Advances in Bone Tissue Engineering

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Abstract

Bone deficiencies, caused by malformations, trauma or adverse effects from medical treatments, are a clinical challenge and often associated with reduced physical function and quality of life. Autologeous bone grafts can be used to reconstruct skeletal defects, but the right size and quality of bone might not always be available and even so, donor-site morbidity might follow. A pre-fabricated or tissue engineered material has been proposed as an alternative means of addressing these limitations. By combining bone forming cells, growth factors and scaffolding materials, this technique has the potential to generate custom made bone grafts.

The main objectives for this thesis were to optimize the conditions for bone tissue engineering, to introduce new perspectives and to gain further understanding of the involved components. In study I, the scaffolding material hydroxyapatite was coated with fibronectine and serum to augment the materials bioactivity and cell carrying capacity. Cell attachment and growth were significantly enhanced by the surface manipulation *in vitro*. Similar trends were found for *in vivo* cell delivery, but the difference was not statistically significant compared to the controls. In study II, mesenchymal stem cell (MSC) growth was accelerated in 2-D and 3-D cultures by transient downregulation of cell cycle regulator p21, using short interfering RNA. In study III, MSCs were transduced with adenoviruses to express BMP2 and VEGF. An interesting interaction was discovered, where VEGF was shown to inhibit simultaneous BMP2 expression. In study IV, a periosteum-like graft was engineered, using the dermal matrix AlloDerm and seeded MSCs. When the seeded cells were transduced to express BMP2, the created periosteum proved capable of inducing ectopic bone formation in muscle and healed a critical-sized bone defect in rat mandible.

Collectively, the presented studies highlight important aspects and current limitations of bone tissue engineering. A new approach was introduced in facilitating the process of bone formation and regeneration, and valuable insights were gained regarding stem cell manipulation and behavior. Furthermore, a novel strategy to induce bone formation and healing through the use of a manufactured periosteum was presented.

Contents

List of Publications

Study I

Schönmeyr B, Wong A , Li S, Gewalli F, Cordeiro P, Mehrara B. Treatment of Hydroxyapatite Scaffolds with Fibronectin and Fetal Calf Serum Increases Osteoblast Adhesion and Proliferation *in vitro.* Plast Reconstr Surg. 2008 Mar;121(3):751-62.

Study II

Plasilova M, Schönmeyr B, Fernandez J, Clavin N, Soares M, Mehrara. Accelerating Stem Cell Proliferation by Down-Regulation of Cell Cycle Regulator p21. Plast Reconstr Surg. 2009 Feb;123(2 Suppl):149S-57S.

Study III

Schönmeyr B, Soares M, Avraham T, Clavin N, Gewalli F, Mehrara B. Vascular Endothelial Growth Factor Inhibits Bone Morphogenic Protein 2 Expression in Rat Mesenchymal Srem Cells. Tissue Eng Part A. 2010 Feb; 16(2): 653-662.

Study IV

Schönmeyr B, Clavin N, Avraham T, Longo V, Mehrara B. Synthesis of a Tissue-Engineered Periosteum with Acellular Dermal Matrix and Cultured Mesenchymal Stem Cells. Tissue Eng Part A. 2009 Jul;15(7):1833-41.

Abbreviations

2-D	Two-dimensional
3-D	Three-dimensional
AdV	Adenovirus
ALP	Alkaline phosphatase
BrdU	Bromodeoxyuridine
BMP	Bone morphogenic protein
BRAH	Bioengineering and Research to Aid the Handicapped
CT	Computer tomography
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FN	Fibronectin
GFP	Green fluorescent protein
H&E	Hematoxylin and eosin
MOI	Multiplicity of infection
MSC	Mesenchymal stem cells
OCT	Optimal cutting temperature compound
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PFU	Plackforming units
rtPCR	Real-time polymerase chain reaction
siRNA	Small interfering RNA
si-p21	Small interfering RNA against p21
TGF-β	Tumor growth factor beta
VEGF	Vascular endothelial growth factor

Preface

The idea of creating and replacing body parts has long intrigued both scientists and science fictionists. Not until recently, however, has this area of medicine shown promise of becoming a reality. During the past decades, biomedical labs around the world have managed to reproduce a number of tissues, including skin, myocardial muscle, uriepithelial tissue, fat, cartilage and bone [1-6]. Even though the generated tissues at this point are immature and often lack full cell composition and vascular supply, it seems for the first time as if the goal of creating biological substitutes for clinical use is within reach.

The term "tissue engineering" was coined in the mid 80s, when scientists, with backgrounds in engineering, medicine and biology, started to seriously engage themselves in the concept of tissue formation and improvement. An attempt was made in 1985 by bioengineer Yuan-Cheng Fung to found a research center entitled "Center for the Engineering of Living Tissues" [7]. Even though the proposal was rejected, the subject started to gain more and more interest within the medical research community. In 1987, Fung once more surfaced the topic at a panel meeting for the Bioengineering and Research to Aid the Handicapped (BRAH) program. This time he used the term "tissue engineering" to crystallize the concept [8]. Since then, the term has been used with increasing frequency and several definitions have been suggested over the years. Allen Zelman, a Program Director for BRAH, characterized the term later on in 1987 as "a new inter-disciplinary initiative which has the goal of growing tissues or organs directly from a single cell taken from an individual" [9]. A broader and more frequently cited definition was proposed by Robert Langer and Joseph P. Vacanti in a review paper, published in Science in 1993: "Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function" [10].

Background

The skeleton constitutes the internal framework of the body and offers structure, posture and protection. If our bones become damaged by trauma or by adverse effects from medical treatment, such as tumor surgery or radiation therapy, physical function and appearance often becomes markedly hampered. Muscles and tendons will loose their function, once the area of origin or insertion fails to offer support. Bone defects in the extremities or head and neck region will therefore often lead to significant reduction in quality of life. The literature reports of physical, social and psychological impairments associated with osseous defects [11-13]. Previous studies at our institute have shown that inadequate reconstruction of bone deficiencies in the jaw will lead to inability to chew, swallow and talk as well as severe distortion of the face [14].

At this point, skeletal reconstruction of segmental bone defects has been performed using metal plates or allogenic and autologeous bone grafts. However, due to high incidence of plate exposure and inadequate healing, [15-17] few proponents still support nonvascular reconstructive techniques. Free vascular bone grafts have proven successful in many aspects but there are still several shortcomings associated with these procedures. The right dimensions and quality of bone might not always be available, and moreover, donor-site pain, instability and fractures might follow [18-21].

A pre-fabricated or tissue engineered material has been proposed as an alternative means of addressing these limitations. By combining bone forming cells, growth factors and scaffolding materials, this technique would allow for manufacturing of a graft based on what is required rather than what is available. Furthermore, donor site morbidity could be reduced.

Bone created by stem cell contribution has at this point only been used in select clinical cases. In 2004, Warnke et al. reported a case study, where an extended mandibular discontinuity was repaired using a custom made bone transplant, composed of mineral blocks, bone marrow and the stimulatory growth factor bone morphogenic protein (BMP) 7 [22]. The contents were contained within a titanium mesh and implanted in the latissimus dorsi muscle of the patient. This allowed time for bone formation and bloodvessel infiltration. After seven week, the vascularized composite graft was transplanted to the jaw using microsurgical techniques. Since Warnke's report, a handful of other cases have been described in the literature. Lendeckel and colleagues healed a calvarian defect with the use of resorbable macropourous sheets and autologeous fat derived stem cells [23]. Mesimäkis research group used a similar approach as Warnke when producing an ectopic bone flap following a hemimaxillectomy [6].

Some investigators question the need for stem cell transplantation and believe that adequate delivery of growth factors will recruit enough local or migrating precursor cells. Moghadam et al. showed in 2001 that a critical-sized bone defect in fact can be

healed with the sole use of growth factors and scaffolding materials [24]. Arnander et al. investigated this approach further when creating a composite graft suitable for microsurgical transfer, using an acellular degradable scaffold containing BMP2 [25].

Although these reports are exciting, the follow-ups have been limited and the quality and composition of the created bone are at this point uncertain. Furthermore, many aspects of the physiological processes involved are just beginning to be unwound and there are still much to be learned regarding cell recruitment, differentiation and behavior. There are other aspects that limit bone tissue engineering from becoming a routine in clinical practice as well. One obstacle is the lengthy culture and manufacturing periods. In the above mentioned case studies, the created grafts required up to eight months to form [6, 22, 25]. These long construction times are not oncologically safe for rapidly growing malignancies, since surgical resection and reconstruction need to be performed soon after diagnosis. Similarly, long culture periods are a significant drawback to the use of tissue engineered bone for traumatic defects, as it would require prolonged open wound care and convalescence. Another evident impediment for tissue engineering purposes is the need for a vascular supply at an early stage. A graft with compromised blood supply is restricted in size and prone to infection or resorption [26-28]. This, in turn, is a limiting factor for construct preparation *in vitro* and graft formation *in vivo*. Taken together, means of accelerating osteogenesis as well as angiogenesis would be a significant advance in this field of research.

Tissue engineering in general

With the goal set to produce a wide range of human tissues for regenerative and reconstructive purposes, most investigators approach the matter by bearing certain fundamentals in mind. Just like endogenous tissues rely on a well balanced composition of cells, signaling molecules and extracellular matrix, researchers involved in tissue engineering must take all these elements into consideration. Cells provide mechanical, endocrine and reparative function and are carefully regulated by autocrine and paracrine growth factors. The matrix surrounding the cells offers support and make up barriers, separating one tissue or structural unit from another. Consequently, by combining the right selection of cells, cytokines and scaffolding materials, various tissues could be mimicked and reproduced. Even though well integrated cells naturally engage themselves in matrix formation and cytokine production, cellular support and stimuli need to be provided during the early phases of de novo tissue formation. Thus, when aiming to repair significant tissue defects by tissue engineering principles, selecting cells, stimulants and appropriate scaffolding materials is a crucial step.

The selected cells should be autologeous to avoid immunologic rejection and either well differentiated or undifferentiated cells can be used. Undifferentiated cells, such as stem cells, are generally easier to harvest (e.g. through bone marrow aspirate, liposuction or skin harvest) but need to be differentiated toward a cell lineage

capable of forming the tissue in mind. Driving the cells towards a specific lineage is achieved by supplementing the cells growth media *in vitro* or by local administration of growth factors *in vivo*. Alternatively, cells could be genetically modified to secrete growth factors for autocrine stimulation. This is most commonly performed by transducing the cells with a replication deficient virus, carrying the gene for the selected growth factor. Autocrine stimulation is often considered advantageous, since the growth factor will be secreted at a fairly steady concentration over a prolonged period of time. Locally administered stimulants and growth factors would, in contrast, soon diminish through degradation and diffusion.

