymal lineages could hamper functional regeneration. For example, osteogenic differentiation of cells may cause unwanted matrix calcification, and myofibroblast differentiation may cause extensive contraction and premature rupture of the engineered tissue.[22,23]

Here we study the biocompatibility of EDC-crosslinked electrospun gelatin (cESG), and its effects on cell differentiation. For this purpose, we seeded cESG meshes with human adipose-derived stem cells (hASCs). These cells proliferate rapidly and show multi-lineage differentiation potential.[24] Moreover, these cells are candidates for connective tissue engineering, as we previously showed they produce large amounts of collagen in-vitro, and were shown to improve Achilles tendon healing in-vivo.[22,25] We investigated hASC survival and migration on cESG, as well as their metabolic activity, proliferation, and extracellular matrix remodeling by hASCs. To elucidate the effects of cESG on hASC differentiation, we compared gene-expression of hASCs on cESG with undifferentiated hASCs on tissue culture plastic

MATERIALS & METHODS
Unless specified otherwise, all chemicals were obtained from Sigma Aldrich, and used as received. Materials were obtained from Life Technologies, Bleiswijk, the Netherlands, and the manufacturer's instructions were followed.

Electrospinning
Type B gelatin (GelB) isolated from bovine skin was kindly supplied by Rousselot, Ghent, Belgium. A solution of 12% (w/v) GelB in 1,1,1,3,3-hexafluoro-2-propanol (HFIP) was prepared. The solution was poured in a plastic syringe and connected to the spinneret via Teflon tubing. The polymer solutions were injected through the spinneret with a constant flow rate of 1 ml/hour, using a New era Pump Systems LLC pump. A voltage of 18 kV was applied at the G21 tip of the spinneret and the fibers were collected on a grounded collector (DC high-voltage supply, Glassman High Voltage LLC). The distance between the tip of the spinneret and the collector was 12 cm. The meshes were collected from the collector plate after 8 hours of electrospinning, and were vacuum dried overnight to remove any residual solvents.

Crosslinking of GelB fibers

The electrospun GelB meshes were crosslinked with N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) solution in acetone/distilled water. The crosslinking solution was prepared by mixing 1 ml of a EDC solution (1 mg/ml EDC in acetone/distilled water) into 100 mL acetone/distilled water (9/1 v/v) obtaining a final concentration of 0.01 mg/ml EDC in acetone/distilled water. The GelB meshes were crosslinked in this solution for 4 hours at room temperature. Unbound excess EDC was removed by rinsing 2-3 times with distilled water, followed by incubation in distilled water at 37°C overnight. The products were then freeze-dried and sterilized using ethylene oxide gas (cold cycle, AZ Sint Jan, Brugge, Belgium).

Fiber and pore morphology

To observe if EDC crosslinking altered fiber and pore dimensions, non-crosslinked and crosslinked meshes were coated with gold through plasma magnetron sputter coating. Fiber and pore morphology was studied with scanning electron microscopy (SEM) using the JSM 5600 SEM (JEOL, Peabody - MA, USA), in the high vacuum mode. Fiber diameter and pore surface area were determined with ImageJ (NIH, USA). The pore diameter (r) was calculated from the surface area with the formula $r=\sqrt{(surface/\pi)}$, on the assumption that pores represent perfect circles.

Mechanical testing

The ultimate tensile strength, ultimate tensile strain and elastic modulus of the crosslinked meshes were determined using an Instron 6022 machine. Meshes were wetted with water for 20 minutes, cut to bone shape of 6*2 cm and fixated in sandpaper-glued clamps. The clamps were attached to a load cell of 50 N, machine speed was set at 1 mm/min. Single meshes were stretched to rupture at room temperature.

Human adipose-derived stem cells (hASCs)

Subcutaneous adipose tissue was harvested from the abdominal wall of 5 healthy women (age = 44.8 ± 8.35 years; range 33-54 years) undergoing elective abdominal wall correction at the Tergooi ziekenhuizen Hilversum, The Netherlands. All participating donors provided their written informed consent. The written informed consent and study protocol were reviewed and approved by the Ethics Review Board of the VU University Medical Center, Amsterdam, The Netherlands.[26] The vascular stromal fraction, which contains the hASCs, was obtained as described previously.[26] The cells were pooled and the plastic adherent fraction was cultured up to passage 4 in basic culture medium: minimal essential medium alpha (αMEM) with 2% human platelet lysate (PL), 10 IU/ml heparin (Leo Pharma, Amsterdam, The Netherlands), 100 IU/ml penicillin, 100 IU/ml streptomycin and 0.25 IU/ml fungizone (1% P/S/F).[22]

Cell seeding and culture

The crosslinked electrospun gelatin (cESG) meshes were cut with sterile scissors to fit the wells of standard 24 and 96 wells culture-plates. Before cell seeding, the meshes were incubated for 1 hour in αMEM + 1%P/S/F. hASCs were detached with phosphate-buffered saline (PBS) containing 0.5 mM EDTA/0.05% trypsin, suspended in basic culture medium and seeded on the cESG meshes at 5,000 cells/cm2. Cells seeded at 5000 cells/cm2 on tissue culture plastic (TCP) served as controls. Cells were allowed to attach for 3 days, where after the basic culture medium was supplemented with 50 mg/ml ascorbic acid. The cells on meshes and controls were cultured up to 21 days in a humidified incubator at 37˚ C and 5% CO2. Culture medium was refreshed two times per week.

Histology and immuno-histology

At culture days 7, 14 and 21, cells on meshes were fixed in PBS containing 4% formaldehyde and stored in 70% ethanol. To observe the cells' actin skeleton and nuclei, meshes were rehydrated and washed with PBS. Then they were incubated for 50 minutes with 7.5 units/ml phalloidin Alexa-488 in PBS. Subsequently, meshes were washed with PBS, and incubated for 15 minutes with 1:500 DAPI in PBS. Meshes were washed with PBS and mounted on glass microscopy slides.

To observe type I collagen production, meshes were rehydrated and washed with PBS. Thereafter, they were incubated for 1 hour in PBS containing 1% bovine serum albumin (1%BSA/PBS) and 20% normal rabbit serum. The meshes were incubated for 1 hour with goat-anti-human antibody for type I collagen at 1:200 in 1%BSA/PBS. Subsequently, meshes were washed with PBS, and incubated for 1 hour with 1:200 rabbit-anti-goat-Alexa-488 antibody in 1%BSA/PBS. Cell nuclei were stained with DAPI as described above. Wide-field mages were captured with the Leica DRMA HC-F14 microscope and Qwin software (Leica). Z-images were captured with the Axio Zoom.V16 equipped with Apotome.2 and Zen software (Zeiss).

Metabolic assay and DNA quantification

At culture days 1, 3, 7, 14 and 21, 20 µl Alamar blue solution was added to 200 µl culture medium and incubated for 4 hours. After incubation, 75 µl of the reaction product from each well was transferred into a 96-well ELISA plate. Fluorescence was read at 530/25, 590/35 nm in the Synergy multi-plate reader (Biotek systems, Bad Friedrischshall, Germany).

DNA was quantified by CyQuant assay. After the Alamar blue assay, cells were washed with PBS, lysed with 100 µl CyQuant lysis buffer and frozen over night at -20 ˚C. Per sample, 50 µl lysate was transferred into a 96-well ELISA plate and conjugated with CyQuant GR dye. After incubation, fluorescence was read at 480/520 nm in the Synergy multi-plate reader. DNA content was determined

from a standard curve included in the kit.

RNA-isolation and cDNA-synthesis
At culture days 7, 14 and 21, cells on meshes and controls were lysed with RLT lysis buffer (Qiagen, Venlo, the Netherlands). RNA was isolated with RNeasy mini spin columns (Qiagen). cDNA was synthesized from 250ng RNA with the superscript VILO cDNA synthesis kit.

Quantitative polymerase chain-reaction (qPCR)
qPCR was performed as described previously, to determine the RNA-expression of markers of ECM remodeling, osteogenesis, chondrogenesis, adipogenesis, and myofibroblast differentiation.[22] The primer-sequences are listed in appendix 2. All genes were expressed relative to a normalized housekeeping (HK) gene, which was calculated from the genes UBC and YWHAZ with the formula HK = $\sqrt{(UBC*YWHAZ)}$.

Statistical analysis
Prism 5 (GraphPad) was used for statistical analysis. The student's T-test was used to compare RNA expression between cells on cESG and TCP at the different time points. Changes in RNA expression over time were analyzed with one-way ANOVA with Bonferonni multiple comparisons post-hoc test. Each n represents an independent experiment, P-values <0.05 were considered significant.
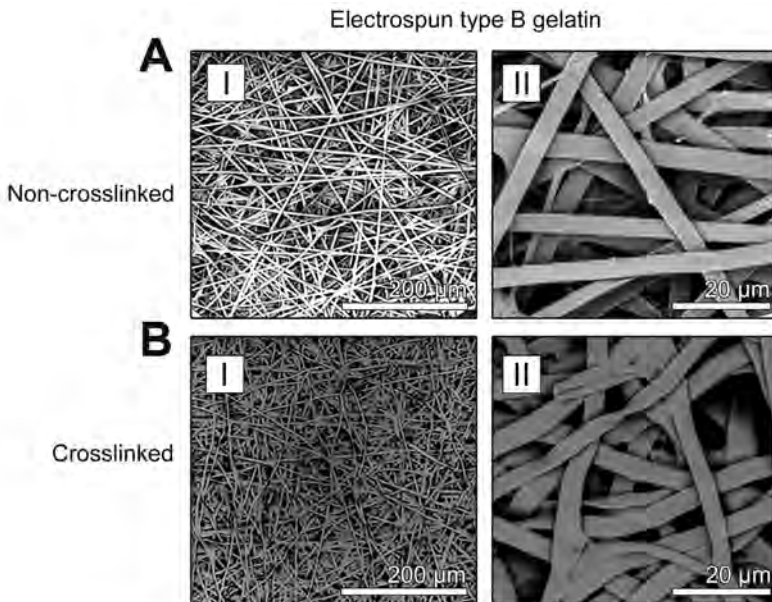

Figure 1: Scanning electron microcopy images of electrospun gelatin
A) The non-crosslinked electrospun fibers show a flattened morphology.
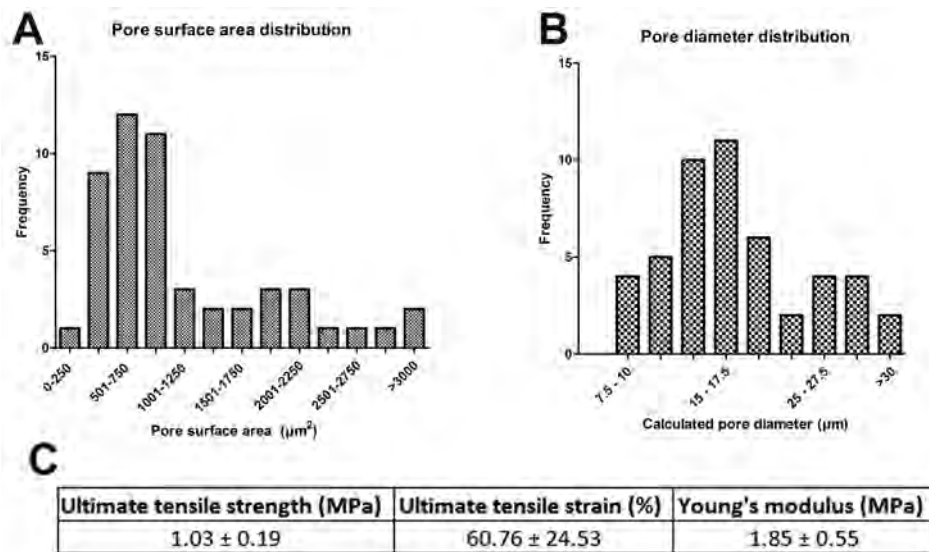B) The morphology of the fibers and pores is not altered by crosslinking.

figure 2: Distribution of pore surface and size, and mechanical properties of crosslinked meshes
A) Measured pore surface area distribution
B) Calculated pore size distribution
C) Mechanical properties of crosslinked meshes

RESULTS

Electrospinning, crosslinking and mechanical tests

After 8 hours of electrospinning, a mesh of approximately A4 paper size (210*148 mm) was obtained. The mesh had a thickness of 55 ± 7 µm, with a fiber thickness of 3.8 ± 0.6 µm. The fibers show a flattened appearance. After crosslinking and drying, fibers retained their shape and dimensions (figure 1). The median pore surface area was 790 µm2 (range 220-3446 µm2), which corresponds to a calculated median pore diameter of 15.9 µm (range 8.4-33,1 µm) (figure 2a-b). The crosslinked meshes showed a linear elasticity profile without plastic deformation before rupture. The meshes had had an ultimate tensile strength of 1.03 ± 0.19 MPa, ultimate tensile strain of 60.74 ± 24.54 % and Young's modulus of 1.85 ± 0.55 MPa (figure 2c).

The hASCs attach to, and proliferate on cESG meshes

The cells attach to the cESG and infiltrate the mesh (figure 3a-b). The distribution of the hASCs appears homogeneous throughout the scaffold. From the actin staining it is visible that the cells show a stellate fibroblast-like morphology (figure 3c).[27] The hASCs remained viable and metabolically active during 21 days of culture, and increased in numbers at a rate that is comparable to cells on TCP (figure 4a and supplemental figure 1). After 21 days of culture, the cESG meshes were intact and showed no macroscopic signs of degradation (data not shown).
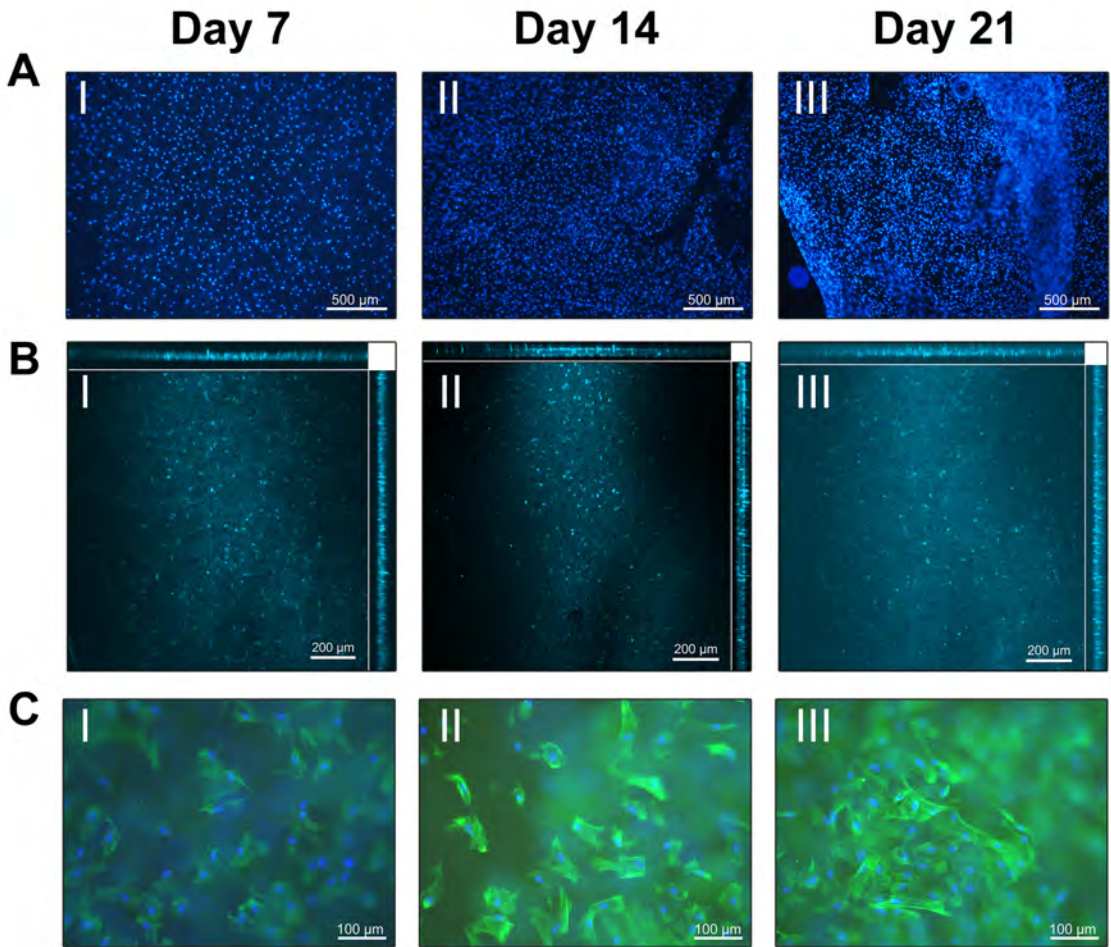
Figure 3: Images of cell attachment, cytoskeletal morphology, and cell infiltration of human adipose-derived stem cells seeded on crosslinked electrospun gelatin

