CHAPTER 1

GENERAL
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The developing fields of bone tissue engineering and regenerative medicine have identified skeletal defects as attractive translational targets, and several clinical applications have been proposed. Bone tissue engineering comprises stem/progenitor cells, biomaterials, which should preferably be biodegradable, and biologics (natural/biological stimuli to trigger or prime differentiation of stem/progenitor cells). These three building blocks (biomaterials, biologics, and cells) shall, either grown in vitro and subsequently transplanted, or after direct placement in vivo, regenerate and/or remodel the desired tissue, in order to repair, replace, maintain, or enhance the tissue’s function, for bone regeneration in the orthopaedic practice (e.g. spinal interbody fusion).

Spinal interbody fusion is performed in patients with various spinal disorders including degenerative disorders of the lumbar spine, in which non-surgical treatments fail. This surgery aims for stabilization of the spinal column, realigning of the spine and restoring intervertebral disc height thereby relieving the pain. The current clinical practice is using non-biodegradable interbody fusion cages, made of e.g. stainless steel, titanium, carbon fiber, PEEK, although biodegradable cage materials are also evaluated (reviewed in Wuisman and Smit, 2006). Usually the cages are filled with autologous bone graft taken from the iliac crest.

There are certain disadvantages associated to this surgical set-up for spinal interbody fusion. Metal-based interbody cages are too stiff, hampering bone graft remodeling, and radio-opaque, which is eclipsing the radiographic judgment of fusion. In addition, they reside as a foreign body in the spine, which might be a focus for, e.g. infections or foreign body reactions, and the cages might migrate over time. Secondly, the harvesting of iliac crest bone results in temporary or persistent serious donor site morbidity in 10-30% of the patients undergoing spinal interbody fusion for 2 years or longer. Using alternatives for autogenous bone graft can circumvent donor site associated problems: at present allogenic grafts, calcium phosphate based synthetics, and biologics such as BMP-2 and BMP-7 are under clinical and preclinical investigation. In addition, autologous adult mesenchymal stem cells (MSCs) seeded on a synthetic bioresorbable scaffold could also be a vital and attractive alternative used to replace bone graft material. Adult MSCs provide an alternative source for the ethically controversial embryonic stem cells for bone tissue engineering (Chapter 2, Fig 1). MSCs derived from the stromal vascular fraction of adipose tissue serve as a source of autologous adult MSCs, as well as MSCs
derived from bone marrow.\textsuperscript{1,15-19} Advantages of using MSCs from adipose tissue over bone marrow are their easy accessibility, minimal morbidity upon harvest, and a higher frequency of MSCs with a high proliferative activity.\textsuperscript{1,15,16} Adipose tissue-derived mesenchymal stem cells (AT-MSCs) or adipose stem cells express surface markers similar to those expressed by MSCs from bone marrow, including CD105, SH3, Stro-1, CD90, and CD44.\textsuperscript{15,20} After lineage-specific stimulation AT-MSCs can differentiate i.e. down the adipogenic, myogenic, chondrogenic, and the osteogenic pathways.\textsuperscript{15,17,18,21} In addition, AT-MSCs exhibit an \textit{in vivo} osteogenic potential, comparable to that of bone marrow-derived MSCs, and almost comparable to that of osteoblasts.\textsuperscript{1,16} Thus, AT-MSCs provide an extraordinarily promising source of stem cells for the regeneration of functional bone tissue.\textsuperscript{15}

Functional bone tissue provides physical support of the body, protection of vital organs, attachment sides for skeletal muscles, and plays an important role in calcium homeostasis.\textsuperscript{22} In order to efficiently bear prevailing mechanical loads bones need to adapt their mass, shape and trabecular structure, to provide proper resistance to mechanical failure.\textsuperscript{23} Osteocytes are the proposed sensors of changes in the mechanical demands.\textsuperscript{24} They are the mature long-lived, terminal differentiation stage of the osteoblasts embedded in the calcified matrix, and they orchestrate bone remodelling accomplished by bone depositing osteoblasts and bone resorbing osteoclasts.\textsuperscript{24,25} Osteocytes sense the flow of interstitial fluid through the lacuno-canalicular network after mechanical load-induced deformation of bone. Upon this flow of fluid osteocytes subsequently produce signaling molecules, such as nitric oxide (NO) and prostaglandins.\textsuperscript{26,27} These two signaling molecules are essentially involved in the adaptive response of bone to mechanical stimulation.\textsuperscript{24}