The scaffold or matrix serves as a vehicle for cell delivery and the shape of the scaffold constitutes a mould for the generated tissue. A scaffold composed of a biocompatible and degradable material will eventually be replaced by host tissue. This is beneficial, since foreign materials within a wound often leads to delayed healing, fibrous encapsulation and may be a nidus for infection. A strong and durable scaffold is many times harder for the cells to degrade, but may on the other hand stabilize tissue defects in need of physical support. It has also been shown that scaffolds can play an endocrine role during tissue formation. Growth factors and stimulants can be incorporated into a scaffold or matrix and slowly released through diffusion and scaffold degradation [29-31].

Physiologic bone

Physiologic bone harbors three cell types which synthesize, remodel and maintain the intercellular mineralized matrix. Osteoblasts produce the organic part of the matrix (composed of type I collagen, proteoglycans and glycoproteins) and contributes to the deposition of the inorganic components as well. Osteoclasts are multinucleated giant cells, responsible for resorption and remodeling of the matrix - a constantly ongoing process in viable bone. Osteocytes are matured osteoblasts, locked within cavities of the bony matrix, and are actively involved in the maintenance of the matrix. The osteocytes are connected by a network of small canals (canaliculi) enabling cellular communication through thin cytoplasmic extensions. Nutrients and oxygen are thereby past along the cells from the blood vessels transversing the matrix. Bone forming cells are not only involved in matrix turnover, but also in the production and secretion of regulating growth factors.

All bones are lined with a periosteum on the outer surface and an endosteum along the bone marrow cavity. These well vascularized and sensate connective tissues contain bone progenitor cells, capable of differentiation towards osteoblastic lineage and secretion of growth factors. Fresh cells can thereby be recruited all along the bone surface, and the periosteum and endosteum consequently play an essential part in bone turnover and healing. In the case of a fracture, the periosteal response is to a large extent triggered by the fracture hematoma. The vascular disruption leads to platelet aggregation and degranulation of platelet-derived growth factor (PDGF) and

fibroblast growth factor (FGF)-2. This, in turn, activates the periosteal cells [32] and stimulates release of BMPs [33, 34] and vascular endothelial growth factor (VEGF) [35].

Tissue engineered bone

When tissue engineering principles are applied to create bone substitutes or enhance osseous healing, various cell types, scaffolding materials and growth factors could be considered. As mentioned previously, cells chosen for these purposes could either be of well differentiated nature or possess multipotency. Well differentiated osteogenic cells can only be found within the bone and may be reluctant to proliferation, due to senescence associated with terminal differentiation. Therefore, multipotent progenitor cells are usually preferred, since they are easier to harvest and expand. Mesenchymal stem cells (MSCs) are most commonly used for these purposes. These cells can be found in various tissues but the full characteristics of MSCs have not yet been elucidated. There are at this point no full understanding of variations within this group of cells in terms of surface markers and differentiation potential. MSCs are most commonly harvested from the bone marrow or adipose tissue, but recent reports tell of cells with similar characteristics found in the muscle and skin [36, 37]. Whether one of these cell types are more appropriate for bone regeneration is at this point unknown. Nevertheless, some investigators claim that fat-derived stem cells are less responsive to osteogenic stimuli compared to MSCs from the bone marrow [38-41].

When choosing a scaffold for bone tissue engineering, there are both biodegradable and permanent materials available. Biodegradable scaffolds, derived from biological tissues, generally consist of extracellular matrix molecules such as collagen and fibrin. There are also absorbable synthetic scaffolds available, made up of organic compounds such as polyglycolic acid (PGA) or polycaprolactone (PCL). A degradable scaffold will gradually be replaced by new tissue, generated by the delivered or native cells. The rate of degradation and the number of natural cell binding sites varies amongst the various materials. An important downside to the degradable scaffolds is that they tend to be too soft to support the mechanical load of a skeletal defect. Materials that are unpliable, such as titanium and hydroxyapatite, may offer necessary initial support, but may on the other hand be less prone to biointegration. Metals or alloys will not be replaced by host tissue over time and may eventually brake or become rejected. Hydroxyapatite is a calciumphosphate, naturally occurring in bone and dentine, and is often brought up in bone tissue engineering contexts. This material is already in clinical use as a bone void filler, and has been suggested to promote osseous integration when used as coating on orthopedic implants [42-44]. However, hydroxyapatite lacks natural binding sites for delivered or native cells and follow up studies have shown that the material will only partially be replaced by natural bone over time [45-48]. This is a potential problem, since the material lacks an organic component, making it brittle and prone to fatigue and breakage over time.

Various hydroxyapatite surface manipulations involving electrical polarization, altered topography and chemical composition have been investigated to enhance the material's bioactive properties. Many agree that an electrically charged hydroxyapatite surface promotes bone ingrowth by accumulation of calcium ions and other bioactive molecules [49-51]. Furthermore, the coarseness of a ceramic's surface and its chemical composition is believed to affect cell attachment, proliferation and differentiation [52-54]. The net effect of these parameters is however still under debate, and the optimal conditions for cell attachment, spread and bone deposition have not yet been established.

Scaffolds should preferably contain pores or be composed of a matrix that allows for cell and tissue ingrowth. The dimensions of the pores have been subjected to several investigations. Even though some inconsistencies exist in the literature, most investigators believe that a pore size >100μm is necessary for adequate bone ingrowth and that optimal osteogenesis and vascular infiltration occur with a pore size >300μm [55].

Among the cytokines involved in bone formation and healing, the BMPs, belonging to the transforming growth factor beta (TGF-β) family, are considered to be the most influential. These proteins are therefore often used to guide and enhance bone tissue engineering. However, the BMPs and growth factors in general, will not work unless there are responder cells present. The BMPs potential is therefore limited in an acellular or devascularized area. Angiogenic growth factors are consequently also of interest for bone tissue engineering, since these cytokines can facilitate the establishment of a vascular network and thereby promote construct formation and viability. Furthermore, angiogenesis and osteogenesis are closely linked and one process can possibly potentiate the other. VEGF-A is the most well known member of the VEGF family and considered to play a pronounced role in vascular development and maintenance. This factor has been found to differentiate and guide endothelial cells to form new capillary networks (vasculogenesis) and capillary branches from preexisting vessels (angiogenesis) [56]. VEGF-A (from here on referred to as VEGF) has therefore been suggested as a particular candidate to enhance vessel ingrowth and formation in tissue engineered grafts [57-60].

Aim

The main objective of this thesis was to optimize the conditions for bone tissue engineering, with emphasis on acknowledged predicaments in this field. Focus was also directed towards further understanding of cell behavior and the consequences of altered gene expression in bone forming cells.

The specific aims were:

1. To assess cell attachment, growth and delivery, using a hydroxyapatite scaffold coated with FN and serum **(study I)**.

2. To enhance mesenchymal stem cell expansion in 2-D and 3-D cultures, by transient downregulation of cell cycle regulator p21, using siRNA **(study II)**.

3. To evaluate the *in vitro* and *in vivo* effects of induced simultaneous expression of BMP2 and VEGF in MSCs **(study III)**.

4. To create a periosteum-like material, using acellular human dermis and cultured bone forming cells. Furthermore, to evaluate the generated materials potential to form ectopic bone and contribute to bone healing **(study IV)**.

Materials and Methods

Cell harvest

Osteoblasts
Primary rat calvarial osteoblast were harvested from the frontal and parietal bones of newborn Fisher 344 rats **(study I** and **IV)**. The calvaria were serially digested in a collagenase-dispase solution and the purified osteoblasts were resuspended, plated and grown in Dulbecco's Modified Eagle Medium with 10% fetal calf serum (FCS).

Bone marrow derived mesenchymal stem cells
For *in vitro* studies, MSCs were harvested from Lewis rats (LEW/SsNHsd) **(study II** and **III)** and for *in vivo* studies, cells were harvested from green fluorescent protein (GFP) expressing transgenic mice (C57BL/6-Tg(ACTbEGFP)1Osb/J) and rats (LEW-Tg [EGFP] F455/Rrrc) **(study I, III** and **IV)**. Endogenous GFP-expression in MSCs has previously been shown not to interfere with the cells osteogenic capacity [38]. For p21 trials **(study II)**, cells from wild type B7129PF1/J mice and p21 knockout mice (based on the same strain) were used.

The cells were harvested from the femurs and tibias by disrupting the bone marrow from the diaphysis. The cell suspension was then centrifuged and plated in MesenCult media (StemCell Technologies, Vancouver, Canada), containing stimulatory supplements designed to optimally initiate and maintain MSC proliferation. MSCs were isolated from hematopoetic cells by plastic adhesion. MSC phenotype was confirmed by ensuring the cells potential to differentiate to bone lineage or by determining antigen expression of CD 105, CD 29, Sca-1 and antigen negativity for CD45 and CD34, using flow cytometry.

Cell culture

Cells were cultured at 37°C in a humidified, 5% carbon dioxide atmosphere, and when 90% confluence was reached, they were split in a 1:2 ratio using 0.25% trypsin and 0.03% ethylenediaminetetraacetic acid (EDTA). Media changes were performed regularly and early passage cells were used for all experiments.

Scaffolds and scaffold preparation

Hydroxyapatite disks
Porous hydroxyapatite disks (Pro Osteon 500, Interpore International, Irvine, CA), measuring 5mm in diameter and 2mm in thickness, with an average pore diameter of 0.5 mm, were used in **study I**. The disks were prepared in four different ways. One set was placed in 40µg/ml human fibronectine (FN) at room temperature for two

hours and then placed in FCS at 4°C overnight on a shaker. Another group was placed in 40µg/ml FN at room temperature for two hours and then placed in phosphate-buffered saline (PBS) at 4°C overnight. A third set was placed in FCS overnight at 4°C, and the last group was only exposed to PBS. The following day all hydroxyapatite disks were seeded with $1x10^5$ cells. Cells were allowed to adhere and then kept at the air –medium interface with regular media changes.

Collagen scaffolds

Three-dimensional collagen composite scaffolds (BD Biosciences, Bedford, MA), composed of collagen I and III derived from bovine hide, were used in **study II** and **III.** The scaffolds measured 5mm in diameter and 3mm in thickness and had an average pore size of 100–200µm. For *in vivo* studies **(study III)** a central circular pore was created in the scaffolds, using a 1mm hole punch, to prepare for incorporation of a vascular pedicle. Scaffolds were hydrated and seeded with 5-5.5 $x10^5$ MSCs. Cells were allowed to adhere and then kept at the air–medium interface with regular media changes.