A) Cell distribution is homogeneous over the electrospun gelatin. [Wide field view, DAPI staining: blue = cell nucleus]

B) Cells are able to migrate, and are present throughout the entire gelatin mesh. [Z view, DAPI staning: blue = cell nucleus]

C) Images of actin cytoskeleton of cells on electrospun gelatin. From day 7 to day 21, the cells retain a stellate, fibroblast-like morphology. [Wide field view, Phalloidin Alexa-488 staining: green = actin, DAPI staining: blue = cell nucleus]

Gene-expression for proliferation markers reduces, but increases for markers of matrix remodeling

The expression of the early regeneration marker type III collagen (COL-3) increases in hASCs on cESG from day 7 to day 14, and is higher in cells on cESG than on TCP. From day 14 to 21 COL-3 expression in hASCs on cESG reduces to levels similar to that of hASCs on TCP (figure 4b). The expression of the extracellular matrix protein type I collagen (COL-1) is lower in cells on cESG than in cells on TCP on day 7. On day 14 however, COL-1 expression is similar

DNA content & Metabolic activity

A

I DNA content

II Metabolic activity per ng DNA

- ● Tissue culture plastic
- △ Electrospun gelatin

Gene-expression

B

I Type III collagen

II Type I collagen

III Gelatinase A (MMP-2)

IV Gelatinase B (MMP-9)

- ● Tissue culture plastic
- △ Electrospun gelatin

C

I Alkaline phosphatase

II RUNX-2

III Osteopontin

IV alpha smooth muscle actin

V PPAR-γ

- ● Tissue culture plastic
- △ Electrospun gelatin

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◄ Figure 4: DNA content, metabolic activity, and qPCR data
A) I) The amount of DNA is similar for electrospun gelatin and tissue culture plastic. II) The metabolic activity of cells on electrospun gelatin is similar to that of cells on tissue culture plastic. [n=3]
B) I,II) Gene expression for the extracellular matrix proteins type III and type I collagen increase for cells on electrospun gelatin from culture day 7 to day 14, and decrease from day 14 to day 21. III) Gelatinase A gene expression shows a similar pattern. IV) Gelatinase B gene expression does not change over culture time. [n=4]
C) I) Gene expression for alkaline phosphatase is higher for cells on electrospun gelatin, but does not change over culture time. II) RUNX-2 gene expression increases for cells on electrospun gelatin from day 7 to day 14, but is lower than in cells on tissue culture plastic. III) osteopontin gene expression does not change over culture time. IV) Alpha smooth muscle actin gene-expression decreases over culture time. V) PPAR-γ expression does not change over culture time for cells on electrospun gelatin. [n=4]
[Effect over time: * P<0.05, ** P<0.01, *** P<0.001; Difference between TCP and ESG: # P<0.05, ## P<0.01]

between cells on cESG and TCP. On day 21 hASCs on cESG show reduced levels of COL-1 (figure 4b). Gelatinase A (MMP-2) shows a pattern of expression similar to COL-3 and COL-1, it is upregulated in cells on cESG from day 7 to day 14, and downregulated from day 14 to day 21 (figure 4b). The expression of gelatinase B (MMP-9) does not change over time for cells on cESG or TCP (figure 4b).

hASCs on cESG do not show a preferential differentiation route
The expression of the early osteogenic differentiation marker alkaline phosphatase (ALP) is higher for cells on cESG than on TCP on days 14 and 21. However, neither for cells on cESG or TCP does ALP gene-expression change during culture time (figure 4c). Cells on TCP express higher levels of the early osteogenic marker runt-related transcription factor 2 (RUNX-2) (figure 3c). The expression of the late osteogenic marker osteopontin does not change over time in cells on either cESG or TCP. However, on day 21, osteopontin expression is higher for cells on cESG than for cells on TCP (figure 4c). Expression of alpha smooth muscle actin (αSMA), a marker for myofibroblast differentiation, is higher in hASCs on TCP than on cESG on day 7.[23] From day 7 to day 14, αSMA expression decreases in cells on TCP, but increases in cells on cESG. On day 21, αSMA expression decreases in hASCs on cESG to levels similar to that of hASCs on TCP controls (figure 4c). The expression of the adipogenic marker PPAR-γ remained unaffected in cells on cESG, but decreased over culture time in cells on TCP (figure 4c). The expression of the adipogenic differentiation marker adiponectin, as well as the chondrogenic differentiation markers type II collagen and SOX-9, were detectable but not quantifiable with qPCR (data not shown).

hASCs on cESG produce increasing amounts of type I collagen
On day 7, some cells show positive signal for type I collagen, which is found only
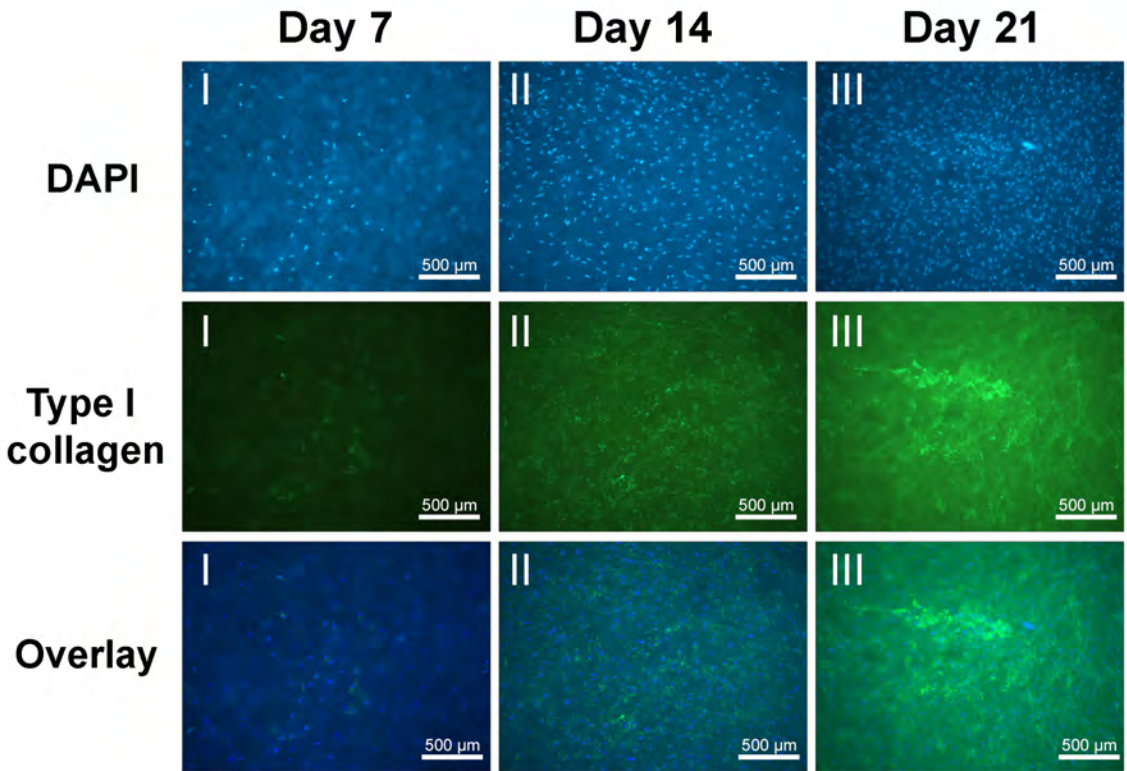
Figure 5: Immunolocalization of type I collagen on electrospun gelatin mesh

I) On day 7, some cells show positive signal for type I collagen, which is found only intracellularly.

II) On day 14, almost all cells show positive signal for type I collagen, which is found in and around the cells.

III) Large amounts of extracellular type I collagen are deposited on the electrospun gelatin on day 21.

[DAPI staining: blue = cell nucleus, Anti-COL1 Alexa-488 staining: green = type I collagen]

intracellularly (figure 5). On day 14, almost all cells show positive signal for type I collagen, which is found in and around the cells (figure 5). Large amounts of extracellular type I collagen are deposited on the electrospun gelatin on day 21 (figure 5).

DISCUSSION

In this study we investigated the biocompatibility of EDC-crosslinked electrospun gelatin (cESG) and its effects on cell differentiation when combined with human adipose-derived stem cells (hASCs). We show that cESG facilitates hASC attachment, proliferation and metabolism similar to tissue culture plastic (TCP). The porous structure of the cESG allows hASCs to migrate through the full thickness of the material. We show that the hASCs retain a fibroblast-like morphology, and that cESG does not stimulate the hASCs to differentiate towards the osteogenic, chondrogenic, adipogenic or myofibroblast lineage. The

cells express increased levels of markers for matrix remodeling, and deposit large amounts of type I collagen.

Biocompatibility of materials for tissue engineering is key, as this will determine the host response after implantation, and survival and function of regenerative cells. Here we show excellent biocompatibility of cESG, despite the toxicity of the spinning solvent HFIP and crosslinking agent EDC.[19] This excellent biocompatibility makes cESG a suitable candidate for tissue engineering. cESG allows the creation of three-dimensional constructs, because cells are able to attach to and migrate through the material. Furthermore, the fibrous and porous structure of cESG makes the material suitable for the engineering of connective tissues. However, for this application it is important that cESG is not instructive for differentiation, and that regenerative cells retain a fibroblast-like phenotype. To test the effects of cESG on cell differentiation, we chose hASCs, as they show multi-lineage differentiation capacity.[28] To elucidate the distinct effects of cESG on hASC differentiation, the culture medium was kept as basic as possible, and specifically free of chemical inducers of differentiation. As a control, hASCs were cultured in similar conditions on TCP, and considered undifferentiated.[29] Our data show that the hASCs do not show differentiation towards other mesenchymal lineages when cultured on cESG. This is in accordance with the results of Sisson et. al, as they reported that electrospun fibers with a diameter similar to ours do not enhance differentiation.[21] Also phenotypically and functionally, the hASCs on cESG appear fibroblast-like. Histology shows that these cells retain a fibroblast-like morphology throughout the culture, and that they produce increasing amounts of type I collagen, which is the main constituent of connective tissue matrix.[30] qPCR shows that with increased expression for the regeneration markers type III and type I collagen, also gelatinase A is upregulated. This suggests that the hASCs are able to remodel the cESG to a collagenous matrix.[31,32] This data combined shows that cESG has a broad application potential for connective tissue engineering. Especially since the material may be combined with factors that stimulate differentiation before application.[33] However, if left unaltered, it is likely that the stromal surrounding of the cESG provides tissue-specific signals to the regenerative cells after implantation.

Importantly, we show that the meshes' fiber and pore morphology was not altered by crosslinking with EDC. This ensures that predefined fiber and pore dimensions are retained, and that further post-processing of the materials is not necessary. The concentration of EDC was optimized for the crosslinking solution (see supplemental figure 2). Only at 0.01 mg/ml EDC concentration, the fibers retained their dimension and shape as before the crosslinking process. They were also easier to handle and flexible when placed in water, as they did not aggregate together as they did for higher EDC concentrations

(i.e. > 0.1 mg/ml). For smaller concentrations of EDC (i.e. < 0.01 mg/ml), the fibers solubilized due to the water part of the solvent mixture and their appearance after crosslinking resembled a very thin 2D film.

The gelatin fiber meshes were collected from the collector plate after 8 hours of electrospinning with an average mat thickness of 55 μm. These results are in agreement with previous studies, where 10 hours of electrospinning yielded 50 μm thick meshes.[34] After crosslinking and drying, the fiber meshes were not as pliable as the non-crosslinked ones. It was noticed that when placed in water the crosslinked meshes regained their flexibility. Therefore, it can be concluded that water acts like a plasticizer.

The ultimate breaking strength and elastic modulus of cESG match those of commercially available mesh materials for hernia repair.[35] However, the maximum breaking strain is lower, most likely because our material shows no plastic deformation before rupture. Undoubtedly the mechanical properties of the meshes will have changed as a result of the crosslinking process. However, it was not possible to reliably compare the mechanical properties of non-crosslinked versus crosslinked meshes. Crosslinked meshes require wetting before mechanical testing, which is impossible with non-crosslinked meshes as they rapidly dissolve in water. We only report the mechanical properties of the crosslinked meshes, as these are the most relevant.

In this study we did not vary the dimensions of the fibers and pores, as this was beyond the scope of our research question. However, such dimensions are easily tunable by altering the electrospinning parameters.[15] Our crosslinking protocol may be used in future studies to further optimize the meshes' characteristics for tissue engineering of different connective tissues.

We chose gelatin as a biomaterial because it is cheap and abundant, and requires no specialized production facilities. Our in-vitro results show great potential for tissue engineering with cESG. It is of importance that the in-vivo performance of cESG is determined before clinical application, however the translation from 'bench-to-bedside' of this material should be easily made, as non-crosslinked gelatin is already approved for clinical use.[10,11,36] The clinical potential of cESG, combined with the easy accessibility and abundancy of hASCs provides a strong combination for connective tissue engineering.