Bone remodelling requires the deposition of appropriate extracellular matrix by the osteoblasts, to obtain the strength and resilience for optimal load bearing.\textsuperscript{28} Bone extracellular matrix is composed of anorganic calcium phosphate in the form of hydroxyapatite, and organic collagen, mainly type I collagen, as well as non-collagenous proteins, such as osteopontin, osteocalcin, and growth factors.\textsuperscript{22,29} Collagen needs to be post-translationally modified in order to give bone its characteristic elasticity.\textsuperscript{29} Hydroxylation of collagenous proline and lysine residues by lysyl and prolyl hydroxylases, and the oxidative deamination of collagenous lysine residues by lysyl oxidase are a prerequisite for the generation of stable cross-links within the collagen network to provide the physical and mechanical properties of bone (reviewed by Gelse \textit{et al.}, 2003).\textsuperscript{29}
Bone does not only contain mineralized collagen, it also serves as reservoir for growth factors, i.e. of the transforming growth factor-β (TGF-β) super family. Proteins of the TGF-β super family, such as TGFβ1, bone morphogenetic protein-2 (BMP-2), and BMP-7 (also known as osteogenic protein-1), gain more and more significance for bone tissue engineering, due to their osteoinductive activity.\textsuperscript{12,30} BMP-2 and BMP-7 are currently approved by the Food and Drug Administration (FDA) and are already in clinical use.\textsuperscript{12,30}

This thesis concentrates on the utility of AT-MSCs for bone tissue engineering, thereby providing insight into stem cell biology, and proposes a novel and challenging concept for a one-step spinal interbody fusion surgery (chapter 2) by addressing the following questions:

- Do the retrieval methods for adipose tissue affect yield and growth characteristics, thus quality and quantity of AT-MSCs?
- Do AT-MSCs undergoing osteogenic differentiation respond to mechanical loading by pulsating fluid flow in a bone cell-like manner?
- Do prostaglandins PGE\textsubscript{2}, PGI\textsubscript{2}, and PGF\textsubscript{2α}, which are locally produced in bone, affect osteogenic differentiation of AT-MSCs?
- Are AT-MSCs able to correctly modify their own produced collagen matrix in tissue engineering applications after treatment with BMP-2 or TGF-β1?
- Can a short treatment (30 min) the polyamine spermine trigger AT-MSCs in their osteogenic differentiation?
- Can a short treatment (15 min) with the growth factors BMP-2 or BMP-7 trigger AT-MSCs in their osteogenic or chondrogenic differentiation?

The following section of the thesis briefly explains the proposal of a one-step surgical procedure for spinal interbody fusion and addresses these questions.

Spinal interbody fusion is a well-known and applied treatment modality for patients with, e.g. degenerated disc disease that experience severe conservative therapy-resistant low back pain with or without irradiating pain to the extremities. The surgery aims for fusion of a spinal segment by reaming of the degenerated intervertebral disc, restoring intervertebral disc height to release the pressure on the nerve roots, and to establish stability of the spinal segment, which is turn facilitates bony fusion. Chapter 2 proposes an alternative treatment modality to the current
clinical practice, which is associated with the disadvantages described above using a bone tissue engineering approach.

Adipose tissue for bone tissue engineering purposes can be harvested by using resection, tumescent liposuction, and ultrasound-assisted liposuction.\textsuperscript{31,32} These surgical procedures may detrimentally affect the functional characteristics of AT-MSCs. For tissue engineering, however, quality and quantity of the cellular material should be clearly investigated and defined. Therefore, chapter 3 discusses the effects of these three commonly used adipose tissue-harvesting methods on quality and quantity of AT-MSCs by determining their yield and growth characteristics.

Bone tissue engineering aims for the regeneration of functional bone. The adaptation of bone mass, shape, and trabecular architecture is crucial for the mechanical performance of the skeleton.\textsuperscript{23} This adaptation is accomplished by the orchestration of osteoblasts and osteoclast by mechanically stimulated osteocytes.\textsuperscript{24,25,33} The osteocyte is the fully differentiated osteoblast and the mechanosensory cell par excellence residing within bone, thereby governing the process of bone remodelling.\textsuperscript{24,25,33} Therefore we address in chapter 4 whether AT-MSCs in the course of their differentiation towards mature bone cells are capable of sensing a bone specific mechanical stimulus and whether they respond to this stimulus in a bone cell-like manner.

Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), PGI\textsubscript{2} and PGF\textsubscript{2α} are produced by osteocytes upon mechanical stimulation and are key players in the adaptation of bone to the mechanical demands of the environment.\textsuperscript{34,35} PGE\textsubscript{2} recruits osteoprogenitor cells from the bone marrow space and induces their differentiation towards osteoblast-like cells.\textsuperscript{36,37} PGE\textsubscript{2} also induces osteogenic differentiation of bone marrow stromal cells.\textsuperscript{38} Whether PGE\textsubscript{2}, PGI\textsubscript{2} and PGF\textsubscript{2α} affect AT-MSCs in their osteogenic differentiation is addressed in chapter 5. This might give insight into how factors produced in the in vivo environment bone, could influence differentiation of AT-MSCs during tissue engineering applications.

Besides responsiveness to bone specific factors, AT-MSCs should be able to fulfill osteoblast function, i.e. the deposition of bone extracellular matrix. Bone extracellular matrix contains mainly organic type I collagen and non-collagenous proteins, such as growth factors, e.g. BMPs or TGF-β1 and other proteins, e.g. osteopontin or osteocalcin, and anorganic calcium phosphate in form of hydroxyapatite. Bone extracellular matrix provides the resilience to mechanical stress, thereby preventing mechanical failure.\textsuperscript{29} To accomplish this, it is constantly
remodeled according to changes in the mechanical environment. Type I collagen needs to be post-translationally modified to provide the mechanical resilience of organic bone extracellular matrix. In chapter 6 we compare the effects of BMP-2 and TGF-β1 on gene expression of collagen modifying enzymes, i.e. lysyl hydroxylases and lysyl oxidase by AT-MSCs. The expression of these enzymes is an essential prerequisite to give rise to functional bone tissue desired in bone tissue engineering.

After the validation of the biological functionality of AT-MSCs for the regeneration of bone and the determination of their yield and growth characteristics, the concept of a one-step surgical procedure (chapter 2) requires induction of osteogenic differentiation of AT-MSCs in a short time frame. In order to achieve this we need to develop a protocol to trigger osteogenic differentiation of the AT-MSCs present in the stromal vascular fraction of adipose tissue within 15-30 min as described in Phase III in the proposed concept [see also chapter 2, Fig 2]. Chapter 7 and chapter 8 discuss three possible compounds, BMP-2 or BMP-7 and the polyamine spermine to accomplish the desired initial “osteogenic triggering” of AT-MSCs.

Polyamines are organic cations involved in cell proliferation and differentiation, which have been linked to bone growth and development. Tjabringa et al. (2006) have shown that the polyamine spermine might modulate the response of AT-MSCs to bone cell-specific mechanical loading. Therefore, spermine could be a candidate, which provides the initial short trigger for osteogenic differentiation of AT-MSCs during a one-step surgical procedure for spinal interbody fusion. The potential use of spermine in a short (30 min) treatment of AT-MSCs to trigger osteogenic differentiation is investigated in chapter 7.

BMPs are growth factors involved in bone development and induce differentiation of multipotent mesenchymal stem cells towards the osteochondrogenic lineage. Especially, BMP-2 and BMP-7 are under evaluation for tissue engineering targeting skeletal defects and already available as clinically approved recombinant proteins. Therefore, chapter 8 discusses the effect of a short (15 min) treatment with BMP-2 and BMP-7 on osteogenesis and chondrogenesis of AT-MSCs and the potential use of these growth factors in the proposed one-step surgical procedure.

Finally in chapter 9 we discuss the functionality of AT-MSCs and their feasibility for bone tissue engineering, i.e. for spinal interbody fusion, and the concept of a one-step surgical procedure. We describe in chapter 9 the procedure of a one-step surgery for spinal interbody fusion in the Dutch milk goat using bioresorbable,
radiolucent 70/30 poly(L-lactide-co-D,L-lactide) interbody fusion cages filled with a bioresorbable biphasic calcium phosphate carrier either or not seeded with AT-MSCs, which are either treated or not treated with BMP-2 for 15 min (chapter 8) to induce osteogenic differentiation of the AT-MSCs.
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