AlloDerm

AlloDerm (LifeCell Corporation, Branchburg, NJ) is an acellular human dermis derived from donated human skin and was used to construct a periosteal-like membrane in **study IV**. The dermal allografts are decellularized using a proprietary technique, leaving a material composed of extracellular matrix molecules. AlloDerm is approved by the Food and Drug Administration and has been used clinically with low rates of complications [61, 62]. 0.012-0.015″ thick 12x8mm AlloDerm pieces were hydrated and seeded on the dermal side with $1x10^5$ cells. Cells were allowed to adhere and then kept at the air–medium interface with regular media changes.

Transduction, transfection and differentiation

Viral transduction

For viral transduction, replication-deficient E1/E3-deleted adenoviruses were used, encoding the gene of interest under the control of a cytomegalovirus promoter. The adenoviruses were amplified in 293 cells and purified using a ViraBind™ Adenovirus Purification Kit (Cell Biolabs Inc., San Diego, CA). The viruses were quantified using a plaque-forming assay on 293 cells. Viruses encoding the genes for LacZ, VEGF or BMP2 were used in **study III** to transduce cells, growing in culture dishes or on collagen scaffolds. Various ratios and combination of viruses were used to make up a total multiplicity of infection (MOI) of 100 plackforming units (PFU) per cell. Viruses encoding the genes for LacZ, GFP or BMP2 were used, at a MOI of 100 or 200 PFU/cell, in **study IV** to transduce cells growing on AlloDerm.
In general, viruses were added in a low volume and allowed to adhere for 1h before more media was added. Analyses or implantation was performed 48-72 hours after the viral transduction.

siRNA transfection

SMARTpool small interfering (si) RNA (Millipore, Billerica, MA) was used to cause short-term inhibition of p21 expression in MSCs **(study II)**. P21 siRNA and nonsense siRNA controls were used to transfect cells. Cells for 2-D culture were transfected by electroporation using a Nucleofector® kit (Amaxa Biosystems, Koeln, Germany) and cells grown on collagen scaffolds were transfected by lipofection using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) in accordance with the provided protocols.

Differentiation

Cell differentiation towards bone lineage was performed by either adding recombinant BMP4 (10 ng/ml) or 5mM b-glycerophosphate, 10^{8-9} M dexamethasone and 0.28mM ascorbic acid to the culture media.

Animal procedures

Ethical approvals

Animals were used in accordance with the guidelines of the American Veterinary Medical Association, and all *in vivo* experiments were approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee. The animals were housed in a temperature- and light-controlled facility and food and water were permitted ad libitum. For surgical and imaging procedures, animals were anesthetized using isoflurane inhalation, and postoperative pain was alleviated using Buprenorphine 0.01mg/kg subcutaneously. All procedures were conducted under sterile conditions. Animals were euthanatized in a humane manner, using carbon dioxide overdose.

Hydroxyapatite implantation

For hydroxyapatite experiments **(study I)**, disks seeded with GFP-positive MSCs were implanted in adult male athymic mice (NCr-nu/nu) **(Figure 1)**: Incisions were made along the femoral vessels and bilateral muscle pockets were bluntly prepared in the adductor magnus muscle. One coated disk (i.e. FN/FCS) and one control disk (PBS) were implanted in each animal. The disks were harvested three and ten days after implantation.

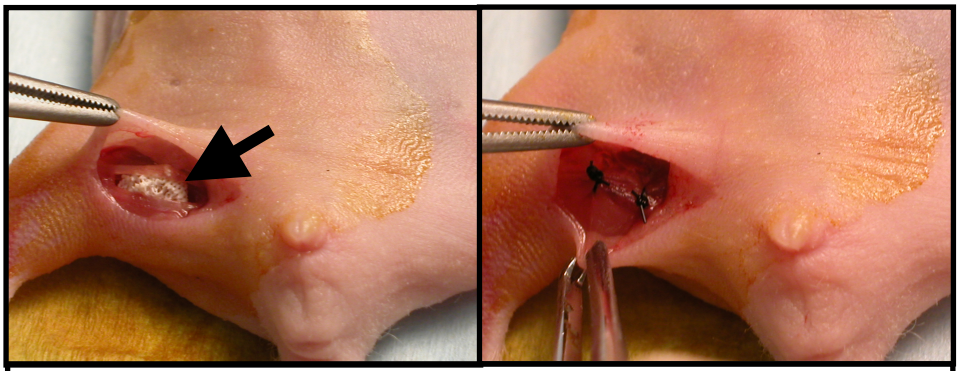

Figure 1. Implantation of prepared hydroxyapatite disk(arrow) in nude mouse

Collagen scaffold implantation

Cell-seeded and virally transduced collagen composite scaffolds were implanted in syngeneic non-GFP rats (LEW/SsNHsd) **(study III)**. A vascular bundle was surgically incorporated into the construct, using a previously described model [63]: Incisions were made along the femoral vessels and the superficial inferior epigastric artery and vein were located and carefully freed from the groin fat pad. The pedicle was distally ligated and pulled through the center of the perforated collagen scaffold **(Figure 2)**. The construct was then covered by the groin fat pad and the incision was closed. Four weeks after implantation, imaging with computer tomography (CT) was conducted and the scaffolds were harvested.

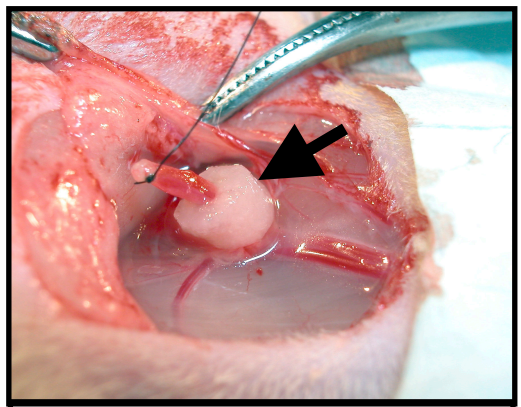

Figure 2. Implantation of prepared collagen scaffolds (arrow) around a distally ligated pedicle in rat.

AlloDerm implantation

AlloDerm with cultured GFP-positive MSCs were implanted around the adductor muscle in syngeneic adult mice (C57BL/6J) **(study IV)**: Incisions were made along the femoral vessels and a portion of the adductor muscle, measuring approximately 5mm in cross-section diameter, was separated along the length of the muscle fibers. The AlloDerm constructs were then wrapped around the muscle, with the cellular surface oriented toward the muscle **(Figure 3)**. Specimens were harvested after one, three, seven and 14 days and analyzed with fluorescent microscopy. AlloDerm seeded with GFP-positive MSCs and transduced with viral vectors, carrying the gene for BMP2 or LacZ, were implanted in a similar fashion. Analyses for bone formation with CT and histology were performed three weeks after implantation.

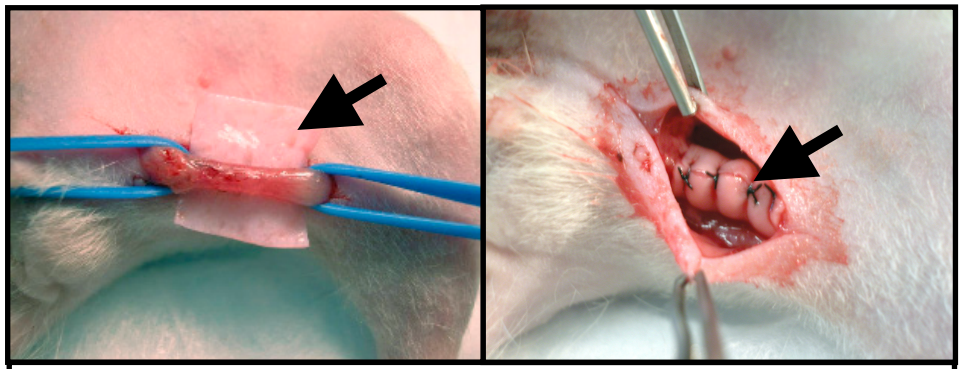

Figure 3. Implantation of prepared AlloDerm (arrow) around adductor muscle in mouse

Virally transduced AlloDerm-constructs were also used to treat critical-sized mandibular defects in adult athymic rats (Cr:NIH-RNU): After sparing the masseter muscle, 4mm circular defects were created in the posterior ramus of the mandibles, using a diamond burr at slow speeds **(Figure 4A)**. After irrigation with PBS to remove residual bone chips, the defects were covered with the prepared AlloDerm **(Figure 4B)**. Analyses with CT were performed four and six weeks after implantation. Specimens were harvested for histological evaluation at the last time point.

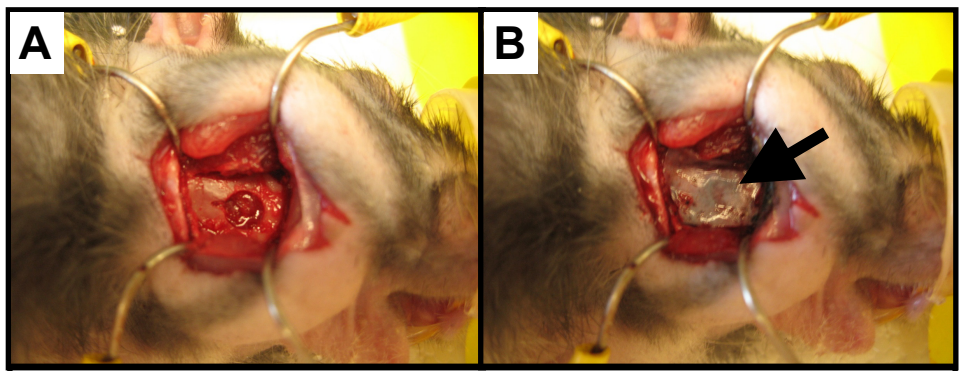

Figure 4. Critical-sized defects were created in the posterior ramus of rat mandibles **(A)**. Prepared AlloDerm (arrow) was used to treat created defects **(B).**

Assays and analyses

Cell proliferation

Cellular proliferation was assessed by various means. Trypsinized cells were manually counted in bright-field microscopy with a hemocytometer **(study I-II)**. Trypan blue staining was used to exclude non-viable cells. To determine rate of proliferation, the relative cell number was assessed at even intervals using the

CellTiter96® Aqueous Assay (BD, Franklin Lakes, NJ) according to the manufacturer's instructions **(study II-III)**. This assay relies on the mitochondrial conversion of MTS to formazan, a reaction directly proportional to the number of living cells in the culture. To detect proliferating cells and assess the potential for cell growth on AlloDerm and collagen scaffolds *in vitro* **(study II, IV)**, cells were labeled with the thymidine analogue bromodeoxyuridine (BrdU). BrdU was added to the media at a concentration of 10μmol/l for 4-6h. The AlloDerm constructs were then fixed in 4% paraformaldehyde (PFA) and paraffin embedded. 5μm thick sections were immunostained using the BrdU Staining Kit (Zymed, San Francisco, CA) in accordance with the provided protocol. Cells cultured on collagen scaffolds were trypsinized and BrdU-positive cells were identified using a Becton Dickinson FACScalibur™ flow cytometer and MultiCycle software for Windows (Phoenix Flow Systems, San Diego, CA).