SUPPLEMENTARY FIGURES
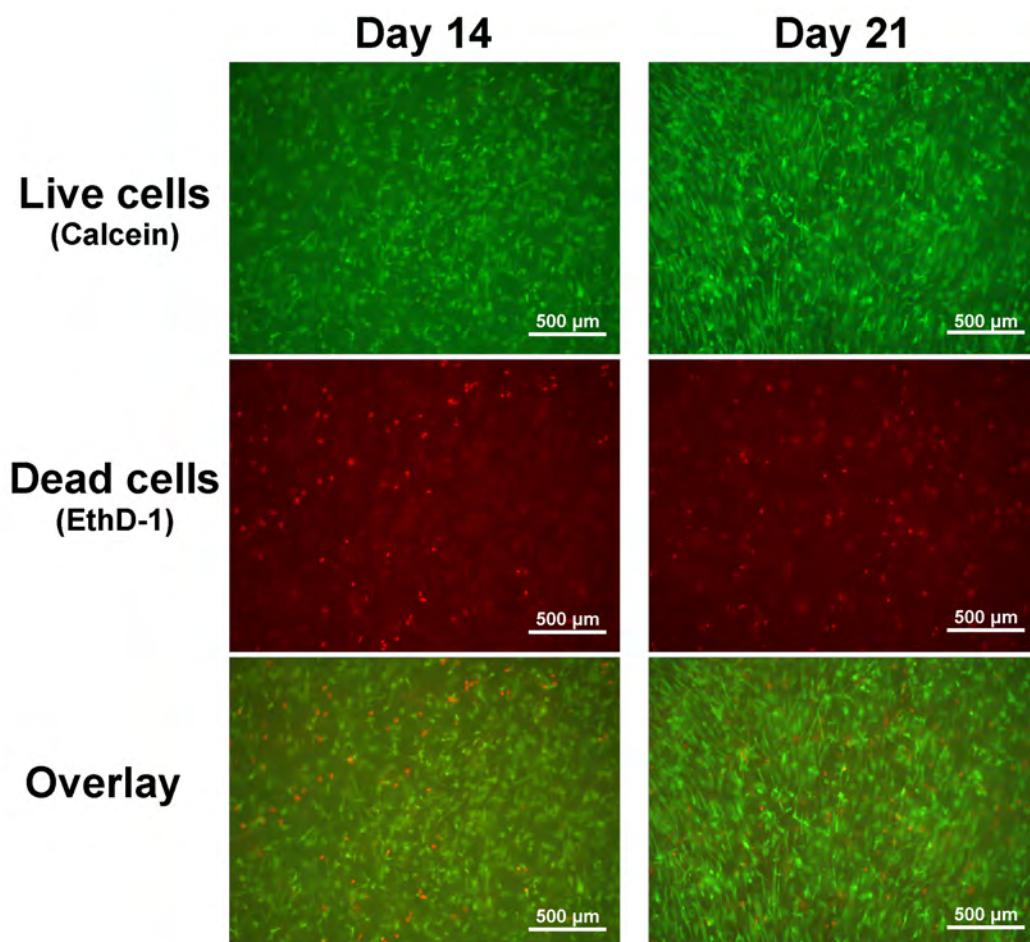
Supplementary figure 1: Live/dead staining of hASCs on crosslinked meshes.
On day 14 and day 21 predominantly live cells are found on the meshes. The gelatin shows some auto-fluorescence in the red channel, however dead cells can clearly be distinguished.
[Calcein/Ethidium homodimer-1 Live/Dead staining: Green = live cells, Red = dead cells]

non-crosslinked
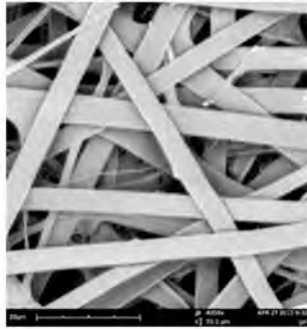
1 mg/ml EDC in acetone/water
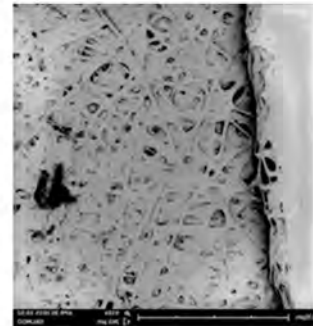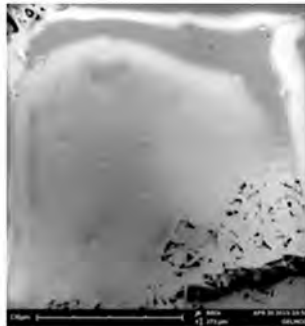
0.05 mg/ml EDC in acetone/water
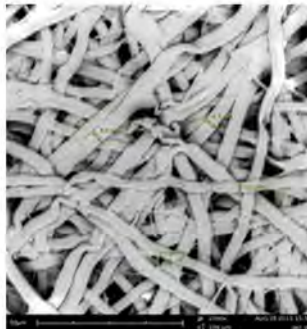
Supplementary figure 2: Scanning electron microcopy images of electrospun crosslinked with different concentrations of EDC.
At 1 mg/ml EDC the fibers aggregate and melt together to form a 2D film. At 0.05 mg/ml EDC most fibers retain their shape and dimensions, however some aggregation occurs.

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Chapter VI

Adipose-derived stem cells form collagenous fibers in fibrin matrices anchored to crosslinked electrospun gelatin meshes

Thijs de Jong, Astrid D. Bakker, Theodoor H. Smit

ABSTRACT

Introduction
Teeth are supported and protected by the periodontal ligament (PDL), which absorbs the high impact loads from mastication, prevents bacterial progression, and provides sensory input. These capacities are lost when teeth are replaced with dental implants, which are placed into the alveolar bone to osseointegrate. This results in unbuffered loading from mastication, and unhampered bacterial progression along the implant surface, which commonly leads to bone loss. As a solution for these problems, we propose a concept for the tissue engineering of a PDL around dental implants. This concept is derived from natural PDL development, and consists of a fibrin-matrix that is seeded with human adipose-derived stem cells (hASCs), which mimics the stromal tissue from which the PDL fibers are formed. We previously showed that fibrin stimulates the production of collagen fibers by the hASCs. However to be functional, attachment of these fibers to the implant surface is necessary. In order to facilitate such attachment, we propose a mesh of crosslinked electrospun gelatin (cESG), that may be wrapped around the implant. Such a fibrous surface mimics the non-mineralized dentin matrix, to which the initial PDL fibers are attached during tissue development.
Here we investigate how to achieve the best connection between the fibrin and cESG, and if hASCs must be seeded in the fibrin, on the gelatin, or on both materials.

Materials & Methods
hASCs were seeded at 5,000 cells/cm2 on cESG, or at 200,000 cells/ml in fibrin. Four groups were created: 1) Cells seeded on cESG, covered with cell-free fibrin. 2) Cells seeded on cESG, covered with cell-seeded fibrin. 3) Cell-free cESG, covered with cell-seeded fibrin 4) Cell-free cESG covered with cell-free fibrin. During 14 days of culture, cell position, and type I collagen production was assessed histologically (n=3).

Results
After 7 days of culture, cells seeded only on cESG did not survive, and only background signal was observed for type I collagen. When both materials are seeded, cells reside in the cESG and fibrin matrix up to the 7th day of culture. Thereafter, only cells can be observed in the fibrin matrix. After 7 days of culture, cESG and fibrin detached from each other in all three experiments. Positive signal for type I collagen is mainly found in the fibrin matrix, with the highest intensity at the interface of fibrin and cESG. When only fibrin is seeded, cells are dispersed evenly throughout the fibrin matrix. Some cells reside at the interface of fibrin and cESG. Throughout 14 days of culture, cESG and fibrin remain attached to each other. Positive signal for type I collagen is mainly found in the fibrin matrix, with the highest intensity at the interface of fibrin and cESG.

Discussion
Our concept for PDL tissue engineering around dental implants shows feasibility, in-vitro. To maintain a close attachment of fibrin and cESG, hASCs should be seeded only in the fibrin matrix.

INTRODUCTION

The periodontal ligament (PDL) is an aligned connective tissue that constitutes the connection between the tooth and the alveolar bone. It provides stability and protects the tooth and bone from the high forces of mastication by acting as a shock absorber.[1] Furthermore the PDL is a physical barrier that prevents the invasion of pathogens from the oral cavity, and acts as a sensor for the mastication system.[1] Dental implants do not have a PDL, as they are placed directly into the alveolar bone where they need to osseointegrate.[2] To improve the osseointegration, the implant surface roughness is highly increased.[3] However, increased surface roughness also enhances biofilm attachment, and aids in bacterial colonization.[4] Such bacterial colonization triggers an inflammatory response that leads to irreversible bone loss, and ultimately to implant loosening.[5,6] Furthermore, direct implant placement in the alveolar bone causes loads from mastication to be unbuffered, and forces to be transmitted directly to the bone. This may lead to micro-fractures of the alveolar bone, which allows further bacterial invasion and increases bone loss.[7]

Such problems with dental implants are common, and often lead to implant loss.[8] To overcome them, we are developing a tissue engineering strategy to engineer a PDL around dental implants that aims at rapid clinical translation. To allow such translation, all materials and cells in our concept should be patient-derived, or off-the-shelf available for clinical use.

In the creation of our concept for PDL tissue engineering, we found inspiration in the natural development of the PDL.[9] We mimic the developing tooth's stromal surrounding with a fibrin matrix that is created with physiological concentration of fibrinogen and thrombin, two proteins found in human blood, which can be derived from the patient's plasma (figure 1).[10] This fibrin matrix may be seeded with the patient's own adipose-derived stem cells derived (hASCs), to be injected at the place of PDL generation. We have shown previously that hASCs extensively produce type I collagen in such fibrin matrices.[10] In our proposed tissue engineering strategy, the fibers produced within in the injected fibrin matrix, will later constitute the non-mineralized part of the PDL, and need then to be firmly anchored to the implant. To achieve this, again we found inspiration in the developing PDL. Fibers of the natural PDL attach to the surfaces of the bone and root cementum via Sharpey's fibers, which constitute the mineralized part of the PDL.[11] Sharpey's fibers are formed by mesenchymal cells residing in the stromal tissue that surrounds the root.[12] These cells attach the initial Sharpey's fiber to the existing non-mineralized collagenous matrix of the root dentin.[12] In the tissue engineering concept we developed, we mimic the non-mineralized dentin matrix with crosslinked electrospun gelatin (cESG) (figure 1).[13] Gelatin can be firmly anchored to chemically modified titanium implants, and may so provide a surface for the attachment of engineered PDL fibers.[14] Moreover, previously we have shown that cESG has excellent biocompatibility
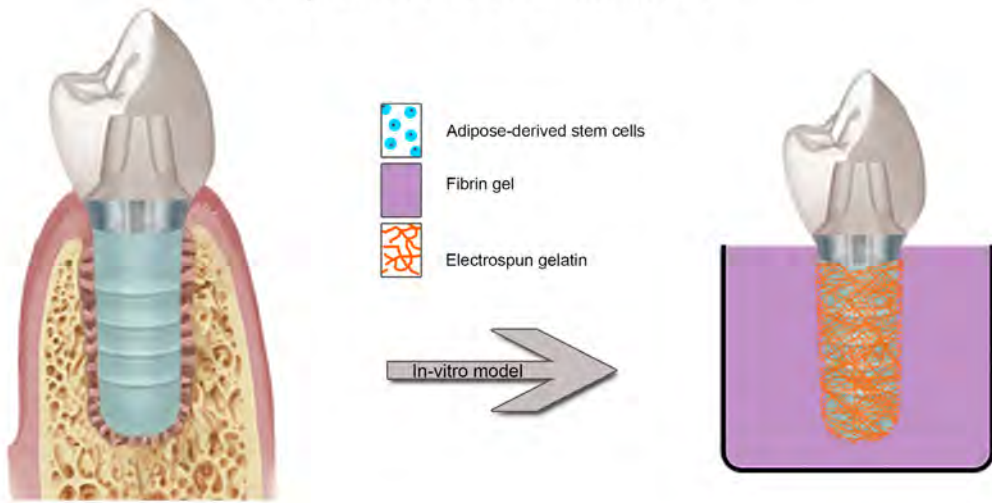
Figure 1: Concept design for periodontal ligament engineering around a dental implant with electrospun gelatin surface, in-vitro.

and stimulates human adipose-derived stem cells (hASCs) to produce type I collagen.[13]

Here we combine three components of our concept: hASCs, fibrin, and cESG, in vitro. We investigate how to achieve and maintain a connection between the fibrin and cESG, and whether hASCs must be seeded in the fibrin, on the gelatin, or on both materials. Furthermore, we investigate collagen type I production by hASCs, with specific focus on matrix production at the interface between fibrin and cESG.

Additionally, as the first step towards in-vitro experiments for PDL regeneration around a dental implant, with sterile materials, we assemble a dental implant coated with a sheet of cESG, and subsequently immersed it in a fibrin matrix. We investigate the possibility to culture this concept in a standard 48 wells plate, and if the combination of materials holds together when it is removed for analysis.

MATERIALS & METHODS

Unless specified otherwise, all chemicals were obtained from Sigma Aldrich, and used as received. Materials were obtained from Life Technologies, Bleiswijk, the Netherlands, and the manufacturer's instructions were followed.

Gelatin electrospinning and crosslinking

Type B gelatin from bovine skin was kindly supplied by Rousselot, Ghent, Belgium. Electrospinning was performed as described previously.[13] Electrospun meshes were vacuum dried overnight and crosslinked with N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) as described previously.[13] The meshes were then freeze-dried and sterilized using ethylene oxide gas (cold cycle, AZ Sint Jan, Brugge, Belgium).

Human adipose-derived stem cells (hASCs)

Subcutaneous adipose tissue was harvested from the abdominal wall of 5 healthy women (age = 44.8 ± 8.35 years; range 33-54 years) undergoing elective abdominal wall correction at the Tergooi ziekenhuizen Hilversum, The Netherlands. All participating donors provided their written informed consent. The written informed consent and study protocol were reviewed and approved by the Ethics Review Board of the VU University Medical Center, Amsterdam, The Netherlands.[15] The vascular stromal fraction, which contains the hASCs, was obtained as described previously.[15] The cells were pooled and the plastic adherent fraction was cultured up to passage 5 in basic culture medium: minimal essential medium alpha (αMEM) with 2% human platelet lysate (PL), 10 IU/ml heparin (Leo Pharma, Amsterdam, The Netherlands), 100 IU/ml penicillin, 100 IU/ml streptomycin and 0.25 IU/ml fungizone (1% P/S/F).[10]

Cell seeding and culture

The crosslinked electrospun gelatin (cESG) meshes were cut with sterile scissors to fit the wells of standard 48 wells culture-plates. Before cell seeding, the meshes were incubated for 1 hour in M199 medium + 1%P/S/F. hASCs were detached with PBS containing 0.5 mM EDTA/0.05% trypsin, suspended in M199 medium and seeded on the cESG meshes at 5,000 cells/cm2. The cells were allowed to attach to cESG for 30 minutes. Subsequently, fibrin matrices composed of M199 medium containing 2mg/ml human fibrinogen and 1.0 IU/ml of bovine thrombin (STAGO BNL, Leiden, the Netherlands) were created as

described previously.[10] Fibrin matrices were created without cells, or with 200,000 hASCs/ml.

Four experimental conditions were created: 1) Cells seeded on cESG, covered with cell-free fibrin. 2) Cells seeded on cESG, covered with cell-seeded fibrin. 3) Cell-free cESG, covered with cell-seeded fibrin. 4) Cell-free cESG, covered with cell-free fibrin constructs served as controls. The fibrin matrices were polymerized for 1 hour at room temperature. Thereafter 400µl of basic culture medium supplemented with 50 mg/ml ascorbic acid was added on top of the fibrin. The cells on meshes and in fibrin were cultured up to 14 days in a humidified incubator at 37˚C and 5% CO_2. Culture medium was refreshed two times per week.

Immunohistology

After culture, fibrin matrices were fixed in PBS containing 4% formaldehyde and embedded in paraffin. 10 µm sections were cut. To observe type I collagen production, sections were rehydrated and washed with PBS. Thereafter, they were incubated for 1 hour in PBS containing 1% bovine serum albumin (1%BSA/PBS) and 20% normal rabbit serum. The meshes were incubated for 1 hour with goat-anti-human antibody for type I collagen at 1:200 in 1%BSA/PBS. Subsequently, meshes were washed with PBS, and incubated for 1 hour with 1:200 rabbit-anti-goat-FITC antibody in 1%BSA/PBS. Cell nuclei were stained 15 minutes with 1:500 DAPI in PBS.

Implant surface coating

Sterile titanium MONDEFIT mini-implants measuring Ø 2.0 x 11 mm (Van Straten Medical, Nieuwegein, the Netherlands), were inserted into a sterilized silicone base (Juwel, Rotenburg, Germany), which was cut to shape to fit into the wells of a 48 wells plate. cESG was cut with sterile scissors in a tapered shape, and subsequently hydrated in sterile phosphate buffered saline (PBS). The hydrated cESG was wrapped around the mini-implants, and fixated with non-resorbable 6.0 nylon suture wire (Ethilon). Subsequently these implants were inserted in a 48 wells plate, and covered with 800 µl of cell-free fibrin matrix, which was created with 2mg/ml fibrinogen and 1.0 IU/ml thrombin as described previously.[10] The fibrin matrix polymerized for 1 hour at room temperature. Images were taken with a Powershot SX210 camera (Canon).

