Stem cell behavior and biomaterials
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CHAPTER 7

GENERAL DISCUSSION AND SUMMARY
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Bone defects in fields such as orthopedics and oral and maxillofacial surgery can occur as a consequence of injury, cancer, or inflammation, and their reconstruction still remains challenging. Bone tissue engineering has become a promising alternative for the use of autologous bone in the reconstruction of bone defects. A combination of scaffolds, cells (including stem cells), and physical/mechanical and/or chemical stimuli is used to improve or replace bone. Scaffolds play a critical role in tissue engineering to provide a microenvironment where cells proliferate, differentiate, and generate the desired tissue. Currently, a wide variety of bone substitutes are commercially available and used as a scaffold for the reconstruction of bone defects with varying degrees of success. Bone substitutes can be categorized into autografts, allografts, xenografts, demineralized bone, ceramics, polymers, and composites. In this thesis, we focused on ceramic biphasic calcium phosphate (BCP), the natural polymer fibrin, and resin-based composites. Adipose stem cells (ASCs) are promising for bone tissue engineering due to their multilineage potential, and fibroblasts have also received attention due to their dynamic lineage potential. ASCs and fibroblasts can be combined with biomaterials. The interaction between these cells and their microenvironment results in dynamic changes in the composition, stiffness, and architecture of the microenvironment. The cell-matrix interaction regulates processes such as cell morphology, adhesion, migration, differentiation, and gene expression.

The main goal of this thesis was to investigate stem cell behavior in/on biomaterials with implications for bone tissue engineering in oral regenerative medicine. The interaction of stem cells with biomaterials by using different cell types and biomaterials for bone tissue engineering was addressed. The aims of the studies presented in this thesis were:

1. To investigate whether differences exist in the response to mechanical loading by pulsating fluid flow of human ASCs (hASCs) on nanocomposite or micro-hybrid composite;
2. To study the mechanical adaptation of fibrin networks due to contractile stress generated by fibroblasts at the micron scale;
3. To test the osteogenic and/or vasculogenic differentiation potential of composites consisting of hASCs seeded on BCP with a hydroxyapatite (HA)/β-tricalcium phosphate (β-TCP) ratio of 60/40 (BCP60/40) or BCP with a HA/β-TCP ratio of 20/80 (BCP20/80) incorporated in fibrin gels as well as fibrin gel degradation;
4. To test the effect of hypoxia on the osteogenic and/or vasculogenic differentiation potential of composites consisting of hASCs seeded on BCP60/40 or BCP20/80 incorporated in fibrin gels as well as fibrin gel degradation;
5. To test whether differences exist in osteogenic and/or angiogenic potential of BCP60/40 or BCP20/80 in patients undergoing maxillary sinus floor elevation.
For bone tissue engineering, it is important that stem cells differentiate into bone cells and display a bone cell-like response to mechanical loading. We found that hASCs display a bone cell-like response to mechanical loading by pulsating fluid flow. Earlier pulsating fluid flow has been shown to increase nitric oxide production, which is implicated in mechanical adaptation of bone, and RUNX2 expression, which is a transcription factor expressed in hASCs during early stages of osteogenic differentiation.

In chapter 2 the response to pulsating fluid flow by hASCs seeded on nanocomposite or micro-hybrid composite was compared. hASCs on nanocomposite and on micro-hybrid composite showed a different bone cell-like response. hASCs on nanocomposite, but not on micro-hybrid composite, increased nitric oxide production, while hASCs on micro-hybrid composite, but not on nanocomposite, increased RUNX2 expression in response to pulsating fluid flow. The differences in mechanoresponse of hASCs is possibly due to the composition of the materials. Cells can attach via a protein layer to a material. This protein layer is dependent on the underlying chemistry of the material. Therefore, the underlying chemistry of the different materials used in mechanoresponsiveness studies with hASCs might be an important parameter for the mechanoresponse of hASCs. Mechanical loading did not affect mean cell orientation and shape index of hASCs on both composites. The different mechanoresponsiveness of hASCs on both composites might have important implications for bone tissue engineering in oral regenerative medicine, such as the choice of material to fixate dental implants or to close a bone defect.