Histochemical staining

Osteoblastic differentiation was assessed by staining cell cultures for alkaline phosphatase (ALP) using a Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich, St. Louis, MO) according to the provided protocol. Formation of calcium depositions in cell cultures was detected using von Kossa staining: Cells were fixed in 4% PFA, and then exposed to 5% silver nitrate and ultraviolet light. After serial rinses in distilled water, 5% sodium thiosulfate (in H2O) was added and after additional rinses, nuclear fast red was added.

To evaluate expression of LacZ **(study IV)**, AlloDerm constructs were fixed with 1% glutaraldehyde for 5 min, followed by staining with an X-Gal staining kit (F. Hoffman-La Roche, Basel, Switzerland) according to the manufacturer's instructions.

Protein expression

Western blot was used to evaluate p21 expression in p21 knockout MSCs and in MSCs treated with siRNA **(study II)**. 30μg of total cellular protein per sample was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Goat anti-mouse p21 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used for the immunoblots. Membranes were developed through the enhanced chemiluminescence method (Ecl- Luminol kit; Santa Cruz Biotechnology). Protein loading was systematically verified by Ponceau S staining and/or actin immunoblotting.
Protein expression of BMP2 and VEGF in virally transduced MSCs **(study III)** was determined using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the provided protocol.

Gene expression

Real-time polymerase chain reaction (rtPCR) was used to assess gene expression **(study II-III)**. Cellular RNA was isolated using RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Quantity and quality of the purified RNA samples was analyzed using a NanoDrop® ND 1000 Spectrophotometer (NanoDrop

Technologies, Wilmington, DE). About 1 mg of total RNA from each sample was reverse transcribed using TaqMan® Reverse Transcription Reagents Kit (Applied Biosystems, Foster City, CA). Rt-PCR primers and probes for VEGF, BMP2, RUNX2, osteocalcin, p21, C/EBP-α, 18s rRNA, and Hexon (Applied Biosystems) were used to perform PCR on an ABI Prism® 7900HT using 2xTaqMan® Universal PCR Master Mix (Applied Biosystems).

For evaluating mRNA stability and degradation **(study III)**, cells were exposed to medium containing 5 ng/ml of RNA-polymerase II inhibitor actinomycin-D prior to RNA harvest. For evaluating posttranslational inhibitory mechanisms, cells were exposed to medium containing 10 ng/ml protein synthesis inhibitor cycloheximide prior to RNA harvest.

Tissue processing and imaging

Fixation and embedding

Harvested constructs **(study I-III)** were fixed in 4% PFA and decalcified in 0.5 M ethylenediaminetetraacetic acid (EDTA) for two to ten days on a rocker at room temperature, protected from light. The implants were then split into two halves. One half was embedded in paraffin, sectioned and used for hematoxylin and eosin (H&E) staining and immunohistochemistry. The other half was transferred to 30% sucrose in phosphate-buffered saline on a rocker at 4°C for two days. An equal volume of optimum cutting temperature embedding compound (OCT) (Sakura Finitek, Torrance, CA) was then added for an additional two hours. The sample was then put in a cryomold with 100% OCT, allowed to solidify on dry ice, and then sectioned and used for fluorescent evaluation.

Immunohistochemistry

Blood vessel infiltration was assessed **(study I, IV)** with immunohistochemical localization of CD34, using the Discovery® XT System and CD34 rat monoclonal antibody (Abcam, Cambridge, MA) in 2µg/ml concentration. Blocking was performed with 10% normal rabbit serum and 2% bovine serum albumin. Biotinylated rabbit anti-rat immunoglobulinG was used as a secondary antibody (Vectastain ABC kit; Vector labs,Burlingame, CA). A DAB detection kit (Ventana, Tucson, AZ) was used in accordance with the manufacturer's instructions. Hematoxylin was used for counterstaining, and sections of embryo and testis were used as positive controls.

For immunostaining of GFP, anti-GFP rabbit polyclonal antibody (Invitrogen) was used in 2µg/ml concentration. The blocking reagent contained 10% normal goat serum and 2% bovine serum albumin. Biotinylated goat anti-rabbit immunoglobulinG was used as a secondary antibody (Vectastain ABC Kit; Vector labs). A DAB detection kit (Ventana) was used in accordance with the manufacturer's instructions. Hematoxylin was used for counterstaining, and tissues from GFP mice were used as positive controls.

Histological imaging
For bright-field imaging, a Zeiss Axioplan 2 microscope with a linked QImaging Retiga EX charge-coupled device digital camera (Opelco, Dulles, VA) was used together with the Volocity 4.0 software (Improvision, Inc., Lexington, MA).

To assess vascular ingrowth in hydroxyapatite disks **(study I)**, ten randomly selected visual fields of immunostained sections were captured at 100x total magnification. The number of stained vascular lumens was then manually counted. Single infiltrating endothelial cells were not included in this assessment. The MetaMorph software (version 7.0r2; Molecular Devices Corp., Downingtown, PA) was used to calculate the area of each image. Only the area within the pores and not the decalcified hydroxyapatite or surrounding tissue was used to normalize the cell counts.

To quantify GFP immunostaining **(study I)**, ten randomly selected visual fields were captured (100x magnification) and the number of stained cells were manually counted and normalized by sample surface area (as described above).

The GFP signal of cryosections was examined using a Zeiss Axioplan 2 microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) with a GFP filter. To verify that the signal was truly of GFP profile rather than from autofluorescence, a confocal microscope (Leica TCS AOBS SP2; Leica Microsystems Inc., Bannockburn, IL) was used to measure the excitation-emission spectra of the cells. The images were furthermore captured using not only a GFP filter, but also a tetra-methyl rhodamine iso-thiocyanate (TRITC) filter. This created a merged image where GFP cells appeared distinctly green and autofluorescent surroundings had a yellowish hue.

To quantify the amount of GFP positive cells in hydroxyapatite samples **(study I)**, randomly selected visual fields of cryosections (100x magnification) were captured using a linked Zeiss AxioCam MRm digital camera and the Zeiss Axiovision 4.6 acquisition software (Imaging Associates, Ltd., Bicester, UK). Only visual fields showing intact tissue morphology were captured. Cells were then manually counted but the digital images were also analyzed using MetaMorph software (version 7.0r2; Molecular Devices Corp., Downingtown, PA), where the percentage of GFP-positive pixels was calculated for each image (decalcified hydroxyapatite excluded).

Radiographic imaging

Ectopic bone formation was radiographically imaged **(study III-IV)** using the CT component of the X-SPEC dual-modality SPECT/CT system (Gamma Medica Ideas, Northridge, CA).

Healing of a mandibular defect **(study IV)** was evaluated with a μCT scanner (Imtek Inc., Oak Ridge, TN), four and six weeks after surgery. Serial images were obtained using 180mm thick slices and images were reconstructed using Cobra Software

(COBRA Solutions, Inc., Tempe, AZ). A fixed region of interest was encircled 4mm from the posterior ramus and 4mm from the inferior edge of the mandible and analyzed using ASIpro software (CTI Molecular Imaging, Inc., Knoxville, TN). The intensity of the CT signal was quantified and compared with background signal. Results were reported as a ratio of these two measurements and referred to as signal intensity.

Statistics

Statistical analyses were conducted using InStat 3.0 (GraphPad Software, Inc., San Diego, CA) software for Windows. Comparative parametric analyses between two groups were performed using an unpaired *t* test. Multiple group parametric comparisons were analyzed by one-way analysis of variance with post hoc Tukey's test. Non-parametric comparisons were performed using Mann-Whitney test for comparisons between two groups and Kruskal-Wallis test for multigroup comparisons. Data were presented as mean ±SD, with $p < 0.05$ considered significant.

Results and Comments

Paper I

The use of hydroxyapatite in bone tissue engineering and skeletal reconstruction is limited by the material's lack of cellular binding sites and slow degradation. In an effort to overcome these shortcomings, porous hydroxyapatite disks were coated with FN and/or FCS and evaluated for cell attachment and growth. The disks were seeded with $1x10^5$ osteoblasts, cultured *in vitro* and evaluated for cell number, 48 hours later. Disks coated with FN and FCS showed the greatest number of attached cells. Whereas disks coated with FN+FCS contained 10.8 ± 1.4 $x10^4$ cells, uncoated disks only contained 3.8 ± 1.7 $x10^4$ cells **(Figure 5A)**. Furthermore, disks coated with FN+ FCS contained significantly more cells than disks coated with FN or FCS alone. When the number of attached cells was assessed after seven days, the cell number had increased significantly in disks coated with FN+FCS to 14.4 ± 3.0 $x10^4$ cells. No such increase in cell number was observed among the uncoated disks (3.4 ± 0.2 $x10^4$ cells), implying that not only cell attachment but also cell growth was improved by coating hydroxyapatite with FN and FCS.

For *in vivo* studies, coated hydroxyapatite disks were seeded with GFP expressing MSCs and implanted into muscle pockets in immunocompromised mice. Disks were harvested, decalcified and sectioned, three and ten days after implantation. GFP intensity and cell number was recorded using fluorescent microscopy and immunohistochemistry. FN+FCS pretreated hydroxyapatite disks showed greater fluorescent intensity and cell numbers compared to the controls, but the difference was less pronounced compared to the *in vitro* results and not statistically significant. After three days, implanted experimental disks contained 122 ± 50 cells/mm^2, whereas the uncoated control discs contained 85 ± 21 cells/mm^2 **(Figure 5B)**. After ten days *in vivo*, the cell numbers had decreased in both groups to 19 ± 11 cells/mm^2 and 12 ± 11 cells/mm^2 respectively. Furthermore, the cells had a tendency of clustering in a less evenly distributed pattern in both groups compared to the early time point. The FN+FCS coating did not affect vessel ingrowth or scaffold vascularization, according to CD34 immunostaining.

Previous reports have indicated that FN and serum proteins have the potential to enhance cell attachment on hydroxyapatite [64-67]. However, due to inconsistent results and lack of *in vivo* confirmation, there has been a need for further investigations. Our study found a strong relationship between FN+FCS coatings and *in vitro* cell attachment and growth. The effect *in vivo* seemed however less clear. This discrepancy is of great importance and emphasizes the fact that current and previous *in vitro* results may not be directly applied to an *in vivo* setting. The factors behind this inconsistency have yet to be determined and calls for additional studies. One may however speculate, that due to a less complex environment *in vitro*, the addition of FN and serum proteins has a greater impact compared to the *in vivo* setting. The *in*

vitro effect could thus be depleted due to a vast number of other stimulants and influencing factors in a physiologic milieu. Furthermore, enhanced competition from native cells, due to the added stimulants on the implanted scaffolds, may offer an alternative rationale. Some inconsistencies within the current study and previous investigations may also be explained by slight variations among the studied cell types.