RESULTS

hASCs should be seeded in the fibrin matrix only

After 7 days of culture, no cells can be found that were seeded on cESG and then covered with cell-free fibrin. On sections of these cultures, only background signal is observed for type I collagen staining (figure 2a).

When cells are seeded on the cESG, and are covered with cell-seeded fibrin, cells reside in the cESG and fibrin matrix up to the 7th day of culture. Thereafter,

only cells can be observed in the fibrin matrix (figure 2b). After 7 days of culture, cESG and fibrin start to detach from each other (figure 2b). Positive signal for type I collagen is mainly found in the fibrin matrix, in the vicinity of cells and areas of local fibrin degradation. The highest staining intensity is found at the interface of fibrin and cESG (figure 2b).

When a cell-seeded fibrin matrix covers cell-free cESG, cells are dispersed evenly throughout the fibrin matrix. Some cells reside at the interface of fibrin and cESG (arrows in figure 2c). cESG and fibrin remain attached to each other throughout 14 days of culture. Type I collagen staining is found mainly in the fibrin matrix, however highest staining intensity is observed at the interface of fibrin and cESG (figure 2c).

*The conceptualized implant for PDL engineering can be created with sterile materials*

In a sterile environment, cESG was successfully wrapped around titanium mini-implants (figure 3b), which was subsequently immersed in a fibrin matrix (figure 3c). After 1 hour, the fibrin matrix was polymerized fully, and could be removed from the 48 wells-plate without breaking (figure 3d).

DISCUSSION

We combined hASCs, cESG and fibrin, three key components of our concept for PDL tissue engineering around a dental implant, in-vitro. We showed that to maintain contact between the cESG, the fibrin, and newly produced extracellular matrix, it is best to only seed the fibrin matrix with cells. Additionally, we showed that our conceptualized implant can be assembled with sterile materials for culture experiments in a standard 48-wells plate. This implant and fibrin matrix can be removed for analysis without breakage.

We envisioned a concept for PDL engineering around dental implants, which allows rapid clinical translation, by the use of only clinically relevant materials and cells. In the initial design, we envisioned a cell-seeded implant surface. This was based on previous experiments, where we showed that hASCs on a thin cESG substrate produce increasing amounts of collagen.[13] However, when only the cESG is seeded and then immersed in fibrin that does not contain hASCs, cells cannot be found with histology after seven days of culture. It is likely that these cells died, however the reason for this is not entirely clear. A lack of nutrients seems obvious, but this is most likely not the case, as cells seem to survive without problems when a cell-seeded fibrin matrix covers the cESG. Even though we seeded similar cell numbers as in previous studies, a lack of cell-cell contact might explain the death of the hASCs on cESG.[13,17] This might be overcome when higher cell numbers are seeded on the cESG, however our data suggests that it is not required to seed cells directly on the cESG. After seven days of culture, delamination between the cESG and fibrin occurs when cells
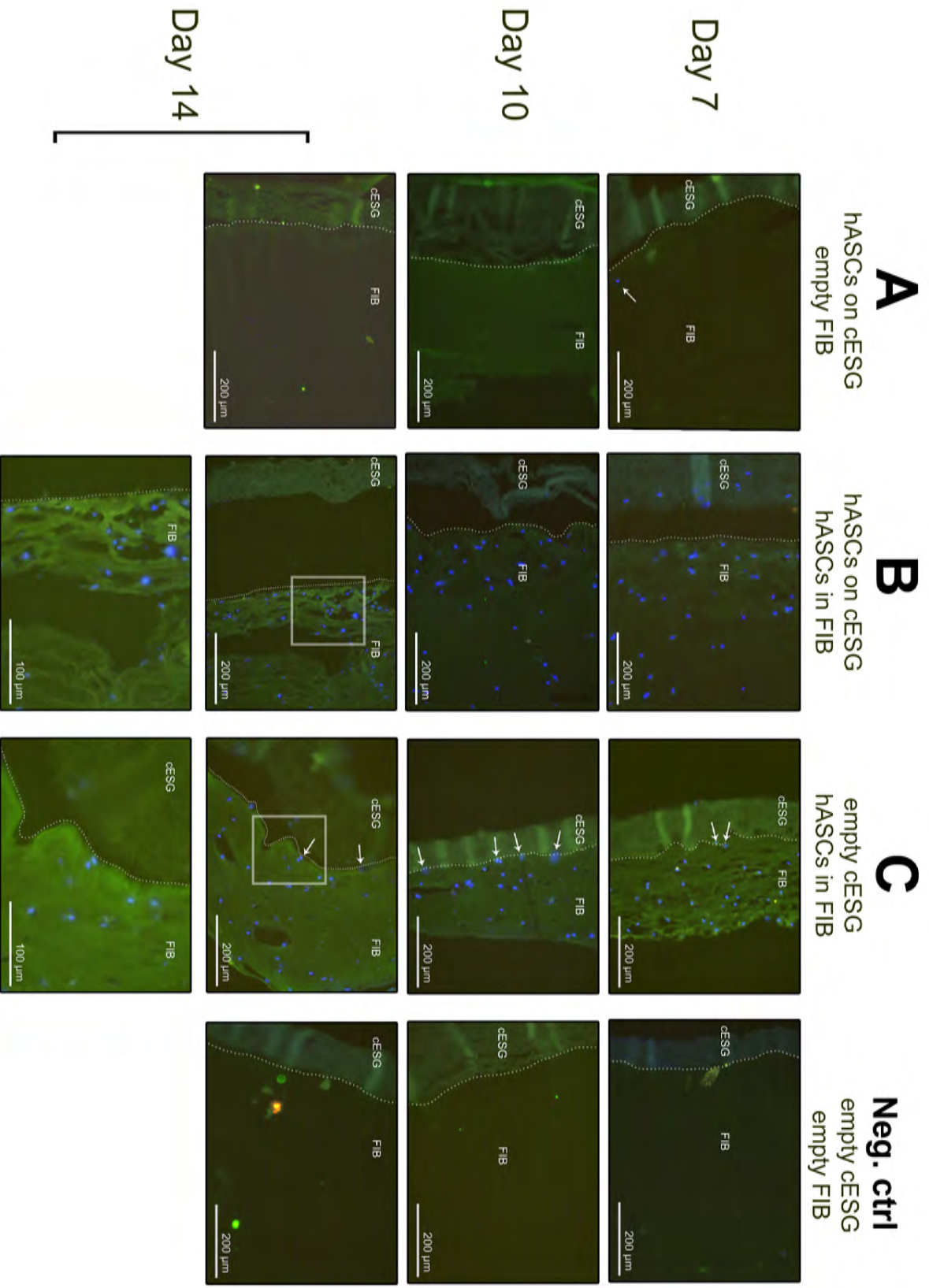
◄ Figure 2: Interface between crosslinked electrospun gelatin (cESG) and fibrin matrix (FIB)
A) cell-seeded cESG + cell-free fibrin: After 7 days of culture, hardly any cells can be found (white arrow). On sections of these cultures, only background signal is observed for type I collagen staining.
B) cell seeded cESG + cell-seeded fibrin: cells reside in the cESG and fibrin matrix up to the 7th day of culture. Thereafter, only cells can be observed in the fibrin matrix. After 7 days of culture, cESG and fibrin start to detach from each other. Positive signal for type I collagen is mainly found in the fibrin matrix, in the vicinity of cells and areas of local fibrin degradation. The highest staining intensity is found at the interface of fibrin and cESG.
C) cell-free cESG + cell-seeded fibrin: cells are dispersed evenly throughout the fibrin matrix. Some cells reside at the interface of fibrin and cESG (white arrows). cESG and fibrin remain attached to each other throughout 14 days of culture. Type I collagen staining is found mainly in the fibrin matrix, however highest staining intensity is observed at the interface of fibrin and cESG.
[n=3; Anti-COL1 FITC staining: green = type I collagen, DAPI staining: blue = cell nucleus]

are present in both materials. This might be caused by matrix remodeling of the cells in both materials, which is concentrated around the interface between them, because of the high cell concentration there.[10,13] Such delamination is undesirable, as it prevents integration of extracellular matrix proteins such as type I collagen with the cESG, and thus hampers integration of the implant with the surrounding tissues.

However, when cells are seeded in the fibrin matrix only, the fibrin and cESG remain attached, and cells either migrate to the interface with cESG, or after fibrin polymerization retain their initial position there. Histology shows disperse staining of type I collagen throughout the fibrin matrix. From our data, it is not fully clear if the collagen interacts - e.g. intertwines or crosslinks - with the cESG. However, such interaction is very likely, as the highest staining intensity for type I collagen is found near the interface between cESG and fibrin. Moreover, seeding cells only in the fibrin matrix, mimics the natural development of the PDL, as the initial Sharpey's fibers are deposited by mesenchymal cells from the stromal tissue that covers the developing root.[9]

Furthermore, only seeding cells in the fibrin matrix, increases the potential for clinical translation of our concept. Mainly because homogeneously seeding cells on a three-dimensional implant is challenging, while homogeneous cell seeding in a fibrin matrix is easily achieved.[10,18]

An obvious limitation of this study is that during culture experiments, the cESG was placed horizontally in a culture plate, instead of attached vertically on the implant surface. Although this experimental setup provides some practical advantages for histology, it was chosen mainly because of time constraint created by delayed supply of the mini-implants. The data presented here provides valuable information, however their outcome needs to be confirmed in an experimental setup as shown in figure 3.

We show that our conceptualized implant can be realized with sterile materials,
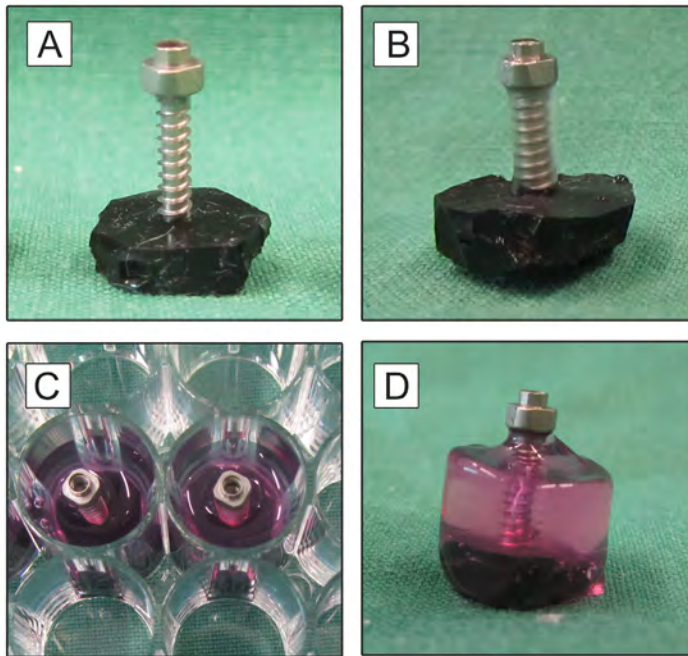
In-vitro concept

Figure 3: Sterile in-vitro concept
A) Titanium MONDEFIT implant inserted into silicone base.
B) Titanium implant coated with crosslinked electrospun gelatin.
C) In-vitro culture system, with gelatin coated implant submersed in fibrin hydrogel in a 48-wells plate.
D) Explant of coated implant surrounded by fibrin matrix.

in a sterile environment, at least in an in vitro setting. This is opens up the possibility for in-vitro experiments to further optimize these implants, a required step towards clinical implementation. However, to allow clinical translation, further optimization of these implants is necessary. An important step towards optimization of these implants is to create a firm attachment of the cESG to the titanium surface, and not to wrap and tie it around the implant. One method to create such an attachment would be a surface coating of poly-dopamine on the titanium, which binds gelatin without compromising biocompatibility.[14] These coated implants could then be used as a collector during the gelatin electrospinning process, where after the gelatin can be crosslinked with the protocol we described previously.[13,16]

Summarizing, we created the concept we designed for PDL engineering around dental implants in-vitro. Our data shows that for further experiments, it is best to combine these implants with cells seeded only in the fibrin matrix. The in-vitro model we created provides a solid basis for tissue engineering of a PDL

around dental implants with clinical translation in mind.

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Chapter VII

General discussion

GENERAL DISCUSSION

In this thesis, we meticulously devised a concept for tissue engineering a periodontal ligament around dental implants with patient friendly materials and cells. We used human adipose-derived stem cells (hASCs), because these regenerative cells proliferate rapidly, and possess multi-lineage differentiation capacity.[1] Furthermore, hASCs have great clinical potential, as they are relatively easily obtainable, in quantities that permit direct application.[2,3] As a biomaterial, we used fibrin, the bodies' own scaffold material after vascular damage, and widely used clinical material that stimulates tissue regeneration and vascular ingrowth.[4,5] We showed that the fibrin matrix' stiffness can be tuned so that it stimulates extensive production of collagen by incorporated hASCs.[6] Such extensive collagen production is essential for the regeneration of the PDL.[7] However, just production is not enough: because the PDL receives high loads on a daily basis, alignment of cells and fibers is essential for increased mechanical strength.[8,9] We showed that hASC-seeded fibrin matrices can be aligned with a straining regime that mimics daily mastication, underlining the potential of these matrices for self-organization according to the local straining conditions. Importantly, the used straining regime does not compromise the hASCs regenerative capacities, but does reduce their potential for matrix calcification, which decreases the chance of unwanted calcification in the engineered ligament.[10]

To attach the thus produced collagen fibers to the implant surface, we envisioned a biological substrate to cover the implants. For this substrate we used gelatin, as it has a similar structure and chemical composition as the intended engineered ligament, and because gelatin degrades in fragments that stimulate the production of new extracellular matrix.[11,12] We used electrospinning to create fibrous meshes of gelatin, which were subsequently crosslinked for stabilization. Importantly, we show that crosslinking does not alter the meshes' structural properties or their biocompatibility. We further showed that hASCs attach and migrate freely through the gelatin, and produce extensive amounts of type I collagen, the protein that makes up the majority of the PDL matrix.[13] We combined the fibrin matrix and gelatin substrate, and showed that type I collagen production by hASCs is highest at the interface between these materials. Furthermore, we showed that the fibrin, gelatin, and newly produced collagenous matrix, remain attached best when hASCs are seeded in the fibrin matrix only. Lastly, we showed that the designed concept could successfully be assembled with sterile materials.

STATE OF THE CONCEPT, AND THE ROAD AHEAD
Further in-vitro optimization

The research in this thesis was designed with materials and cells that allow rapid clinical translation. However, there are several items that need to be addressed before an actual clinical product can appear on the market.

First, a method must be developed to firmly attach the electrospun gelatin to the titanium implant surface, as in this thesis only loose sheets of electrospun gelatin were used. A possible method to achieve this was developed by the Polymer Chemistry and Biomaterials group in Ghent (lead by professor Dubruel), who also aided in the creation of the gelatin meshes used in chapters 5 and 6.[14] These researchers developed a method to functionalize the titanium surface with a polydopamine coating, which binds gelatin without compromising biocompatibility.[15] Such functionalized titanium implants can be used directly as a collector for the electrospun fibers, which results in an electrospun gelatin mesh on the implant.[16] This mesh can subsequently be crosslinked with the protocol described in chapter 5.[14] In our protocol, the fibers are crosslinked with N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), which utilizes free amide residues and carboxyl groups already present in the gelatin.[17] This method of gelatin crosslinking is favorable for biomedical application of the meshes, because it does alter the chemical composition of gelatin, and does not incorporate foreign molecules in the mesh that may compromise biocompatibility.