Fibrin serves as a provisional extracellular matrix for cells such as fibroblasts. Fibrin networks display non-linear mechanics, and thereby become increasingly resistant to further deformation. Fibroblasts can sense and respond to mechanical properties of their surrounding substrate by pulling on the matrix, and at the same time actively change the stiffness and tension of the surrounding substrate by applying localized forces, so-called traction forces, in a mechanical-feedback loop. Fibroblasts affect the macroscopic mechanical properties by actively stiffening of fibrin networks due to generated contractile stress. In chapter 3, a 3D model was used to measure the micromechanical properties of fibrin networks and fibroblasts-seeded fibrin networks by one-particle passive microrheology. Fibroblasts-seeded fibrin networks increased the elastic modulus G' and decreased the viscous modulus G'' compared to fibrin networks without fibroblasts at the micron scale. This suggests that fibroblasts in fibrin networks contributed actively to changes in viscoelastic micromechanical properties of fibrin networks. Therefore, it seems that fibrin networks adapt due to contractile stresses generated by fibroblasts. These data have wide ranging implications for understanding processes for improving wound healing, treating pathological tissue development, and tissue engineering applications. To further unravel the mechanical-feedback loop between local matrix stiffness and cell-generated contractile forces, the viscoelastic properties of the nucleus, and the ability of cells to push against the matrix.
should be taken into account. Deformations of the cell nucleus can have important consequences on cellular function such as cytoskeletal organization and stiffness, and can also stimulate cells to push against the matrix by generating compressive forces. Further studies are needed to investigate the mechanisms underlying nuclear deformations and how cells push against the matrix, and the consequences on cellular processes for tissue engineering applications. In oral regenerative medicine, fibrin might improve healing around implants, and in combination with a bone substitute it might enhance bone regeneration in maxillary sinus floor elevation. The adaptation of fibrin to cell-generated contractile stresses might have implications for maxillary sinus floor elevation. After implantation of fibrin in vivo, cells can invade the fibrin gel and actively stiffen the matrix which can improve local tissue healing, tissue development and homeostasis, and protect local tissues from tearing under high stress.

For efficient scaffold remodeling a BCP with an optimum ratio of HA and ß-TCP is desired. BCP60/40 is successfully clinically used as alternative for autologous bone in patients undergoing maxillary sinus floor elevation. The high percentage of HA may hamper efficient scaffold remodeling. Whether BCP20/80 is more desirable compared to BCP60/40 is still unclear, and BCP20/80 has not been tested before in a clinical human model. A novel bone substitute might offer a framework for regenerating and healing of the host bone as well as formation of blood vessels. Osteogenesis and angiogenesis are tightly coupled processes, and vascular development needs be induced before osteogenesis can take place. This complexity has been a major challenge for engineering viable and functional bone grafts. Cell-based bone constructs might be combined with biomaterials like fibrin to induce vascular development. The cellular response of cell-based bone constructs in fibrin will be influenced by changes in fibrin composition, that will create different matrix stiffness and architectural properties regulating cell differentiation. Since osteogenesis and angiogenesis are crucial for bone regeneration in cell-based bone constructs combined with fibrin, and since degradation of the matrix regulates cell differentiation, the osteogenic and/or vasculogenic differentiation potential of composites consisting of hASCs seeded on BCP60/40 in fibrin gels (BCP60/40-based composites) or BCP20/80 in fibrin gels (BCP20/80-based composites) as well as fibrin gel degradation were investigated in vitro. In chapter 4 we found that osteogenic and vasculogenic differentiation potential as well as fibrin gel degradation was enhanced in BCP20/80-based composites compared to BCP60/40-based composites in vitro. This suggests that BCP20/80-based composites might be more promising for in vivo bone augmentation than BCP60/40-based composites.

After implantation of BCP60/40-based composites and BCP20/80-based composites in vivo, the disrupted blood vessels at the implant site may rapidly lead to a hypoxic microenvironment until neovascularization occurs. In vitro simulation of this hypoxic microenvironment might offer a realistic scenario of how BCP-based composites may
preserve their features and achieve their function *in vivo*. Understanding the role of a hypoxic microenvironment is essential for the development of successful tissue engineering approaches, including bone regeneration, since the physiological oxygen concentration in bone can be as low as 1.3%. Therefore, the effect of hypoxia on the osteogenic and/or vasculogenic differentiation potential of BCP60/40-based composites and BCP20/80-based composites as well as fibrin gel degradation were studied. In chapter 5 we found that hypoxia decreased osteogenic differentiation potential but enhanced vasculogenic differentiation potential of cultured BCP-based composites without affecting fibrin gel degradation. These *in vitro* results implicate that BCP-based composites implanted *in vivo*, might enhance vascular endothelial growth factor production resulting in sprouting and tube formation leading to increased survival of cells in these composites, and stimulation of bone formation.

Finally, the *in vivo* tissue reaction can be influenced by the HA/β-TCP ratio of BCP in patients undergoing maxillary sinus floor elevation. Since BCP20/80 has not been tested before in a clinical human model, patients undergoing maxillary sinus floor elevation were treated with BCP60/40 or BCP20/80, and differences in osteogenic and/or angiogenic potential of both BCP-groups were investigated. In chapter 6 we found that the use of BCP20/80 resulted in higher bone and osteoid volume at the most cranial side of the biopsies than BCP60/40, but no differences in vascularization could be observed. Therefore, BCP20/80 showed enhanced osteogenic potential compared to BCP60/40 in patients undergoing maxillary sinus floor elevation, which might be due to a faster bone remodeling rate, or an earlier start of bone maturation in BCP20/80-treated patients. This demonstrates that BCP20/80 might perform better as a scaffold for bone augmentation in the maxillary sinus floor elevation model than BCP60/40.