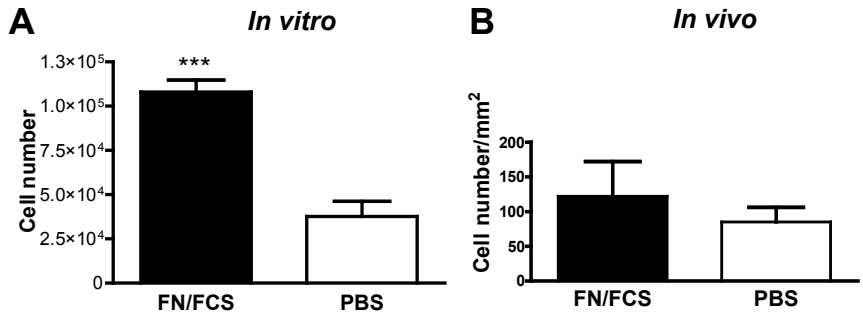

Figure 5. Hydroxyapatite disks were pretreated with fibronectine (FN) and fetal calf serum (FCS) and seeded with bone forming cells. The FN/FCS coating improved cell attachment significantly *in vitro* as compared to uncoated controls (PBS) **(A)**. Cell seeded constructs were implanted in mouse muscle pockets and evaluated for delivered cell number. Three days after implantation, cross sections of pretreated hydroxyapatite disks contained more transplanted cells than uncoated disks, but the difference was not statistically significant **(B).** Results are presented as mean ±SD. *** p <0.001.

Paper II

Cell culture expansion and construct manufacturing for tissue engineering purposes are time consuming processes. To accelerate cell growth and facilitate construct synthesis, short term downregulation of the cell cycle regulator p21 was performed in MSCs *in vitro*. Western blot and rtPCR analyses revealed that p21 expression was diminished after exposure to small interfering RNA against p21 (si-p21). When cells were grown in 2-D culture and evaluated for cell number at various time points, si-p21 treated cells grew significantly faster as compared to untreated cells and siRNA-controls. After four days, there were twice as many cells in si-p21 treated cultures compared to the controls **(Figure 6A)**. After six days, the difference increased further to three times as many cells in si-p21 cultures. Similar effects on cell growth were observed in MSC cultures from p21 knock-out mice.

Si-p21 treated cells, grown on a 3-D collagen matrix, showed increased levels of BrdU incorporation when analyzed by flow cytometry. After nine days, twice as many BrdU-positive cells were found in the p21-silenced 3-D cultures as compared to the controls **(Figure 6B)**.

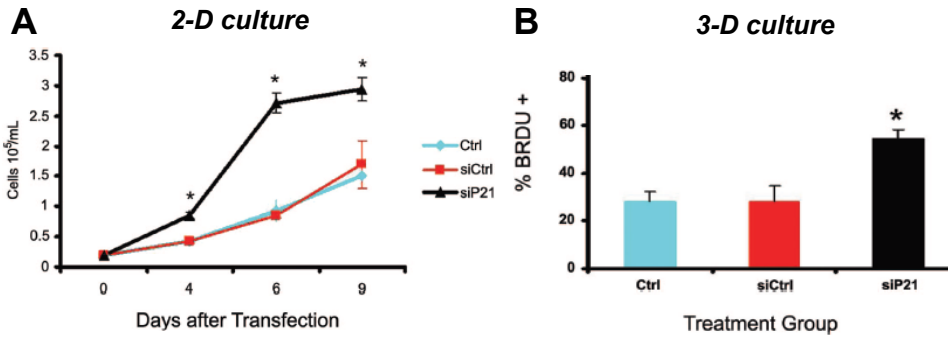

Figure 6. A Mesenchymal stem cells were transfected with p21 small interfering RNA (si-p21). Proliferation was accelerated by the si-p21 treatment in 2-D culture when compared to vehicle control (*Ctrl*) and control siRNA (*siCtrl*) **(A)**. Bromodeoxyuridine (*BrdU*) staining indicated accelerated cell proliferation in 3-D culture among si-p21 treated cells **(B)**. Results are presented as mean ±SD. **p* <0.05

To assess for alterations in stem cell multipotency after p21 manipulation, 2-D cultures were exposed to differentiation media and analyzed for bone and fat differentiation markers, using rtPCR. After 14 days, late markers for fat (C/EPB-α) and bone differentiation (osteocalcin) were elevated in si-p21 cultures as compared to controls. No such increase was found in cultures grown in regular media, undergoing spontaneous differentiation. However, RUNX2, an early marker for bone differentiation, was reduced in si-p21 cells after 14 days of both induced and spontaneous differentiation.

Loss of p21 regulation has previously been shown to accelerate proliferation in several other cell lineages including hepatocytes as well as neural- and hematopoetic stem cells [68-71]. P21 belongs to the Cip/Kip family of cyclin-dependent kinase inhibitors and exerts its effects on cellular proliferation, by affecting cyklin dependent kinase 1, 2, 4 and 6, as well as by binding proliferating cell nuclear antigen. In addition, p21 regulates gene expression and other cellular events through protein–protein interactions. Taken together, p21 is believed to block cell cycle progression at the G1/S, S and G2/M phases and desensitize the cells to apoptotic agents and stimuli [72-75].

Due to its effects on the cell cycle and association with p53, p21 has been regarded as a tumor suppressor [76]. P21 knockout mice spontaneously develop tumors after 16 months and many human cancers are associated with reduced p21 expression [77-81]. This might be a concern when deliberately suppressing p21 expression. However, the role of p21 in tumor formation has not been fully established and it has even been suggested that p21 could be prooncogenic, due to its anti-apototic activity [82, 83]. Furthermore, it is generally agreed that loss of p21 by itself is insufficient to promote malignancies. Secondary mutations are thus necessary in accordance with the multi-hit tumorigenesis theory. It is therefore unlikely that transient down-regulation of

p21 activity would significantly alter the rate of spontaneous tumor formation. Nevertheless, this conclusion requires further analysis.

There might be another concern when repressing p21 expression in stem cells. P21 is likely involved in the regulation of cell differentiation, partly since cell cycle arrest and terminal differentiation are closely linked. The exact role of p21 in these processes has, however, not yet been established and inconsistent results can be found in the literature [68, 84-94]. For instance, Chang et al. found evidence of enhanced osteoblast differentiation due to elevated levels of p21 after BMP4 stimulation [85]. On the other hand, Bellosa and colleagues suggests that p21 is strongly downregulated during differentiation and that p21 null osteoblasts differentiate faster than wild-type cells and are more susceptible to the differentiation promoting action of BMP2 [68]. In **study II** we aimed to gain further insights in this phenomenon, since conservation of the stem cells multipotency and differentiation capability is essential. Both spontaneous and induced differentiation of p21 deficient MSCs were assessed. Since p21 has been claimed to affect osteoblast and adipocyte maturation [68, 85, 94-96], specific differentiation markers for these lineages were evaluated. We found that transient knock down of p21 had little effect on spontaneous differentiation, in terms of expression of bone and fat specific markers osteocalcin and C/EBP-α. On the other hand, gene expression of these markers was elevated in p21 deficient cells compared to controls, after induced differentiation by lineage specific induction media. These findings are supportive of Bellosa et al. [68] and suggest a relative increase in sensitivity to differentiation stimuli among p21 deficient cells. Early bone differentiation marker RUNX2 was on the other hand down regulated in both settings (regular/differentiation media). However this finding needs further investigation, it is possible that RUNX2 expression already had peaked at the time of analysis, since RUNX2 precedes osteocalcin expression [97-99]. Furthermore, RUNX2 could be regarded as less specific than osteocalcin, since RUNX2 is believed to be linked to other cellular processes apart from osteogenesis, processes that might be altered due to the manipulated p21 expression [94, 100-102].

Paper III

Both BMP2 and VEGF play important parts in bone healing and growth. The effects of combining these two growth factors in a tissue engineered setting have, however, not been fully elucidated. Rat MSCs were transduced with adenoviruses (AdV) encoding BMP2, VEGF, or LacZ in a variety of ratios. Simultaneous transduction with AdV-BMP2 (MOI=50PFU/cell) and AdV-VEGF (MOI=50PFU/cell) resulted in a fivefold decrease of BMP2 gene and protein expression, as compared to controls transduced with AdV-BMP2 and AdV-LacZ **(Figure 7A)**. A corresponding inhibition was not observed in dually transduced cells, when evaluating the expression of VEGF. When cells were separately transduced with AdV-BMP2 and AdV-VEGF and then mixed and cultured together, BMP2 expression was inhibited but not to the same extent as in the co-transduction experiment. A dose-dependant decrease in BMP2 expression was observed, when MSCs were transduced with

AdV-BMP2 and then exposed to exogenous VEGF. When cycloheximide was used to block protein translation, BMP2 gene expression was still inhibited in dually transduced cells. This indicates that protein translation is not necessary for VEGF induced inhibition of BMP2 expression and that the inhibition occurs at the transcriptional level.

To assess for potential alterations in MSC differentiation, cells were grown in differentiation media and exposed to recombinant VEGF. After 12 hours, expression of early bone differentiation marker RUNX2 was approximately 50% compared to cell cultures without VEGF supplementation. Furthermore, only cell cultures without VEGF supplementation stained positively for calcium deposition, when evaluated with von Kossa staining.

For *in vivo* studies, GFP-positive MSCs were cultured on collagen scaffolds and transduced with combinations of AdV-BMP2, AdV-VEGF and AdV-LacZ. Scaffolds were then implanted in syngeneic rats and attached to a distally ligated vascular bundle. Constructs transduced with only AdV-BMP2 or co-transduced with AdV-BMP2 and AdV-LacZ produced ectopic bone around the pedicle **(Figure 7B)**. Furthermore, numerous GFP positive osteocytes were identified in these implants, indicating incorporation of implanted MSCs **(Figure 7C)**. In contrast, the combined transduction with Adv-BMP2 and Adv-VEGF in a 1:1 ratio prevented bone formation **(Figure 7D)** and no GFP-positive cells could be identified in the histologic sections.