With the gelatin mesh firmly attached to the titanium implant surface, it is likely that further in-vitro optimization of the conceptualized implants is necessary. In chapter 5 and 6 it is shown that hASCs produce collagen on the gelatin substrate, however these fibers have a random orientation. A more functional, aligned orientation can be achieved by modifying the implant's surface topography, as this has a profound effect on the alignment of cells and extracellular matrix. [18,19] A more functional alignment of newly produced fibers may be obtained by aligning free ends of the electrospun gelatin fibers perpendicular to the implant surface. This mimics the orientation of the cementum matrix on the root surface to which PDL fibers attach during development.[20,21] Such alignment of the free ends of gelatin fibers can be obtained by modifying the electrospinning process with rotating collectors, and by selectively removing fibers during post-processing.[19,22–24]

The research in this thesis mainly revolves around the mechanical and chemical properties of scaffold materials. Therefore, the experiments in this thesis were performed with a very basic culture medium, which next to antibiotics and an anti-fungal agent, contained only the combination of heparin and human platelet lysate as an animal serum substitute.[25] Nevertheless, external stimuli, such as the mechanical signals described in chapter 4, but

also external chemical signals are important determinants of cell behavior and differentiation.[26] By adding specific growth or differentiation factors in the fibrin mixture, or by coupling them to the gelatin, further optimization of the conceptualized implants may be achieved.[27] For example, the growth factors basic fibroblast growth factor (bFGF) and fibroblast growth factor-2 (FGF-2) have been shown to improve periodontal healing.[28,29] Factors such as vascular endothelial growth factor (VEGF), and platelet-derived growth factor (pDGF) have been shown to increase vascular ingrowth and attachment of PDL fibers to mineralized surfaces.[29,30]

In our own attempt to achieve chemical optimization of the implant surface, we performed pilot experiments (not shown), in which increasing concentrations of alkaline phosphatase (ALP) were added to the electrospun gelatin, to create a mineralized cementum-like substrate. We cultured these meshes for 7 days in calcification medium, which contained 10 mM β-glycerol-phosphate. With increasing ALP concentrations, we observed increasing intensity of Alizarin Red staining (which stains deposited calcium) throughout the gelatin mesh. Though these experiments have to be repeated to find the optimal ALP concentration, this pilot-data may provide a basis for future experiments towards implant surface optimization.

Once the surface of the implants has been optimized, further experiments should be performed to determine the appropriate loading pattern to exert three-dimensional alignment of fibers around the implant. In chapter 4 it was shown that a strain pattern that mimics daily dental loading from mastication (four times for 90 seconds per 24 hours) aligns the fibrin matrix and hASCs. However, the strain as described in chapter 4 is unidirectional, and applied on hASC-seeded fibrin matrices only. Dental loading is a multidirectional process, and moreover, specific loading directions differ between dental elements, e.g. incisors, cuspidates and molars.[8] Thus, loads, specific for the dental element replaced, should be exerted on the implant, as this will create a multi-directional strain field on the fibrin matrix that surrounds the implant.[31] First experiments to create such multi-directional strain may be performed with the micro-mover, a very precise motion and load-controlled actuator system that was developed by our group.[32] However, in its current form, also the micro-mover is capable of applying only uni-directional loads, and several adaptations to the machine are necessary before multi-directional simulation of element-specific loading is possible. However, based on the potential for self-organization of hASC-seeded fibrin matrices, we speculate that experiments with multi-directional loading will show that the matrix will align according to the local straining conditions. [32–34]

Finally, in the introduction of this thesis, we envisioned that our concept is suitable for one-step surgery. The idea for a single-step surgery procedure was

based on a study by members of our group, and consists of isolation of the adipose-derived stem cells prior to the implantation procedure, so that they can be injected with the fibrin matrix after the implant has been placed.[3] However, all experiments in this thesis were performed with a purified pool of hASCs from multiple donors. In one-step surgery, regenerative cells need be obtained from the stromal vascular fraction (SVF) from the adipose tissue of a single donor, i.e. the patient. In the SVF only 0.1% of the cells is characterized as an adipose-derived stem cell, which may necessitate costly and time-consuming cell purification on site.[35] The outcomes of the experiments in this thesis, based on experiments with a pool of cultured ASCs, provide a proof of principle. However, an important step before actual clinical translation can be made is the repetition of our experiments with both the SVF and purified cells from individual donors.

In-vivo testing will prove the value of the conceptualized implants
The next step towards proving the value of implants with an attached PDL is in-vivo testing. However, these experiments should commence only when the implants for PDL generation have been optimized fully in-vitro. Societal demand for alternative methods to animal experiments is growing.[36] One such alternative that receives much attention is the lab-on-a-chip concept, which consist of an in-vitro system that can mimic a tissue and its physiology by housing several distinct layers of cells, and incorporation of relevant chemical and biomechanical signals, including fluid flow and strain.[37,38] Also the author is of opinion that animal testing should be kept to a minimum, and that alternatives such as the lab-on-a-chip are valuable, for example in drug-screening.[39] However, the author is also of opinion that in-vitro models can never fully replace animal experiments when designing tissue engineering constructs for implantation in humans. In-vivo experiments are essential, because in-vitro it is difficult to predict the multi-facetted responses of the tissues surrounding the implant, which include tissue healing, vascular and nervous ingrowth, invasion of regenerative cells and immune cells, possible pathogenic infections, differences in pH and oxygen concentration, etcetera. [40]
The animal model for the in-vivo tests must as close as possible resemble the human anatomy and pattern of mastication. The pig is such a model, which is often used for dental implant research.[41] Furthermore, pigs are suitable to test the merit of the designed implants, as protocols have been developed to experimentally induce peri-implantitis-related bone loss in these animals.[42] To reduce the use of animals, for initial tests, 3D-printed human skulls or cadavers may be used, for example to develop a surgical protocol for implantation.[43] Such a surgical protocol is likely necessary, because these implants are not suitable for conventional implantation methods, in which an implant is screwed into a pre-drilled socket in the alveolar bone.[44] This surgical protocol must

CONCEPT DESIGN
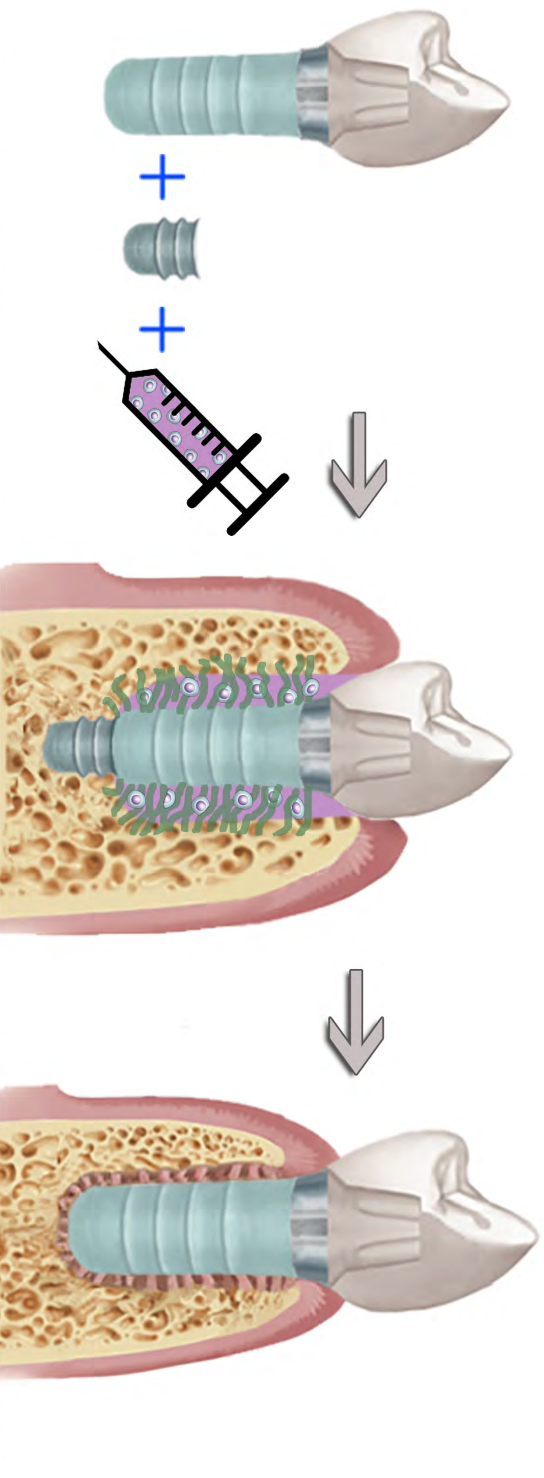
The implant is combined with a resorbable screw and subsequently implanted in the recipients alveolar bone. The fibrin gel containing adipose stem cells is injected arround the implant.

The screw is resorbed and the implant with ligament is incorporated in the alveolar bone.

Figure 1: Concept for in-vivo fixation of a dental implant on which an engineered PDL might be attached.

also account for initial implant stability, as it will take several weeks before the tissue is healed, and before the matrix created by the hASCs can provide mechanical stability to the implant.[45] Figure 1 shows a potential solution for this problem: by attaching a resorbable screw to the implant, initial stability can be provided, while the fibrin matrix is being remodeled. Another possibility would be to use an orthodontic retainer for initial implant stability, which is removed when the tissue has been functionally remodeled.[46]

In-vivo experiments will provide knowledge on the performance of hASCs and the fibrin matrix

As described above, the extracellular environment in-vivo radically differs from the in-vitro conditions of the experiments presented in this thesis. For example, a hematoma will result from the implant procedure, which not only contains a plethora of cells, but also an abundancy of inflammatory factors.[47,48] Inflammatory cytokines, such as tumor necrosis factor alpha, and the interleukins IL-5, IL-17F and IL-4 are known mediators of cell differentiation, however their specific effect on differentiation depends on multiple factors, including their concentration, and specific time of release.[49] Furthermore, hematomae generally have a hypoxic core.[47] How hypoxia affects hASC differentiation, is currently under investigation by members of our group. Their results show that hypoxia reduces the osteogenic potential of hASCs seeded in fibrin matrices, and that these cells upregulate vasculogenic markers (van Esterik et. al, manuscript submitted for publication).[50] Both these aspects may be beneficial for PDL regeneration, however it remains to be investigated how hypoxia influences collagen production by hASCs seeded in fibrin matrices. Also another important aspect of our concept, the rate of fibrin degradation, is likely affected by the hypoxic conditions in-vivo. Preliminary data shows that hypoxia does not change the rate of fibrin degradation by hASCs (van Esterik et. al, unpublished data). However, it is has been shown that hypoxia enhances fibrinolysis by other cell types, such as endothelial cells and macrophages, which will likely invade the fibrin matrix after implantation.[51,52]

We specifically chose fibrin as a biomaterial, because as the natural wound closing scaffold it stimulates vascular ingrowth, and it allows other regenerative cell types to invade the matrix.[5] However, these capacities may also be the Achilles heel of our concept, as too fast fibrin degradation could result in a loss of contact between the implant and surrounding tissues. Although the fibrin matrix may be stabilized with anti-fibrinolytic enzymes such as aprotinin, or the lysine analogues tranexamic acid, and epsilon aminocaproic acid, the use of these molecules is controversial, because they have been associated with an increased risk for renal and cardiac failure.[53,54]

Summarizing, clinical implementation is still far away, and will only be possible when results have been obtained from human trials, and multiple

years of patient follow up. Also, the outcome of human trials will determine approval from governing agencies. From a materials perspective, it is likely that permission will be granted for the biomaterials we chose, because clinical approval already exists for titanium, for fibrin and non-crosslinked gelatin, and because EDC-crosslinking of electrospun gelatin does not compromise in-vitro biocompatibility.[14,55]

CONCLUSIVE REMARKS

We created a concept for the generation of a PDL around dental implants, with clinical translation in mind. The goal for this thesis was to:

Take the first steps towards the generation of a periodontal ligament around dental implants with patient-friendly materials and cells, specifically adipose-derived stem cells, fibrin and gelatin.

The data in this thesis combined shows the feasibility of our concept, in-vitro, and supports its intended clinical translation. Furthermore, the high plasticity of the materials and cells we chose allows self-organization in-situ, which broadens their applicability. For example, parts of the concept may be translated to periodontal ligament regeneration around natural teeth, or ligament generation around other hard implants such as orthopedic prostheses. The research presented here may provide a solid foundation for further development of implants with an engineered ligament attached.

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Chapter VIII

Summary

SUMMARY

The periodontal ligament (PDL) is of essential importance for the physiology of natural teeth. It consists of an aligned fibrous network that is interposed between, and attached to, the alveolar bone and the tooth root. The PDL provides mechanical stability and acts as a shock absorber for the high forces of mastication.[1] Furthermore, it prevents the invasion of pathogens from the oral cavity, and acts as a sensor of the masticatory system.[2]

These capacities are lost when the PDL is damaged, or when teeth are replaced by dental implants. Because dental implants are placed directly in the alveolar bone to osseointegrate, their loading is unbuffered, which can create micro-fractures of the alveolar bone.[3] Furthermore, the rough surface of dental implants, which aids in osseointegration, also enhances bacterial invasion.[4] These two aspects combined result in a high post-implantation complication rate of dental implants.[5] Especially peri-implant bone loss is an often recurring and irreversible problem.[6] With the increasing popularity of dental implants, novel strategies to prevent such complications are necessary.[7]

In chapter 1, the introduction of this thesis, such a strategy was presented in the form of a concept for the tissue engineering of a periodontal ligament around dental implants. The premise of this concept is to enable rapid clinical translation, by using biomaterials and regenerative cells that are clinically relevant. This means that the materials must be either patient-derived or approved for clinical use, and that regenerative cells can be obtained with minimal patient discomfort and donor-site morbidity.

The development of the natural PDL provided a basis for our concept. This development has been reviewed in chapter 2, with specific focus on the formation of PDL fibers and their attachment to bone and cementum. Furthermore, this review considers the spatial arrangement of the fibers during development, and the key role of mechanical forces to modulate this arrangement.[8] We describe that the formation of the PDL is a self-organizing process, and that many essential processes for PDL formation occur on the interface of materials with different stiffness. For example, fiber attachment to the cementum and bone, and fiber growth by lateral and longitudinal apposition of collagen fibrils in the non-mineralized central part of the PDL.[9–11]

The interfaces of different stiffness between tissues in PDL development, and external mechanical forces, formed the pillars of the concept we presented. Electrospun gelatin, attached to a dental implant, mimics the non-mineralized cementum matrix, to which the initial PDL fibers are attached during tooth development.[11] A fibrin matrix that was seeded with human adipose-derived

stem cells (hASCs) mimics the stromal tissue that surrounds the developing tooth root, from which the fibroblasts arise that functionally shape the PDL. [12] These regenerative cells and biomaterials were chosen for their favorable biological characteristics, tunable mechanical properties, and potential for fast clinical translation.[13–15] We combined these materials, whose Young's moduli differ approximately 1000-fold, resulting in in the creation of a distinct interface between them.[16,17] Such differences in stiffness may trigger a self-regulating alignment-response of regenerative cells and extracellular matrix perpendicular to the surface of the material.[18] However, as is the case during natural PDL development, we also apply external mechanical forces to facilitate matrix alignment further away from the surface.[8,19]

In chapter 3 we showed that the stiffness of the fibrin matrices is decisive for matrix remodeling by hASCs, and for their differentiation fate. In matrices with an average Young's modulus of 2.1 kPa, matrix remodeling and collagen production was substantially higher than in softer matrices (Young's modulus <1.5 kPA). We suggest that the fibrin matrix with the stiffness of 2.1 kPa is more suitable for PDL tissue engineering, because of the extensive collagen production by the hASCs in these matrices. However, also the hASCs' expression of osteogenic differentiation markers is enhanced in these fibrin matrices, which results in generalized matrix calcification.