Chapters 4, 5, and 6 provide insight whether BCP20/80 is more desirable for maxillary sinus floor elevation than BCP60/40. In general, BCP20/80 compared to BCP60/40 enhanced bone formation and vasculogenic differentiation potential in a BCP-based composite *in vitro*. In human maxillary sinus floor elevation model, BCP20/80 compared to BCP60/40 enhanced bone formation, but not angiogenic differentiation potential. Hypoxia enhanced vasculogenic differentiation potential in both BCP-based composites *in vitro*. This suggests that incorporation of BCP20/80, compared to BCP60/40, in fibrin gel implanted in patients undergoing maxillary sinus floor elevation might enhance angiogenic differentiation potential. This is crucial for osteogenesis, and therefore, BCP20/80 or BCP20/80-based composites might be more promising for *in vivo* bone augmentation in maxillary sinus floor elevation than BCP60/40 or BCP60/40-based composites.
Chapter 7

Concluding remarks
In this thesis we studied behavior of hASCs and fibroblasts in/on nanocomposite and micro-hybrid composite, as well as on BCP and in fibrin. We demonstrated that hASCs seeded on nanocomposite and micro-hybrid composite show a bone cell-like response to mechanical loading by pulsating fluid flow in vitro, but differences exist in the mechanoresponsiveness of hASCs on both composites which is important for stem cell differentiation into bone cells. Fibrin networks adapt actively to fibroblast-generated contractile forces in vitro which is important to resist tissue deformation in vivo. hASCs on BCP20/80 incorporated in fibrin gels enhance osteogenic and vasculogenic differentiation potential as well as fibrin gel degradation compared to hASCs on BCP60/40 incorporated in fibrin gels in vitro. This suggests that BCP20/80-based composites might be more promising for in vivo bone augmentation than BCP60/40-based composites. Hypoxia decreased osteogenic differentiation potential of hASCs cultured on BCP60/40 or BCP20/80 incorporated in fibrin gels, but enhanced vasculogenic differentiation potential. These in vitro results implicate that BCP-based composites implanted in vivo might enhance vascular endothelial growth factor production resulting in sprouting and tube formation leading to increased survival of cells in these composites, and stimulation of bone formation. Patients undergoing maxillary sinus floor elevation treated with BCP20/80 show enhanced osteogenic potential compared to BCP60/40, but no differences in angiogenic potential could be observed. This demonstrates that BCP20/80 might perform better as a scaffold for bone augmentation in the maxillary sinus floor elevation model than BCP60/40. The outcome of these and future studies will have pivotal implications for bone tissue engineering in fields such as orthopedics and oral and maxillofacial surgery. These results provide more insight in stem cell behavior in/on biomaterials to create tissue-engineered materials for bone regeneration. Although there is a plethora of cell-based constructs for bone tissue engineering, further long term preclinical and clinical studies are needed to assess the efficacy of the stem cell-based composites that we have developed, and to unravel the underlying mechanisms behind the formation of new bone. The stem cell-based composite stimulates osteogenesis and angiogenesis, and is therefore promising for bone tissue engineering. Patients worldwide will likely benefit from the stem cell-based composite for reconstruction of bone defects.

Future directions
A stem cell-based composite consisting of freshly isolated autologous stromal vascular fraction on BCP20/80 incorporated in a fibrin gel that stimulates osteogenesis and angiogenesis may become an interesting alternative to autologous bone for dental and orthopedic applications. Future directions in bone tissue engineering for oral regenerative medicine should consider:
1. The physiological loading regime including culture time prior to mechanical loading, higher/lower magnitude and/or frequency, duration of stimulation by mechanical loading and/or post-incubation of hASCs on nanocomposite and micro-hybrid composite to investigate whether differences exist in the mechanoresponse of these cells on both composites.\textsuperscript{27,28}

2. The primary cilium of hASCs on nanocomposite and micro-hybrid composite, especially its length, since this cilium has been implicated as mechanosensor in different cell types including hASCs. Osteogenically differentiated hASCs exhibit longer cilia than undifferentiated hASCs.\textsuperscript{29} hASCs on micro-hybrid composite, but not on nanocomposite, have reached an early stage of osteogenic differentiation, which suggests differences in length of the primary cilium.

3. The mechanism underlying nuclear deformation of cells in gels, and how cells push against the fibrin gel to unravel the mechanical-feedback loop between cells and matrix.

4. The effect of hypoxia on formation of tubular structures in BCP60/40-based composites and BCP20/80-based composites after co-culture with primary human endothelial cells. VEGF production in BCP60/40-based composites and BCP20/80-based composites under hypoxia was upregulated suggesting an increase in sprouting and tube formation under hypoxia.

5. The freshly isolated autologous stromal vascular fraction seeded on BCP with different HA/ß-TCP ratios incorporated in a fibrin gel for a one-step surgical procedure in e.g. maxillary sinus floor elevation. The stromal vascular fraction consists of a heterogeneous mixture of cells including endothelial cells and lineage-committed progenitor cells, and will fit in a one-step surgical procedure.

6. The appropriate composition of the stem cell-based composite to be used in bone tissue engineering for e.g. maxillary sinus floor elevation. The biomaterials BCP20/80 and fibrin are promising for \textit{in vivo} bone regeneration.
REFERENCES