Thus, we have shown that VEGF prevents osteogenic commitment, by inhibiting BMP2 expression at the mRNA level. In contrast, Peng et al. have suggested a synergistic relation between BMP and VEGF in certain settings [103, 104]. Peng showed that when a BMP2:VEGF ratio of 5:1 was used, bone formation was augmented rather than impaired. However, higher levels of VEGF resulted in decreased bone formation, suggesting an inverse correlation between the degree of bone formation and the ratio of VEGF to BMP2. The inconsistencies between our and Peng's results could to some extent be explained by the fact that Peng used separately transduced cells. Our results indicate that VEGF inhibition is more pronounced in co-transduced cells compared with separately transduced but co-cultured cells. Li and colleagues have, in consistence with our findings, showed that a continuous co-expression of VEGF and BMP4 in pluripotent cells, reduces bone formation *in vitro* and *in vivo* [105]. Our results are further supported by Geiger et al., who demonstrated that elevated levels of VEGF drastically impaired healing of a critical-sized bone defect [106].

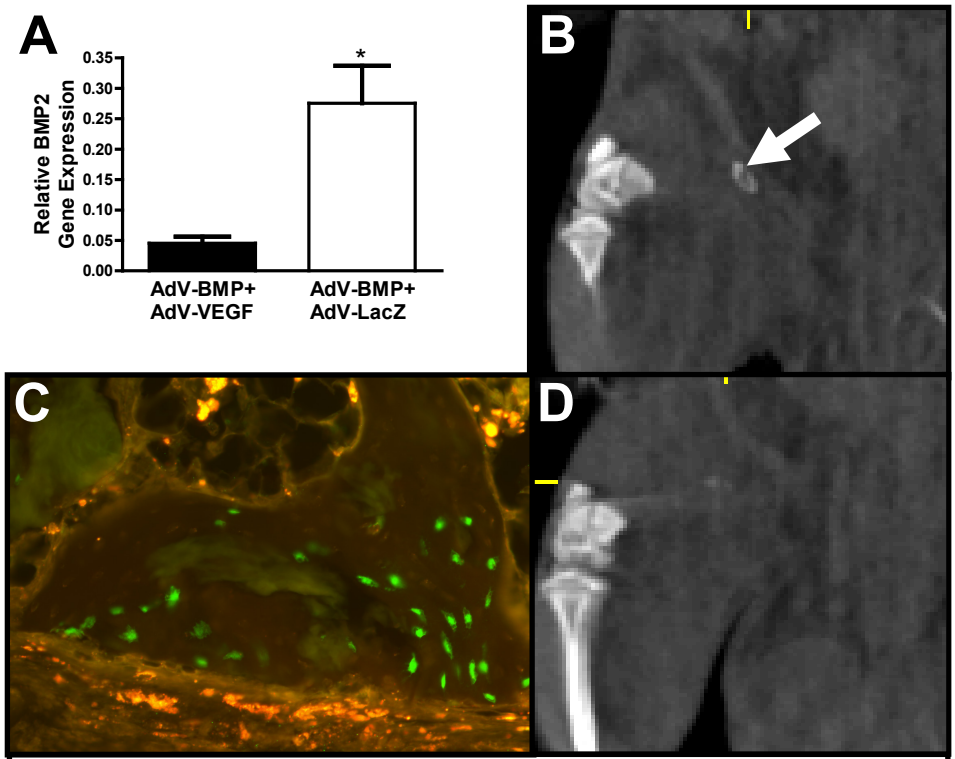

Figure 7. Mesenchymal stem cells were dually transduced with adenoviruses (AdV) coding for BMP2 and VEGF or BMP2 and LacZ. BMP2 gene expression was inhibited by simultaneaous VEGF expression **(A)**. Dually transduced GFP-positive cells were grown on a collagen scaffold and implanted in rats. Constructs transduced with AdV-BMP2 and AdV-LacZ formed bone (arrow) after four weeks **(B)**, and the transplanted GFP-positive cells were found within the generated osseous tissue **(C)**. Little or no bone was formed in constructs transduced with AdV-BMP and AdV-VEGF **(D)**. Results are presented as mean ±SD. *p <0.05

Paper IV

The periosteum is an essential structure in viable bone. A tissue engineered periosteum could potentially aid in the repair of bone defects and facilitate skeletal reconstruction. In **study IV**, a periosteum-like graft was generated, using the dermal matrix AlloDerm and seeded bone forming cells. BrdU immunostaining and ALP enzymatic staining showed that osteoblasts readily grew and differentiated on AlloDerm *in vitro*. Furthermore, cells were transduced with adenoviral vectors while growing on AlloDerm and showed convincing expression of GFP and LacZ transgenes. AlloDerm pieces were seeded with $1x10^5$ GFP-positive MSCs and implanted around the adductor muscle in syngeneic mice. Fluorescent microscopy of sectioned implants showed that GFP-positive MSCs gradually decreased in number

over time. Nevertheless, the cells persisted in the tissue for at least three weeks. H&E staining showed that both seeded and unseeded pieces of AlloDerm were infiltrated by mononuclear and polynuclear cells, and successively replaced by fibrovascular tissues. Constructs were fully vascularized by day 14, according to CD34 immunostaining.

AlloDerm constructs were virally transduced to express BMP2 (MOI=100PFU/cell) and again implanted around an adductor muscle in mouse. Three weeks after implantation, CT detected calcified tissues around the muscles of the treated animals **(Figure 8A)**. H&E staining of harvested and decalcified constructs confirmed that the interface between the muscle and the AlloDerm had transformed into trabecular bone **(Figure 8B)**. The tissue engineered periosteal constructs were then used to cover 4mm circular defects in the mandibles of nude rats. Four weeks after implantation, CT imaging revealed that the defects healed in animals treated with constructs composed of AlloDerm and MSCs expressing BMP2 **(Figure 9A)**. The defects did however not heal in animals treated with AlloDerm and MSCs expressing the control gene LacZ **(Figure 9C)**. The CT findings were confirmed with histological evaluation upon harvest **(Figure 9B** and **9D)**.

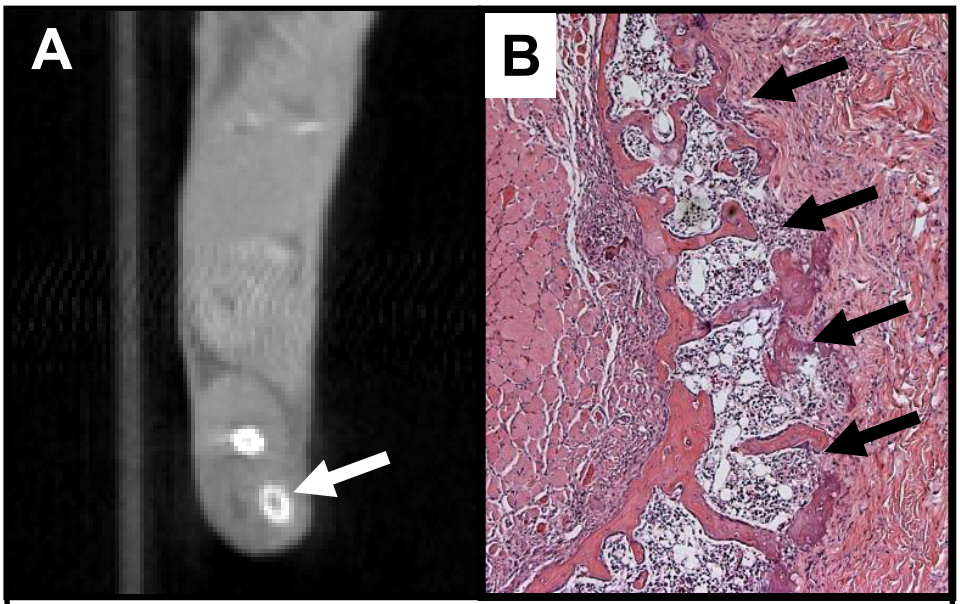

Figure 8. Mesenchymal stem cells were cultured on AlloDerm and transduced to express BMP2. Constructs were implanted around an adductor muscle in mouse. CT-imaging (sagittal plane) showed bone formation (arrow) around the muscle, three weeks after implantation **(A)**. Histology of sectioned specimens confirmed the radiographic findings and trabecular bone formation (arrows) was detected in the muscle-AlloDerm interface **(B)**.

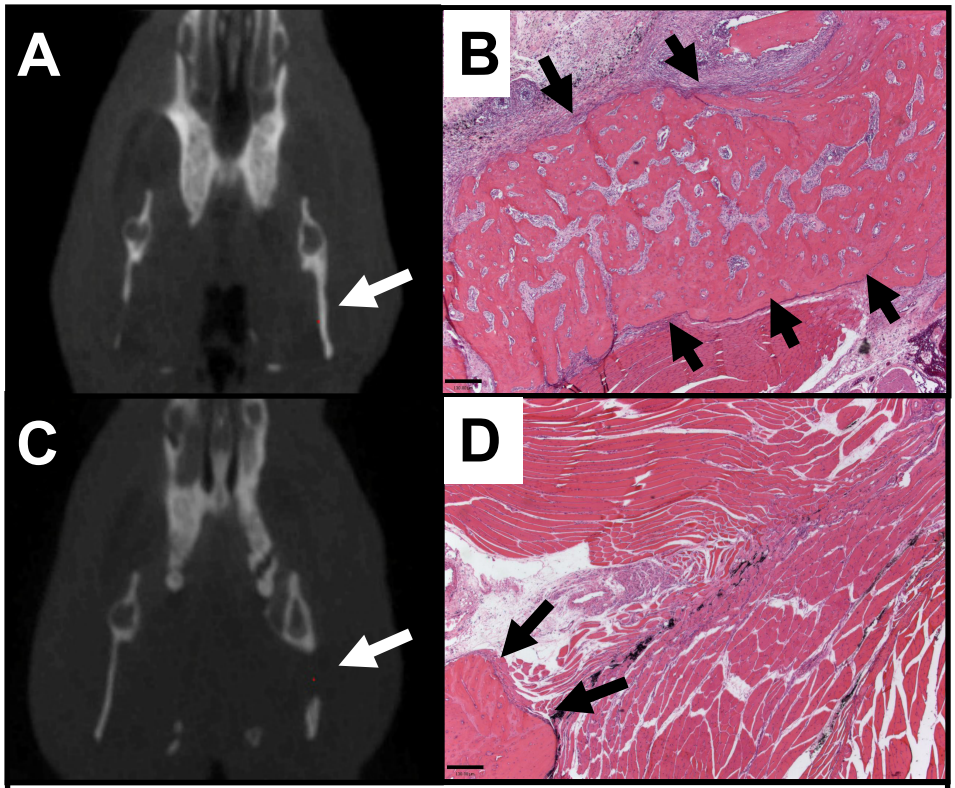

Figure 9. Mesenchymal stem cells were cultured on AlloDerm and transduced to express BMP2. Constructs were used to treat a critical-sized mandibular defect in rat. Defects healed (arrows) in animals treated with constructs composed of AlloDerm and MSCs expressing BMP2, as shown by CT-imaging **(A)** and histology **(B)**. The defects (arrows) did not heal in animals treated with AlloDerm and MSCs expressing the control gene LacZ **(C,D)**.