Matrix calcification is unfavorable for PDL regeneration, as it may lead to ankylosis, a pathological condition in which the tooth fuses to the alveolar bone.[20] Furthermore, though collagen is extensively deposited, it has a random orientation, which results in a reduced mechanical strength in comparison to aligned fibers.[21] Although these aspects are problematic for PDL tissue engineering, they can be overcome by the application of external mechanical loadings. As stated above, mechanical loads are essential for PDL development and function, because their magnitude and direction stimulate the PDL fibroblasts to accordingly remodel and align the PDL fibers.[22] Furthermore, mechanical loading is essential to prevent ankylosis, as it reduces the calcification potential of mesenchymal cells.[23,24]

To reduce the calcification potential of hASCs, and to align the cells and fibers in our tissue engineering concept, we introduced an external mechanical regime to the hASC-seeded fibrin matrices in chapter 4. This regime mimics the strain on natural PDL fibers from daily mastication, and consists of 8% continuous strain, with four dynamic loading periods per 24 hours in the form of a sinus displacement of 8 to 15%.[25–28]. With this straining regime, the fibrin fibers and hASCs aligned in the straining direction. Furthermore, this straining regime reduces the expression of calcification-associated markers by the hASCs, without compromising these cells' regenerative potential.

Chapter 3 and 4 yielded a suitable fibrin matrix composition, cell concentration, and a method to align produced fibers. In the second part of this thesis, a method was studied to modify the implant surface, which should allow engineered PDL fibers to be attached to a titanium implant. During PDL development, the initial root-bound fibers are attached to the non-mineralized dentin matrix.[29,30] We mimic this soft substrate with electrospun gelatin, which is used to cover the titanium implant. In <u>chapter 5</u>, we created porous fiber meshes of gelatin by electrospinning. However, to prevent the gelatin from dissolving in water, these meshes need crosslinking. We show that crosslinking of electrospun gelatin with N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) does not alter the meshes' structural properties, and that EDC crosslinking does not compromise the biocompatibility of electrospun gelatin. When hASCs are seeded on this material, they survive, attach well, and are able to migrate through the pores in the gelatin mesh. On this material, hASCs do not differentiate, retain a fibroblast-like morphology and function, and over time produce increasing amounts of type I collagen.

In <u>chapter 6</u>, we showed in experiments with combinations of gelatin and fibrin that it is best to seed cells only in the fibrin matrix, and not on the gelatin in order to favor cell survival. When cells are seeded only in the fibrin matrix, the interface between fibrin and crosslinked gelatin stays intact during 14 days of culture. Throughout the fibrin matrix, hASCs produce type I collagen, but especially on the interface between fibrin and crosslinked gelatin, a high concentration of type I collagen is observed. Lastly, with the exception of a mechanical loading system, chapter 6 shows the completed concept in-vitro: a titanium implant that was coated with crosslinked electrospun gelatin, submersed in a fibrin matrix.

Taken together, with clinical translation in mind, we created a concept of a dental implant with an engineered periodontal ligament attached. The research in this thesis combined shows the feasibility of our concept, which was created with patient-friendly materials and cells, in-vitro. Furthermore, we show that parts of our concept may be translated to periodontal ligament regeneration around natural teeth. Though further optimization of our concept is necessary for clinical translation, this thesis may provide a solid foundation for the development of a dental implant with an engineered periodontal ligament attached.

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Chapter IX

Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

De tand is verbonden met het kaakbot via het parodontale ligament (PDL). Het PDL wordt gevormd door een uitgelijnd vezelig netwerk dat stevig is verankerd in het cementum op de tandwortel en in het omringende alveolaire bot van de kaak.[1] Het PDL biedt mechanische stabiliteit en functioneert als een schokbreker om de tand en het kaakbot te beschermen tegen de zeer hoge krachten die tijdens het kauwen worden uitgeoefend.[1] Bovendien vormt het PDL een barrière tegen de invasie van pathogenen zoals bacteriën en virussen en is het een belangrijke sensor van het kauwsysteem door de vele zenuwuiteinden die tussen de PDL-vezels zitten.[2] Deze uiterst belangrijke beschermende eigenschappen gaan verloren als het PDL beschadigd raakt of als de tand wordt vervangen door een tandimplantaat. Tandimplantaten worden direct in het kaakbot geplaatst en ontlenen hun stabiliteit aan de aangroei van bot op het implantaatoppervlak. Om te zorgen dat het omringende bot optimaal aan het implantaat vastgroeit, wordt het implantaatoppervlak extra ruw gemaakt. Een nadelig neveneffect is dat dit ruwe oppervlak ook bacteriële aanhechting en ingroei bevordert.[3] Bovendien worden bij directe plaatsing de hoge krachten van het kauwen via een tandimplantaat ongebufferd doorgegeven aan het kaakbot, waardoor daarin microscopische breukjes kunnen ontstaan.[4] Deze twee aspecten leiden tot een hoge mate van complicaties na plaatsing van tandimplantaten.[5] Een veel voorkomende complicatie is peri-implantitis: een hardnekkige bacteriële infectie van het implantaat en zijn omringende weefsels die leidt tot botverlies rondom het implantaat. Botverlies na peri-implantitis is niet te genezen. Bovendien is peri-implantitis een veelvuldig terugkerend probleem, zelfs na goed schoonmaken van het implantaatoppervlak.[6] Doordat de populariteit van tandimplantaten sterk stijgt zal ook het aantal patiënten met deze complicatie toenemen.[7]

Om de problemen rondom tandimplantaten aan te pakken, presenteerden we in hoofdstuk 1, de inleiding van dit proefschrift, een innovatief concept voor een nieuw soort implantaat. In dit concept streven we naar de (re)generatie van een parodontaal ligament op het implantaatoppervlak zodat bacteriële ingroei wordt geremd en de belasting op het implantaat gebufferd wordt. Ons doel is dat dit concept uiteindelijk leidt tot een nieuw type implantaat dat kan worden geplaatst in een klinische praktijk. Om snelle vertaling naar de kliniek mogelijk te maken hebben we het concept opgebouwd uit biomaterialen die ofwel patiënt-eigen zijn of al goedgekeurd zijn voor klinische toepassing. De regeneratieve cellen die we in ons concept voorstellen zijn stamcellen uit vetweefsel. We hebben dit celtype gekozen omdat vetstamcellen bij kunnen dragen aan weefselherstel, zeer snel groeien en de mogelijkheid hebben te

differentiëren naar andere weefselspecifieke celtypes zoals bloedvat- bot- en ligament-cellen.[8] Bovendien kunnen vetstamcellen geoogst worden met minimale complicaties en pijn bij de patiënt en in hoeveelheden die directe toepassing mogelijk maken.[9]

De inspiratie voor de vormgeving van het concept haalden we uit de embryonale ontwikkeling van het natuurlijke PDL, welke is uiteengezet in hoofdstuk 2. We beschrijven deze ontwikkeling met specifieke focus op de vorming van de PDL-vezels en hun hechting aan het bot en cementum. Bovendien definiëren we de rol van mechanische krachten in de ruimtelijke vormgeving van het PDL. [10] We beschrijven dat de vorming van het PDL een zelfregulerend proces is en dat verschillende essentiële stappen in PDL-formatie plaatsvinden op het grensvlak van materialen met verschillende stijfheid. Voorbeelden hiervan zijn de aanhechting van vezels aan bot en cementum en vezelgroei naar het ongemineraliseerde centrale deel van de PDL-ruimte.[11–13]

De grensvlakken van materialen met verschillende stijfheid en externe mechanische krachten vormen de hoekstenen van ons concept. Zo bootsen we de embryologische ontwikkeling van het PDL na door het grensvlak te imiteren dat ligt tussen het ontwikkelende bindweefsel en het ongemineraliseerde wortel-cementum waaraan de jonge PDL-vezels vasthechten.[13] In ons concept wordt het ongemineraliseerde wortel-cementum geïmiteerd door een maaswerk van elektro-gesponnen gelatine dat is vastgehecht aan het implantaatoppervlak. We imiteren het ontwikkelende bindweefsel rondom de tandwortel met een gel van het bloedstollingseiwit fibrine, welke is gezaaid met menselijke stamcellen uit vetweefsel (mVSC).[14] In ons concept worden deze materialen gecombineerd. Omdat hun stijfheid met een factor 1000 verschilt, wordt een duidelijk grensvlak tussen hen gecreëerd.[18,19] Op dit grensvlak zal een zelfregulerende uitlijning ontstaan van regeneratieve cellen en extracellulaire matrix welke loodrecht staat op het stijfste materiaal, de gelatine.[20] Daarnaast bieden we ook externe belastingen aan zoals het geval is tijdens de natuurlijke ontwikkeling en functie van het PDL. Hierdoor realiseren we verder weg van het implantaat oppervlak ook uitlijning van de cellen en de matrix.[10,21]

In hoofdstuk 3 hebben we laten zien dat de stijfheid van de fibrine matrix beslissend is voor de mate waarin mVSC de matrix remodeleren. Bovendien heeft de matrix-stijfheid invloed op hoe deze cellen differentiëren. De mate van remodeleren is substantieel hoger in matrices met een Young's modulus van 2.1 kPa dan in matrices met een lagere stijfheid (<1.5 kPa). We suggereren dat een fibrine stijfheid van 2.1 kPa het meest geschikt is voor PDL tissue engineering omdat de mVSC in deze fibrine matrix veel collageen produceren, het bindweefsel waaruit o.a. het PDL is opgebouwd. Echter binnen deze fibrine matrices is in de mVSC ook de expressie van osteogene differentiatie markers,

de eiwitten specifiek voor botcellen, verhoogd. Dit leidt tot een gegeneraliseerde verkalking van de matrix.

Verkalking van de matrix is ongunstig voor PDL regeneratie omdat het kan leiden tot ankylose, een pathologisch proces waarin de tand via een kalkbrug fuseert met het kaakbot.[22] Daarnaast worden de collageenvezels door mVSC neergelegd in een willekeurige oriëntatie waardoor de sterkte van deze vezels inferieur is aan die van vezels die zijn uitgelijnd, zoals bijvoorbeeld de vezels in het PDL.[23]

Bovengenoemde aspecten van de fibrine matrix zijn problematisch voor PDL regeneratie met mVSC. Echter, deze kunnen voorkomen worden door externe mechanische belasting aan te bieden. Zoals hier boven beschreven is, zijn mechanische stimuli essentieel voor de ontwikkeling en functie van het PDL. De omvang en richting van de belasting stimuleren de vezel-producerende fibroblasten in het PDL om de vezels in de juiste richting neer te leggen.[24] Bovendien is mechanische belasting essentieel in het voorkomen van ankylose omdat door belasting het calcificatie-potentieel van mesenchymale cellen, voorlopercellen van het lymfatische- bloedvat- en bindweefselsysteem waartoe ook mVSC behoren, wordt verlaagd.[25,26]

Om het calcificatie-potentieel van de mVSC te verlagen en om de cellen en vezels in ons PDL-regeneratie concept uit te lijnen, hebben we een extern mechanisch regime aangeboden aan de mVSC-gezaaide fibrine matrix in hoofdstuk 4. Dit regime bootst de belasting na van een dagelijks kauwpatroon op natuurlijke PDL-vezels en bestaat uit een continue oprekking van 8% van de mVSC-gezaaide fibrine matrix, aangevuld met vier dynamische oprekkingsperioden per 24 uur van 8-15%.[27–30] Met dit mechanische regime werden de fibrine vezels en mVSC uitgelijnd in de richting van belasting. Bovendien werd de expressie van calcificatie-markers bij mVSC verlaagd zonder dat het regeneratieve potentieel van deze cellen werd verstoord.

Uit hoofdstuk 3 en 4 volgden een geschikte compositie van de fibrine matrix, een juiste concentratie cellen en een methode om de geproduceerde vezels uit te lijnen. In het tweede deel van deze dissertatie hebben we een methode bestudeerd om de geproduceerde vezels te hechten aan een titanium implantaat. Tijdens de ontwikkeling van het PDL hechten de PDL-vezels zich aan de ongemineraliseerde matrix van de tandwortel.[31,32] In ons concept bootsen we het worteloppervlak na met elektro-gesponnen gelatine. In hoofdstuk 5 hebben we door middel van elektrospinning poreuze maaswerken van gelatine gemaakt. Echter om te zorgen dat de gelatine niet oplost in water moet het chemisch worden gecrosslinked. We laten zien dat de structurele eigenschappen van de maaswerken en de biocompatibiliteit van gelatine niet veranderen na crosslinken met N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride

(EDC). We hebben vervolgens mVSC uitgezaaid op het gecrosslinked gelatine. We tonen aan dat de mVSC overleven, goed hechten aan het materiaal en kunnen migreren door de mazen. Op gecrosslinked gelatine differentiëren de mVSC niet en behouden ze de gewenste fibroblastachtige functie en morfologie. Over de tijd produceren deze cellen oplopende hoeveelheden type I collageen.

In hoofdstuk 6 combineren we gelatine, fibrine en mVSC. We laten zien dat mVSC beter overleven als ze alleen in de fibrine worden gezaaid. Als we de cellen alleen in de fibrine matrix zaaien blijft het grensvlak tussen fibrine en gelatine intact gedurende een celweek van 14 dagen. De mVSC produceren type I collageen in de gehele fibrine matrix maar op het grensvlak tussen fibrine en gelatine vonden we de hoogste concentratie collageen. Afsluitend laten we in hoofdstuk 6 zien dat we in staat zijn om ons concept-implantaat in het laboratorium te maken met steriele materialen: een titanium implantaat, bedekt met gecrosslinked elektro-gesponnen gelatine en ondergedompeld in een fibrine matrix.

Samenvattend: we hebben een concept ontwikkeld voor een tandimplantaat met een aangehecht parodontaal ligament. Dit concept werd vormgegeven met patiëntvriendelijke biomaterialen en regeneratieve cellen zodat het snel zijn weg kan vinden naar de klinische praktijk. Het onderzoek in deze dissertatie laat zien dat het haalbaar is om in het laboratorium zo'n implantaat te maken. Bovendien laten we zien dat delen van ons concept vertaald kunnen worden naar PDL regeneratie rondom natuurlijke tanden. Voordat ons concept-implantaat echt zijn weg naar de kliniek kan vinden is verdere optimalisatie nodig. Echter, deze dissertatie biedt een solide fundament voor het doorontwikkelen van tandimplantaten met een aangehecht parodontaal ligament.

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Dankwoord

DANKWOORD

Beste lezer, allereerst wil ik u bedanken voor de tijd en aandacht die u heeft besteed aan het lezen van mijn dissertatie. Echter moet u weten dat dit proefschrift is opgebouwd uit onderzoek dat is uitgevoerd op diverse afdelingen van verschillende universiteiten en dat ik in de afgelopen vier jaar nooit zover was gekomen zonder de hulp van verschillende mensen. Hierbij wil ik een aantal mensen speciaal bedanken.

Promotieteam

Theo, ik kan me haast geen betere promotor voorstellen dan jij. Jouw praktische denk- en werkwijze als ingenieur sluiten erg aan op mijn eigen werkstijl. Daardoor zaten we voor mijn onderzoek meestal snel op één lijn. Heel erg bedankt voor de vrijheid die je me hebt gegeven voor het ontwikkelen van mijn eigen ideeën, voor je begeleiding, je adviezen en de bijzonder prettige samenwerking van de afgelopen jaren.

Astrid, jij was als co-promotor maar vooral als topwetenschapper en mooi mens onmisbaar voor mijn onderzoek. Je enorme kennis van de celbiologie gecombineerd met je kritische niet-lullen-maar-poetsen mentaliteit zorgden er regelmatig voor dat ik mijn (iets te ambitieuze?) plannen kon bijstellen om tot een goed resultaat te komen. Heel erg bedankt voor je begeleiding, je adviezen en natuurlijk voor alle flauwe grappen van de afgelopen jaren.