The importance of a viable periosteum has been underlined by several investigators in the past. Removal of the periosteum has been shown to result in significant attenuation and delay of bone healing [107, 108]. Furthermore, periosteal grafts have been used to aid in fracture repair [109] and vascularized periosteal transfer has resulted in healing of significant bone defects [110-113]. Since there are limited donor sites and potential donor site morbidity, there is, however, no routine practice of periosteal transfer or grafting today. With this in mind, a periosteum-like membrane, containing much of the qualities of a natural periosteum, was generated in **study IV.** The material offered an osteoinducing surface and proved capable of inducing ectopic bone formation as well as healing of a small but critical-sized skeletal defect. The material was furthermore created by simple means and is not limited in size nor associated with donor site morbidity, as are autologeous periosteal grafts.

Discussion

With the ambition to deliver custom made body parts, tissue engineering opens up a new frontier and has the potential to revolutionize reconstructive medicine. However, the concept is still in its infancy and there are many obstacles to overcome. The basic fundaments of this method need to be optimized and new perspectives should be considered, if bone tissue engineering is to offer satisfactory substitutes to autologeous grafts and conventional reconstructive techniques.

Cells

Osteoblasts were used for early attempts at engineering bone but during the past few years, the MSCs have attracted more and more attention. Researchers have favored the MSCs, mainly because of their easy harvest and abundance in several tissues. Furthermore, with the right stimulation, the MSCs are considered to possess an equal bone forming potential as mature osteoblasts. Some researchers even claim that differentiating MSCs prior to use is redundant [114, 115].

Even though many MSC properties have been unveiled lately, there is still much to be discovered. The uniformity among this cell type is uncertain and there is still no established way to fully characterize these cells. One might therefore argue that osteoblasts, due to their homogeneity, are more suitable and reliable for some experimental settings.

Both calvarian osteoblasts and bone marrow derived MSCs were used in the trials presented in this thesis. Because of positive experiences and well established protocols in our lab, osteoblasts were initially used. However, due to the recently gained interest of MSCs and their positive attributes, MSCs was used for later experiments. MSCs were particularly suitable when using GFP-animals and p21 knockout mice. GFP expression proved reliable in the MSC population, and since a large amount of MSCs can be harvested from each sacrificed animal, the transgenic colonies could be kept at a manageable size.

It has previously been reported that the implantation of MSCs can form ectopic bone and aid in the healing of significant bone defects [116, 117]. In **study I, III** and **IV** we, on the contrary, found that the cells seemed incapable of forming bone unless BMP2 stimulation was added. The sole use of MSCs was thus insufficient to heal a critical-sized defect or form ectopic bone. However, the amount and density of transplanted cells is likely to be a critical factor, and this might very well explain differences in various studies regarding transplanted cell contribution.

The GFP-labeling of transplanted cells in **study I, III** and **IV** enabled us to locate the cells at various time points after implantation. We noticed in all *in vivo* experiments that the implanted cells appeared less spread out and decreased in number over time.

What the cell clustering and uneven distribution represents are at this point uncertain, but cell migration or clonal survival and expansion are possible explanations. If the latter is true, the uniformity among MSCs could be questioned.

The gradual diminution of cells in all of our *in vivo* trials indicates that the cells need additional stimuli to survive. Interestingly, when BMP was added (**study III** and **IV**), cell survival seemed improved and cells were found incorporated in the formed bone four weeks after implantation. This suggests that a threshold or lower limit of BMP expression is necessary for survival and incorporation of implanted MSCs at ectopic sites. The fate of transplanted MSCs has at this point not been fully elucidated, but most investigators report of reduction of implanted cell number over time [114, 118, 119]. One may therefore question to what extent implanted cells can contribute to new tissue formation unless their *in vivo* survival and lifespan is improved. Previous studies have found that the addition of growth factors without concurrent cell transplantation can be enough to create bone and promote healing [24, 25]. Others have however shown that in some models bone defects will not heal unless both cells and growth factors are added [116]. Thus, further understanding of delivered cell behavior, survival and contribution is very relevant for this field of research and these issues could be a future target for improvement. Furthermore, little is known of the quality and mechanical properties of bone created by tissue engineering methods. Therefore, the quantity of created bone should not be the only focus when evaluating the contribution of various components involved in bone tissue engineering.

Growth factors

BMP

Although FGF-2 [120], PDGF [121] and TGF-β [122, 123] have been used with some success, the BMPs are believed to possess the most potent bone forming potential among the various factors used for bone tissue engineering. 20 subclasses belonging to the BMP family have at this point been isolated and many of them are considered essential for bone formation and skeletal patterning [124]. BMP2 and BMP7 have been approved by the Food and Drug Administration for clinical use in conditions involving delayed skeletal union.

BMP2 is known to stimulate osteoblastic differentiation and has been used to induce ectopic bone formation and promote fracture healing [125-127]. BMP2 has thus proven suitable for tissue engineering purposes in the past and was selected for use in **study III** and **IV**. The addition of BMP2 proved critical for ectopic bone formation in these studies and was similarly significant for healing of a critical-sized bone defect. As mentioned above, cell transplantation without BMP stimulation was not sufficient to form bone or heal the created skeletal defect.

VEGF

VEGF is likely to be the most recognized angiogenic growth factor and its importance for vascular development and maintenance is well documented [56].

Elevated levels of VEGF will elicit a potent angiogenic response and enhance the vascularity of delivered constructs [57-60]. VEGF is believed to play an important part in osteogenesis as well. Previous studies demonstrate that mineralization of the endochondral mold and osteoblast infiltration is related to VEGF expression and increased vascular ingrowth [128-131]. In **study III**, we hypothesized that elevated levels of VEGF would stimulate angiogenesis within a graft and that VEGF in combination with BMP2 would augment bone formation. An unexpected interaction between VEGF and BMP2 was, however, discovered. VEGF was found to inhibit the BMP2 expression and consequently stem cell differentiation and *in vivo* bone formation. Thus, even though the initial aim of this study was not reached, an interesting biologic phenomenon was highlighted and perhaps one step was gained towards full understanding of the complex physiology behind natural bone formation and development. The relationship between the BMPs and VEGF is indeed multifaceted and the levels of VEGF vary during the bone forming process. On the one hand, proliferating chondrocytes in early osteogenesis secrete angiogenic inhibitors and reduction of VEGF signaling leads to a dose-dependent increase in epiphyseal growth plate area [132-134]. On the other hand, mineralization of the endochondral mold and osteoblast infiltration has been shown to be closely related to increased VEGF expression and vascular ingrowth [103, 128, 135, 136]. Consequently, it is probable that bone forming and angiogenic growth factors interact in feedback mechanisms and that neither a constant over or under expression of VEGF during bone formation and healing is beneficial. Furthermore, angiogenesis and VEGF response precede BMP secretion in normal bone healing, emphasizing the occurrence of various phases in growth factor secretion and concentration during the healing process [137]. Generation of vascular bone probably requires careful optimization of added growth factors, in terms of both dosage and timing. Much like the timing of vascular ingrowth seems pivotal in natural bone formation, a sequential or multiphase expression of angiogenic factors is perhaps preferable in a tissue engineering setting.

Means of delivery

Viral transduction was used to enhance gene expression of BMP2 and/or VEGF among implanted cells **(study III** and **IV)**. Induced gene expression by viral transduction results in continuous growth factor delivery at the site of implantation. This, in turn, leads to stimulation of both native and delivered cells. Various viral vectors can however be used as a vehicle for transduction. We chose an adenoviral vector, since this vector has proven reliable in the past [138]. Furthermore, in contrast to retro- and lentiviral transduction, the replication deficient adenovirus will only have a transient effect [139]. This minimizes the risk for adverse outcomes resulting from long-term expression. Prolonged expression of BMP could lead to unpredictable and excessive bone formation, and VEGF over-expression is known to result in abnormal and unfavorable vascular characteristics [140]. Non-viral vectors could be considered for gene delivery as well, but their potential use is limited by the fact that cellular uptake is estimated to be approximately 10^{-9} that of viral vectors [141].

The concept of dual transduction is a virtually unknown field. According to **study III**, multiple recombinant genes can be expressed simultaneously using viral vectors.

The specific combination of transduced genes seems, however, to have a great impact on the level of expression of each individual gene.

Scaffolds

Hydroxyapatite

Hydroxyapatite is advantageous as a scaffold in some senses, as it is a strong material with the structural integrity to stabilize a skeletal defect. The material needs however to be replaced by native bone over time. This could be accomplished by enhancing the materials bioactivity and increasing the number of bone forming cells at the site of implantation. However, since hydroxyapatite is an inorganic material, it lacks natural binding sites and is a less optimal vehicle for cell delivery. Previous reports have indicated that cell interactions with hydroxyapatite can be improved by coating the material with serum proteins and extracellular matrix molecules. **Study I** aimed to supplement and clarify inconsistencies in previous findings. The combination of FN and FCS improved cell attachment on the hydroxyapatite scaffold manifold compared to uncoated controls *in vitro*. The effect was less evident *in vivo* but both coated and uncoated scaffolds proved capable of delivering cells *in vivo*. However, the delivered cells declined in number over time and no signs of bone formation could be detected in either group. Thus, the *in vivo* part of the study indicates that further manipulations of hydroxyapatite are required to make the material suitable for cell delivery.

There have been efforts to create scaffolds composed of both inorganic and organic components. For instance, synthetic and natural degradable scaffolding materials have been combined with hydroxyapatite crystals to form composites for bone tissue engineering purposes. Even though such scaffolds have been reported to support cell attachment and biointegration, they have yet failed to match the mechanical strength of natural bone [142].

Collagen scaffolds and AlloDerm

In **study II-IV,** we used collagen and AlloDerm as scaffolds and cell carriers. These materials are composed of organic molecules that are naturally found in the extracellular matrix and thus known ligands to the cells integrins [143]. They would therefore, in theory, support cell attachment in a convincing manner. When these scaffolds were used, the implanted cell number was still reduced over time but in a less drastic manner compared to the hydroxyapatite trial. When AlloDerm was used as a carrier, there were a substantial amount of transplanted cells found in the wound after 2 weeks, even without BMP stimulation. This could be compared with just a few dispersed cell clusters found in the porous hydroxyapatite discs, ten days after implantation. The major down-side to using collagen or AlloDerm as scaffolding materials is the lack of mechanical integrity to stabilize a bone defect.