Mijn dank gaat uit naar de leden van de manuscript-commissie die mijn dissertatie hebben beoordeeld: prof. dr. Cees Kleverlaan, prof. dr. Roelof-Jan Oostra, prof. dr. Bruno Loos, prof. dr. Lorenzo Moroni en dr. Marco Helder.

ORM groep

Mijn onderzoek was zeker niet mogelijk geweest zonder de hulp van de Oral Regenerative Medicine groep.

Jenneke, heel erg bedankt voor alles wat je gedaan hebt voor het ORM project en alle AIO's daarin. Je bent een uniek mens met een bijzondere stijl. Ik kijk met een glimlach terug naar onze samenwerking.

Sue, heel erg bedankt voor je adviezen en voor de samenwerking met jou en de mensen in je lab. Ik heb bewondering voor de wijze waarop je in roerige tijden toch het schip van OCB boven water wist te houden.

Cees, heel erg bedankt voor je adviezen in de ORM meetings en voor je hulp in het lab van materiaalwetenschappen. Ik wens je veel succes met je nieuwe baan als de grote baas van de afdeling 'functionele orale anatomie van de celbiologische materiaalwetenschappen'.

Albert, bedankt voor jouw verruimende blik op de wetenschap en de manier waarop je het hokjes-denken in mijn onderzoek hebt voorkomen.

Vincent, bedankt dat ik met je mocht werken in de jaren voor je pensioen. Je adviezen hebben me erg geholpen in het vormgeven van mijn onderzoek.

Behrouz, waar zouden ACTA, OCB en de verschillende AIO's die daar rondlopen zijn zonder jou? Heel erg bedankt voor je adviezen, voor je hulp in het lab, de borrels en natuurlijk voor je geweldige BBQ elk jaar.

Jan-Harm, bedankt voor je hulp tijdens mijn onderzoek en voor het 'uitlenen' van je computer als ik wilde oefenen met eindige-elementen analyse.

Francis, bedankt voor de afgelopen jaren waarin we als AIO's veel tijd hebben doorgebracht in het lab, op meetings en op congressen. Ik wens je al het beste tijdens je nieuwe baan in Japan.

Jeroen, als mede TG'er en AIO heeft onze carrière aardig parallel gelopen. Ik wil je bedanken voor onze samenwerking en voor de gezelligheid tijdens borrels en op congressen. Succes met het afronden van je project en met alles wat er op je pad komt in de toekomst.

Angela, thanks for the nice time we had at ACTA, and during our commute from Utrecht to Amsterdam. Espero que seguimos viendo uno a otro en el futuro. Todo lo mejor para ti y tu familia.

Rita Murti, tijdens de stage die je bij me liep heb je op voortreffelijke wijze de stamcellen gekarakteriseerd. Ik wil je bedanken voor je inzet. Het onderzoek naar distractie osteogenese dat je opzette vormde de basis voor hoofdstuk 4 van deze dissertatie.

Lab technici
Zonder de volgende mensen is het onmogelijk om onderzoek te doen op het lab van OCB, voor mij en voor iedereen die daar rondloopt. Zij vormen het kloppende hart van de afdeling.

Jolanda, de toon was meteen gezet op mijn eerste werkdag. Ik hoop dat ik je in de jaren daarna genoeg prosecco toegeschoven heb zodat je het me enigszins vergeeft dat ik niet bleef eten en slapen. Bedankt voor je hulp, je adviezen, je geweldige inzet voor OCB en de oorverdovende lach die daarbij gepaard gaat.

Cor, je bent een rots in de branding en staat altijd klaar om mensen te helpen. In ons gezamenlijk enthousiasme voor speciale bieren hebben we regelmatig een onontdekte fles gezelligheid open getrokken. Bedankt voor je toewijding, je nuchtere humor en natuurlijk voor de bak met pepernoten die steevast klaar staat als de donkere maanden aanbreken.

Ton, held in het lab, muzikaal talent en allround good-guy. Bedankt voor je hulp, je nuchtere kijk op zaken en de gezelligheid die altijd om je heen hangt. Ik denk met veel plezier terug aan het bodyboarden in Castricum. Daarna wist je ondanks een gekneusde rib toch nog een dikke set te spelen met je band, respect!

Marion, jij bent de stille kracht van OCB. Bedankt dat je altijd klaar stond voor een praatje en voor je hulp met histologie en de microscopen.

Dirk-Jan, ik vond het geweldig om met je te werken voordat je met pensioen ging. Ik hoop dat je geniet van de vrije tijd en van je kleinkinderen.

Samenwerkingsverbanden

Mijn onderzoek is niet alleen op ACTA uitgevoerd. Ik heb gewerkt op verschillende afdelingen van verschillende universiteiten in Nederland en België. Mijn dank gaat daarom ook uit naar alle mensen met wie ik heb mogen samenwerken.

VUMC – afdeling Fysiologie:
Dr. Pieter Koolwijk, bedankt voor je onmisbare materiële en immateriële bijdragen in de begindagen van mijn onderzoek. Het beschikbaar stellen van je lab en je mensen heeft uiteindelijk geleid tot de publicatie van mijn eerste paper.

Dr. Ester Weijers, zonder jou was ik waarschijnlijk nooit gaan werken met fibrine. Heel erg bedankt voor je hulp met mijn eerste experimenten, voor de gezelligheid en voor het helpen afronden van mijn eerste paper. Veel succes met je nieuwe baan in Leiden en met het moederschap.

Marloes van den Broek, bedankt dat je altijd klaar stond voor ondersteuning in het Fysiologie lab.

Radboud UMC – afdeling Biochemie:
Prof. dr. Toin van Kuppevelt, bedankt voor het beschikbaar stellen van uw lab en mensen. Dit was onmisbaar voor het afronden van mijn tweede paper.

Michiel Pot, via jou kwam ik in aanraking met de afdeling biochemie van het Radboud UMC. Jouw ongeremde energie zorgde ervoor dat we menig borrel als laatste hebben afgesloten tijdens onze studie technische geneeskunde in Enschede en op verschillende congressen tijdens onze PhD-studie.

Corien Oostendorp, jouw idee om de Dermatan Sulfaat kleuring te doen was het missende puzzelstukje voor mijn tweede paper. Bedankt voor je hulp met de kleuring, het afronden van het paper en de gezelligheid op de congressen van de NBTE.

Luuk Versteeghden, de middagen achter de Piuma waren niet altijd even productief, maar wel altijd erg gezellig. Ik hoop dat onze inspanning uiteindelijk leidt tot een mooi paper.

Universiteit Gent - Polymer Chemistry & Biomaterials Research Group:
Prof. dr. Peter Dubruel, bedankt voor het beschikbaar stellen van uw lab en mensen. Dit was onmisbaar voor het afronden van mijn derde paper.

Dr. Diana-Elena Mogosanu, many thanks for the time we spend in the lab in Ghent. Our collaboration was better than I could ever have imagined, and ultimately led to two nice collaboration papers. I wish you all the best.

Roomies

In de afgelopen jaren heb ik kamer 11N43 gedeeld met verschillende mensen. Ik herinner de eerste dag dat ik daar binnen liep nog erg goed. Bedankt voor de goede ontvangst en plezierige samenwerking Anna, Petra Janice, Qilong, Shreeda en Joost. Uiteindelijk werd de 'harde kern' van mijn kamer gevormd door maar liefst acht dames en naast mijzelf één man.

Yixuan, many thanks for your kindness and off course for the delicious hot-pot dinner.

Berend-Jan, bedankt voor het lachen, voor de tripjes naar de snoepautomaat op de 7e verdieping en voor jouw mannelijke bijdrage in het vrouwenbolwerk van de 11e verdieping. Ik hoop dat je opleiding heelkunde voorspoedig verloopt en dat we elkaar nog eens tegenkomen in de toekomst.

Sara, bedankt voor de gezelligheid van de afgelopen jaren. Ik heb erg genoten van onze discussies over hoe de wetenschap er uit zou moeten zien en van de delicatessen die jouw land rijk is. Heja Sverige!

Jing, bedankt voor de gezelligheid die je bracht in de kamer. Ik hoop dat je je Nederlandse tandartsdiploma zult behalen en snel weer aan het werk kan.

Carolyn, sjiek is miech dat, als jongste AIO van onze kamer zul jij uiteindelijk het stokje moeten doorgeven. Ik heb er de volste vertrouwen in dat je dat gaat lukken. Bedankt voor de gezelligheid, de vlaaien en de lekkerdere koffie.

Mahshid, thanks for your kindness and the lessons in Iranian cooking.

Beatrice, I'm sorry that your Jesus and I partied when you were away. Many thanks for your enthusiasm and the laughs we had in the past years. Obrigada!

Angela en Francis, mijn mede ORM'ers, erg bedankt voor de gezelligheid in de kamer.

Collega's
De volgende mensen hebben extra kleur gegeven aan mijn tijd op ACTA.

Hessam, jouw levensadviezen zullen me altijd bijblijven. Heel erg bedankt dat je me erop hebt gewezen dat persé willen zitten in de trein echt voor tatas is en dat je liet zien dat channel eaux de toilette een prima vervanger is voor deodorant. Bedankt voor je ongeremde enthousiasme, de grappen die we hebben uitgehaald en natuurlijk dat me als werkpolitie altijd scherp hebt gehouden. Bedankt voor de mooie tijd.

Bas, je bent een mooie baas. Bedankt voor het afplakken van mijn muis, verstoppen van de wieltjes van mijn stoel, stelen van mijn monitor, sturen van pikante foto's op ongepaste momenten en voor je inzet bij de werkpolitie. Maar vooral bedankt voor de goede tijd die we samen hebben gehad.

Sepanta, the only guy in the whole ACTA building who also likes football. Thanks for the past years, the borrels, and the good talks we had about our favorite game. I hope you finish your project soon, and that we'll meet again for drinks and a match in the future.

Mirte, bedankt voor de Brabantse gezelligheid die je op het lab bracht. Ik hoop dat we na de winter onze racefietsen nog eens uit het vet halen om een mooi tochtje te maken.

Ben, bedankt voor je lessen over Hyena's, je enthousiasme en de gezellige filmavonden. Ik hoop dat we snel weer een 'romantische comedy' gaan kijken.

Daffyd, bedankt voor je enthousiasme, je nuchtere blik op de wetenschap en de gezellige treinreizen tussen Utrecht en Zuid. Succes met het afronden van je project. Hopelijk tot ziens in de toekomst.

Christine, bedankt voor de gezelligheid en voor de knakflapjes op mijn afscheidsborrel. Ik hoop dat je je project goed afrond en dat we elkaar in de toekomst nog eens tegen komen op een orthopedische OK.

Ik wil graag de hele afdeling orale cel biologie en de mensen die daar tot het meubilair horen/hoorden bedanken voor hun gastvrijheid, de gezellige lunches, borrels en dagjes uit.
Veel dank Ton B., Hetty, Janak, Jenny, Alejandra, Marjolein, Rosita, Feresteh, Gang, Dong-Jung, Chinan, Tie, Chow, Nawal, Ceylin, Noel, Rosalien, Brit, Patrick, Teun, Ineke, Hans, Geerling, Jack, Daan, Manuel, Niels, grote Ana, en kleine Ana.

Vrienden
Beter een goede vriend dan een verre buur. De volgende mensen waren en zijn ontzettend belangrijk voor mij.

Mathijs, *Shout out naar Armhoefse Akkers groep 6!*. Dit proefschrift draag ik ook op aan je moeder Josephine, ze was als een tweede moeder voor mij. Bedankt dat je mijn hele leven zo'n goede vriend bent geweest. Ik kijk uit naar alle mooie dingen die we in de toekomst gaan meemaken!

Immanuel, bedankt dat je me al sinds de middelbare school rekent tot je hele sterke vrienden. Ik vind het heel tof dat we elkaar nog regelmatig zien, zelfs nu de burgerlijkheid er toch echt in begint te sluipen.

René, de leukste en kutste clown die ik ken, ik kan altijd op je bouwen. Supervet dat we onlangs nog een pint gedronken hebben bij De Boom waar het allemaal begon. Jij weet inmiddels ook dat echte mannen dochters krijgen, ik wens jou en je meisjes al het beste!

Paul, je bent een goede vriend. Bedankt voor mooie tijden op De Flat, op de Damstraat, de Ostadelaan, op de vakanties, roadtrips, weekendjes weg, feestjes, lowlands, etc. etc. etc. Ik vermoed dat we nog wel één of twee pintjes drinken in de komende jaren.

Jason, al sinds ons eerste jaar in Enschede zijn we Jut en Jul. We hebben zoveel vette shit meegemaakt en lijpe dingen gedaan. Super-relaxt dat we niet levend zijn verbrand toen met dat melkpoeder bijvoorbeeld... Bedankt voor alle mooie feestjes, tripjes, rare plannen en goede tijden. Ik hoop dat je snel je PhD afrond, want dan kunnen we eindelijk de Doppenberg & de Jong kliniek beginnen.

Robert, het duiveltje op mijn schouder dat altijd zegt 'zullen er dan nog ééntje drinken?'. Bedankt voor de mooie weken op Holtus eiland, de zeiltripjes, alle feestjes en de foto's van jouw voeten naast een biertje op de tafel op ons dakterras.

Frank, miauw, miauw, prrrr, miauw. Bedankt voor je gezellige Brabantse bijdrage in het nukkige Twente en dat je altijd je haren wast voordat je je hoofd in andermans zakken steekt. Bedankt voor alle feestjes, vakanties, roadtrips, lowlands en de goede tijden.

Guy, de vaste waarde bij elke gezellige gelegenheid. Dude, thanks voor de mooie feestjes, tripjes, lowlands en je onmisbare advies bij alles wat op stroom werkt.

Ik ken ze één voor één, de heren van fatsoen, ik zal ze nooit vergeten zoals ze jou wel doen. Mannen van het goede leven, van het hart, het lied en het bier, bedankt voor de mooie tijd in Enschede en tijdens de VKV-west borrels: Boeijen, Holterman, Verhagen, Bergsma, Prins, Stevens, Doppenberg, Peters, Maatjes, Schweinsteiger, de Wit, Mennes, van Rossum, Vandeursen, Visscher, ten Brinck, van den Bos, van Grunderbeek, van Maanen, de Groot, Evers, Koster, en alle mannen die de mooie VKV tradities voortzetten.

C.V. de Schnordruckers en 't Nipt, Alaaf!

Holtus, kunde um boas?

Bier op de grond voor lil' VoWoW en iedereen die Lowlands fantastisch maakt!

Schoonfamilie
Je partner kun je uitzoeken, je schoonfamilie krijg je erbij. Wat dat betreft had ik geen betere partner kunnen kiezen.

Jos en Annie, bedankt voor het warme nest dat jullie altijd bieden voor je kinderen, kleinkinderen en de koude kant. Bedankt dat ik altijd bij jullie terecht kan voor advies, of gewoon voor een kop koffie of een lekkere borrel.

Robbert, Steven en Maureen, een stel leukere schoonbroers en schoozus had ik me niet kunnen voorstellen. Bedankt voor de gezelligheid in Zwolle en Heino, tijdens feestjes, op de skivakanties, en natuurlijk bij de wedstijden van PEC.

Luuk, en Vik, ik vind het geweldig om jullie te zien opgroeien en kijk uit naar wat de toekomst voor jullie brengt.

Familie

Pap, ik hoop dat ik later als ik groot ben net zo sterk wordt als jij, en dat ik dan net zo goed kan fluiten. Mam, ik ben nog steeds in de running om echt jouw professortje te worden. Bedankt voor al jullie liefde, goede zorgen en steun die jullie me gegeven hebt in de afgelopen jaren. Bedankt voor het openstellen van jullie huis en jullie harten, aan ons, maar ook aan onze vrienden, onze partners en de kleinkinderen. Ik hou van jullie!

Nicky en Janneke, mijn kleine zusjes die inmiddels al best groot zijn. Bedankt voor alle mooie jaren. Ik ben super trots op jullie, en kijk uit naar alle mooie dingen die nog gaan komen.

Pieter en Gijs, betere schoonbroers had ik niet kunnen wensen, fijn dat jullie zo goed voor mijn zusjes zorgen. Bedankt voor de gezelligheid met carnaval, op (ski)vakantie, op feestjes of gewoon bij ons pa en ma aan tafel.

Hidde en Flore, wat een geweldige aanvulling zijn jullie van onze familie. Ik ben erg benieuwd wat voor mooie dingen we nog van jullie gaan zien.

Mijn grote liefdes

Sarah, mijn drolleke, mijn mopje. Jouw komst heeft mij meer gebracht dan ik ooit had durven dromen. Ik ben echt helemaal gek op je! Jouw energie en ondernemende karakter geeft mijn leven een geweldige impuls. Ik hoop dat je opgroeit tot een mooie sterke vrouw maar dat ook dat je voor altijd mijn kleine meisje blijft.

Lieve Laura, zo sta je in je badjas te fluisteren naast een bed en zo woon je samen, krijg je een kind en ben je verloofd. Bij jou ben ik thuis en voel ik me altijd fijn. Bedankt dat je er altijd voor me bent. Ik hoop dat we ons gezin snel zullen uitbreiden en kijk uit naar een geweldige toekomst samen. Ik hou van jou!

Appendices

APPENDIX I

Human adipose-derived stem cell donor-pool and characterization

Human adipose-derived stem cell
Donor pool composition

	Birth year	Gender	% in pool	Total cell no.
50	1960	Female	21%	3,E+06
47	1963	Female	14%	2,E+06
54	1956	Female	20%	3,E+06
33	1977	Female	27%	4,E+06
40	1963	Female	19%	3,E+06
Mean/SD/Range			100%	
44,8				
8,35				

hASC CHARACTERIZATION

Mesenchymal stem cell markers FACS analysis:
Negative for:
CD45 (LCA) = hematopoetic/leukocyte marker.[1-3]
CD34 = cell adhesion factor (attachment to ECM / stromal cells).[1]
CD14 = macrophage/neutrophil factor (detection of bacterial LPS).[1]
CD31 (PECAM-1) = hematopoetic/endothelial marker.[2,3]

Positive for:
CD90 (thy-1) = cell-cell / cell-matrix interactions molecule.[2,3]
CD105 (endoglin) = cell surface glycoprotein.[2,3]

Lineage specific culture conditions:
Osteogenic
-10% FBS, 0.1 mM dexamethasone, 10 µM β-glycerophosphate, and 50 µg/mL ascorbic acid in α-MEM 4
-10% FBS, 0.1 mM dexamethasone, 10 µM β-glycerophosphate, 50 mM ascorbate-2-phosphate in α-MEM 2

Adipogenic
-10% FBS, 1 µM dexamethasone, 100 µg/mL 3-isobutyl-1-methylxanthine, 5 µg/mL insulin, and 60 µM indomethacin in α-MEM [4]
-10% FBS, 1 uM dexamethasone, 200 uM indomethacin, 10 um Insulin, 0.5 mM isobutyl methylxanthine, in α-MEM [2]

Chondrogenic
-1% FBS, 50 µg/ml ascorbic acid, 10 ng/ml TGF-β1 and 6.25 µg/ml insulin in α-MEM [4]
- 15% FBS, 0.1 um dexamehtasone, 50 mg/ml L-ascorbic acid-phospathase, 40 mg/ml L-Proline, 1% insulin tranfeerring selenium (ITS Premix) 100x, 10ng/ml TGF-B3 in high glucose DMEM [2]

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3.   Kroeze, K.L., et al. Chemokine-mediated migration of skin-derived stem cells: predominant role for CCL5/ RANTES. J Invest Dermatol 129, 1569-1581 (2009).
4.   Lee, R.H., et al. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. Cell Physiol Biochem 14, 311-324 (2004).
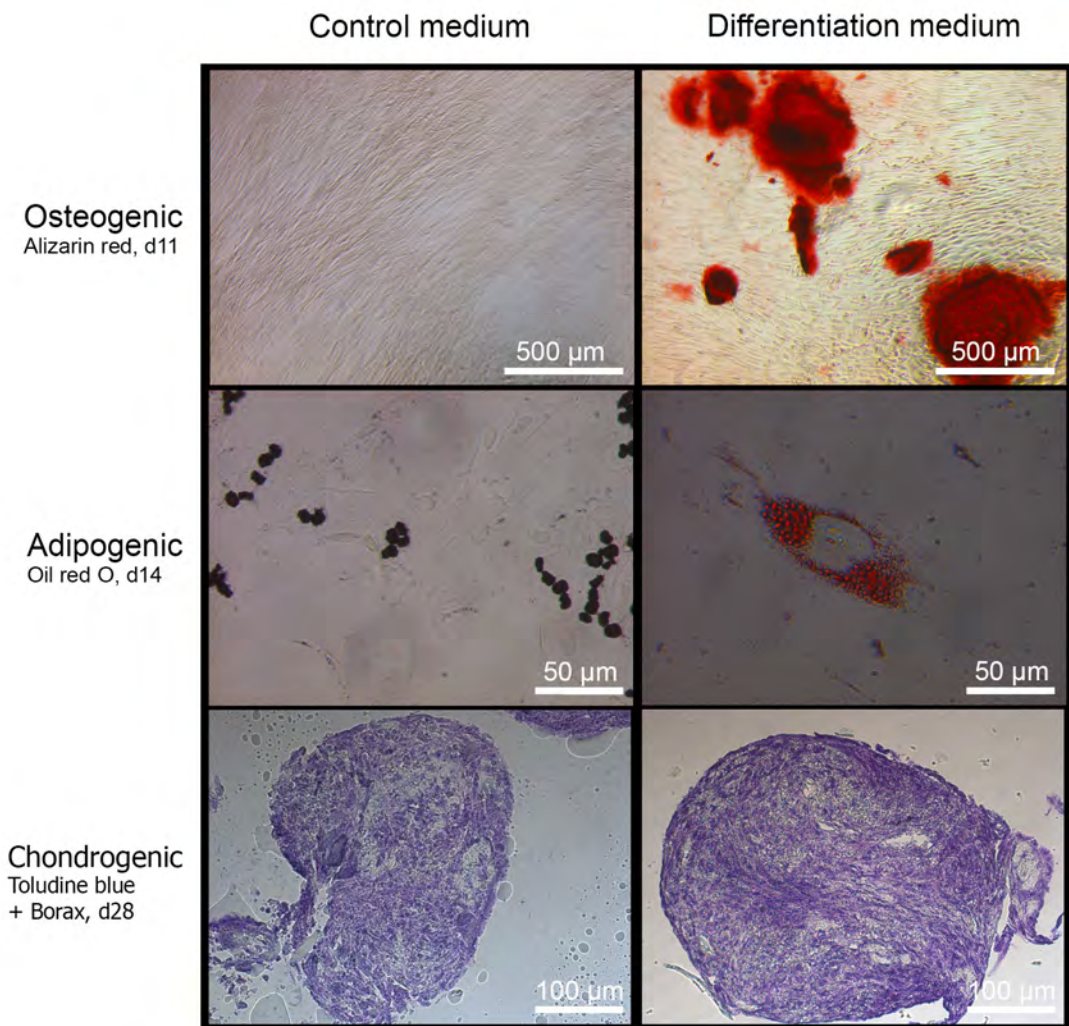
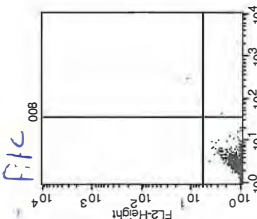Figure 1: Multi-lineage differentiation potential of human adipose-derived stem cells (hASCs)
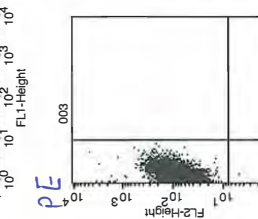
Figure 2: FACS results for surface marker expression
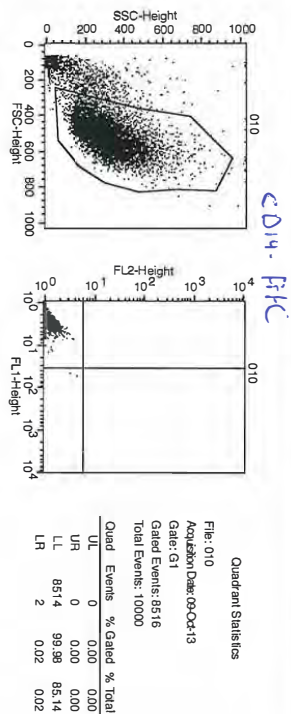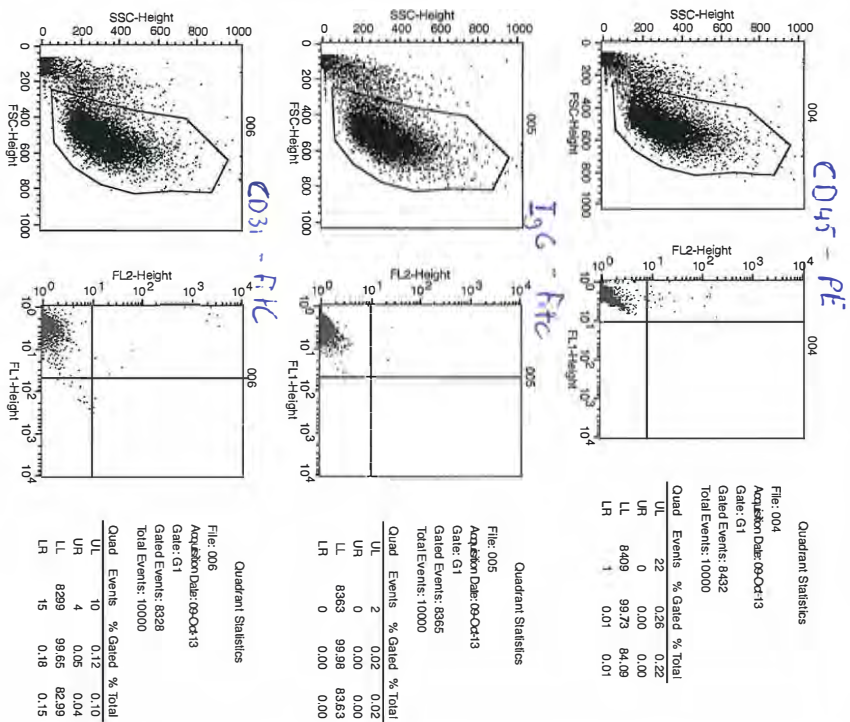
Figure 2 continued: FACS results for surface marker expression

APPENDIX II

Primers for qPCR

YWHAZ	FW	5' GATGAAGCCATTGCTGAACTTG 3'
(House-keeping)	REV	5' CTATTTGTGGGACAGCATGGA 3'
UBC	FW	5' GCGGTGAACGCCGATGATTAT 3'
(House-keeping)	REV	5' TTTGCCTTGACATTCTCGATGG 3'
KI67	FW	5' CCCTCAGCAAGCCTGAGAA 3'
(Proliferation)	REV	5' AGAGGCGTATTAGGAGGCAAG 3'
COL-1	FW	5' TCCGGCTCCTGCTCCTCTTA 3'
(ECM-remodeling)	REV	5' GGCCAGTGTCTCCCTTG 3'
COL-3	FW	5' GATCCGTTCTCTGCGATGAC 3'
(ECM-remodeling)	REV	5' AGTTCTGAGGACCAGTAGGG 3'
COL-5	FW	5' CAGGCCGATCCTGTGGATG 3'
(ECM-protein)	REV	5' GTGGCCTTCTGGAAAGAGT 3'
Elastin	FW	5' TTCCTGGAATTGGAGGCATCG 3'
(ECM-protein)	REV	5' AGCTCCTGGGACACCAACTA 3'
MMP-2	FW	5' GGCAGTGCAATACCTGAACA 3'
(ECM-remodeling)	REV	5' AGGTGTGTAGCCAATGATCCT 3'
MMP-9	FW	5' TGACAGCGACAAGAAGTG 3'
(ECM-remodeling)	REV	5' CGTGGCTCAGGTTCAGG 3'
RUNX-2	FW	5' ATGCTTCATTCGCCTCAC 3'
(Calcification-associated)	REV	5' ACTGCTTGCAGCCTTAAAT 3'
ALP	FW	5' AGGGACATTGACGTGATCAT 3'
(Calcification-associated)	REV	5' CCTGGCTCGAAGAGACC 3'
Osteonectin	FW	5' CTGTCCAGGTGGAAGTAGG 3'
(Calcification-associated)	REV	5' GTGGCAGGAAGAGTCGAAG 3'
Osteopontin	FW	5' TTCCAAGTAAGTCCAACGAAAG 3'
(Calcification-associated)	REV	5' GTGACCAGTTCATCAGATTCAT 3'
PPAR-γ	FW	5' CGACCAGCTGAATCCAGAGT 3'
(Adipogenesis)	REV	5' GATGCGGATGGCCACCTCTT 3'
Adiponectin	FW	5' AGGCCGTGATGGCAGAGATG 3'
(Adipogenesis)	REV	5' CCTTCTTGAAGAGGCTGACC 3'
Col-2B	FW	5' AGGGCCAGGATGTCCGGCA 3'
(Chondrogenesis)	REV	5' GGGTCCCAGGTTCTCCATCT 3'
SOX-9	FW	5' CCCAACGCCATCTTCAAGG 3'
(Chondrogenesis)	REV	5' CTGCTCAGCTCGCCGATGT 3'
αSMA	FW	5'- CCTGACTGAGCGTGGCTATT-3'
(Myofibroblast differentiation)	REV	5'- GATGAAGGATGGCTGGAACA-3

CURRICULUM VITAE

Thijs de Jong werd op 18 december 1985 geboren in Nijmegen. Vanaf zijn 4e levensjaar woonde hij in Tilburg waar hij de basisschool doorliep en vervolgens het VWO afrondde op het st. Odulphus Lyceum.

In 2008 behaalde Thijs zijn bachelor-diploma Technische Geneeskunde aan de universiteit Twente. Zijn opleiding werd gevolgd met een master Technical Medicine - Reconstructive medicine, waarvoor Thijs in 2012 afstudeerde. Na het behalen van zijn master-diploma was Thijs 6 maanden werkzaam als klinisch onderzoeker op de afdelingen reumatologie en orthopedie van het UMC Utrecht. Daarna startte hij met zijn academisch promotietraject aan het academisch centrum voor tandheelkunde Amsterdam (ACTA), waarvan deze dissertatie het resultaat is.

In zijn vrije tijd kookt Thijs graag en brouwt hij bier. Thijs fitnest, zeilt, fietst en snowboardt graag. Samen met zijn grote liefde Laura heeft Thijs een dochter Sarah.

In de toekomst hoopt Thijs werkzaam te zijn op het grensvlak van geneeskunde en innovatieve technologie. Hij wil samen met Laura hun gezin uitbreiden en samen de wereld ontdekken.

LIST OF PUBLICATIONS

Calcium chloride induced shrinkage of collagen scaffolds: morphology, biomechanical characteristics and biocompatiblity
Versteegden LR, Pot MW, Sloff M, Hoogenkamp HR, Pang J, de Jong T, Smit TH, Leeuwenburg SC, Feitz WF, Oosterwijk E, van Kuppevelt TH, Daamen WF.
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Lucila Navarro, Diana-Elena Mogosanu, Thijs de Jong, Astrid D. Bakker, David Schaubroeck, Julio Luna, Ignacio Rintoul, Jan Vanfleteren, Peter Dubruel
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