AlloDerm have unique characteristics as it is a thin pliable sheet rather than a conventional 3-D scaffold. This feature allows for cell and growth factor delivery

over a large surface area. Furthermore, by enveloping a bone defect, AlloDerm can serve as a barrier membrane against scar tissue. This could be of importance, since it has been shown that infiltrating fibrous tissue may hinder osseous healing [144-146]. Both absorbable and non-absorbable materials have previously proven successful in preventing scar tissue from interfering with bone healing [144-149]. Some of these materials are however associated with inflammation, fibrous capsulation, early exposure and infection [147, 150-154]. AlloDerm, on the other hand is well tolerated, rapidly revascularized and has a low rate of infectious complications [61, 62].

Graft formation

By considering and optimizing cells, scaffolds and growth factors, the current limitations of bone tissue engineering could be minimized. The main obstacles to overcome are the need for swift production times as well as the establishment of a vascular network within the forming construct. Reducing the timeframe for cell expansion and graft formation is an essential step when aiming to provide a tissue engineered alternative to conventional reconstructive techniques. The requirement of a reliable blood supply is perhaps even more important, since a functioning vascular network is a prerequisite for formation of strong and viable bone.

Construction time

Time and timing are crucial elements in reconstructive surgery. Immediate reconstruction after tumor surgery or trauma is usually preferred, since secondary surgery is complicated by scarring, fibrosis and distorted anatomical land marks. Furthermore, a delayed procedure could involve additional risks and a less beneficial result, due to prolonged open wound care and hospitalization. Thus, a created replacement tissue needs not only to be refined and individualized, it also requires a fast and efficient manufacturing process. Previous efforts to create bone or heal skeletal defects by tissue engineering principals have generally required a timeframe of several months. For example, Mesimäki and colleagues, who recently created a bone graft for maxillary reconstruction, allowed eight months for the construct to form [6]. Similar durations for graft formation have been reported by Arnander et al [25]. Timeframes like these can only be accepted in a minority of clinical cases and it is therefore of great importance to increase the rate of graft production. Limiting steps include cell expansion and differentiation as well as *in vitro* or *in vivo* formation of the actual construct. These processes could in theory be enhanced by various approaches. The basic fundaments of tissue engineering, e.g. selection and delivery of cells and growth factors, could be optimized and managed in various ways. Furthermore, manipulations can be performed on a cellular level, making cell function and proliferation more effective.

In **study II,** the focus was set on direct manipulation of cell proliferation and growth. There are previous reports of such efforts, where MSCs have been stimulated to expand at a faster pace, using exogeneous FGF-2 and TGF-β gene transfer [155-157]. In **study II,** MSC proliferation was accelerated in a 2-D and 3-D culture by inhibiting

p21 gene expression, using siRNA. Chiou and colleagues found that FGF-2 supplementation resulted in a 1.6-fold increase in cell number after one week compared to untreated controls. Pri-Chen et al. increased the MSC number by a factor 2.4 after two weeks under similar conditions [155, 157]. In comparison, we found a threefold increase in cell number after only six days, using p21 siRNA. Furthermore, MSCs plasticity seemed retained after the p21 inhibition. Thus, short-term alterations of p21 seem to be an efficient way to enhance stem cell expansion, and potentially graft formation and *in vivo* cell survival as well.

Vascularization

Stimulating ingrowth of blood vessels and establishing a vascular network within an implanted scaffold is essential for bone formation and graft integration. In a physiologic setting, native cells are found within 200μm of the nearest capillary (the diffusion limit for oxygen) and similar conditions for delivered or cultured cells are desirable [158]. Improving the conditions for vessel formation and ingrowth as well as the potential for microvascular transfer could be achieved by implanting the forming graft near an existing vessel or pedicle. This strategy along with an attempt to optimize the levels of angiogenic growth factors was used in **study III**. A collagen scaffold, carrying virally transduced MSCs was attached to a distally ligated arterio-venous bundle upon implantation. After four weeks, trabecular bone had formed around the viable pedicle. This method thus seems to be one way to optimize construct vascularization and the conditions for microsurgical transfer. The viability and functionality of a ligated pedicle used in such manner has been investigated previously. Interestingly, this method has been suggested as more suitable, in terms of enhanced graft vascularization, compared to incorporation of an arterio-venous shunt loop [159].

It has recently been shown that endothelial cells, seeded and grown on a scaffold can form primitive vascular networks *in vitro* [28, 160-162], and that these vascularized constructs will form functional anastomosis with the host, when implantated *in vivo* [163]. Future studies should focus on refining or combining the above mentioned strategies for construct vascularization.

It is however worth noting that the periosteum-like graft, created in **study IV,** to some extent circumvents the demand for rapid vascularization. This thin construct is preferably used as an envelope rather than a void filler. The large surface area enables substantial cell and growth factor delivery without an instantaneous need for vascular supply. Due to the cell carrying surface's immediate contact with the host upon implantation, the delivered cells are likely to receive adequate levels of oxygen and nutrients through tissue diffusion.

Conclusion

The presented studies highlight important aspects and current limitations of bone tissue engineering and tissue engineering in general. Valuable insights were gained regarding potential scaffolding materials and further understanding of cell delivery, manipulation and behavior was obtained. A new approach was introduced, making the process of bone formation and regeneration more time efficient. Furthermore, a novel strategy to induce bone formation through the use of a manufactured periosteum was presented.

More specifically,

1. By coating hydroxyapatite with FN and serum, the material's qualities as a cell carrier were improved *in vitro*. Similar trends could be observed *in vivo*, but the effect was less evident and not statistically significant.

2. MSC expansion was accelerated in 2-D and 3-D culture through manipulation of p21 expression, using siRNA. Furthermore, p21 inhibition was performed without adverse effects on MSC plasticity.

3. An interesting and unexpected interaction between BMP2 and VEGF was discovered, where VEGF was found to inhibit simultaneous BMP2 gene expression in rodent MSCs.

4. A tissue engineered periosteum was created, capable of delivering cells and osteoinductive proteins. The manufactured periosteum was successfully used to create ectopic bone and to treat a critical-sized bone defect in rat.

Future directions

There are still many obstacles to overcome before bone tissue engineering constitutes a reliable option for osseous reconstruction. The production of an engineered construct needs to be fast and efficient enough to offer an alternative to immediate osseous reconstruction with autologeous grafts. The outcome of **study II** is promising but the results need to be confirmed *in vivo,* and further analyses are required to eliminate any risk of tumorigenesis. It is moreover likely that additional acceleration of the engineering process is required and that other refinements and new approaches need to be introduced as well.

The necessity of adequate delivery of oxygen and nutrients to cells within a forming construct also requires additional focus. By combining multiple strategies for enhanced vascular ingrowth and vessel formation, the vascularity within a generated graft could be improved. Such strategies include optimization of delivered growth factors, surgical incorporation of vessels and preseeding constructs with endothelial cells. **Study III** emphasizes the need for full understanding of the angiogenic process and underlines the fact that the addition or expression of multiple growth factors can lead to unexpected effects and interactions. This is a valuable insight and further understanding of the combined effect of various growth factors could be used to optimize one or several physiological processes, including angio- and vasculogenesis.

The periosteum created in **study IV** is a novel and promising method for osseous reconstruction. There are several possible study designs for the future, aiming to further investigate the potentials and limitations of this graft. To what extent larger bone defects could be restored using this technique should be determined. The potential to use the periosteum together with other grafts and bone substitutes could also be considered as well as its use for fracture healing and bone augmentation. Additionally, the created periosteum could perhaps be used for applications apart from bone tissue engineering. Other areas of regenerative medicine could benefit from a thin graft, with a large surface area for cell and growth factor delivery. Furthermore, local delivery of drugs and stem cells has recently been in focus, for treatment of a number of disorders, including neurological diseases, articular maladies, cancer and infections, making various means of delivery of high interest [164-171].

Populärvetenskaplig sammanfattning

Skador på skelettet kan uppstå efter bl.a. olyckor, tumörkirurgi eller strålning. Ofta är sådana skador mycket handikappande då de påverkar kroppens funktion, stabilitet och utseende. Om benvävnad saknas eller måste bytas ut använder man sig idag av mikrokirurgiska metoder, där man flyttar kroppseget ben från ett ställe till ett annat. Tyvärr innebär detta att man skapar en ny bendefekt, om än på ett mindre känsligt ställe. Det finns inte heller alltid rätt storlek, form och kvalitet av ben att tillgå och därför skulle det vara ett stort framsteg om man kunde framställa skräddarsydd benvävnad på annat vis. Idag finns förhoppningar om att kunna konstruera nya skelettdelar, i eller utanför kroppen, med hjälp av kroppsegna celler tillsammans med lämpliga bärarmaterial och stimulerande tillväxtfaktorer. Ett fåtal utvalda patienter har genomgått rekonstruerande kirurgi med hjälp av dessa principer men det finns fortfarande alltför många brister och frågetecken för att kunna använda tekniken rutinmässigt. Bland annat är den benbildande processen mycket tidskrävande, vilket innebär försenade rekonstruktioner efter traumatiska skador och tumörkirurgi. Därtill är storleken och kvaliteten på det bildade benet bristfälliga eftersom benet initialt saknar fungerande blodkärl och en etablerad blodförsörjning.

Detta projekt har haft för avsikt att optimera och effektivisera den benbildande processen, att förbättra förutsättningarna för kärlförsörjning av det bildade benet samt att introducera nya koncept och perspektiv inom området.

I det första delprojektet påvisades att det går att odla benbildande celler på materialet hydroxyapatit och att man därigenom kan använda sig av materialet som cellbärare. Hydroxyapatit är ett kalciumfosfat som liknar den icke-organiska komponenten i naturligt ben och har med viss framgång används som bensubstitut. Resultaten från den första studien innebär att celler kan transplanteras tillsammans med hydroxyapatit och därmed underlätta att materialet läker in och ersätts av ben. Vidare konstaterades att cellernas förmåga att fästa till och växa på hydroxyapatit till viss del kan förbättras då man förbehandlar materialet med cellvänliga molekyler.

I den andra studien förbättrades stamcellers tillväxthastighet genom att en bromsande gen (p21) tillfälligt blockerades med hjälp av s.k. inhiberande RNA. Resultaten från studien visade bland annat att cellernas antal i den experimentella gruppen efter en vecka var cirka tre gånger högre än bland kontrollerna. Dessa fynd utgör en modell för hur man tidsmässigt kan effektivisera den benbildande processen genom att cellernas delningshastighet ökas.

I den tredje studien manipulerades stamceller med hjälp av virus till att öka utsöndringen av benbildande och kärlstimulerande tillväxtfaktorer. En intressant interaktion upptäcktes. Den kärlstimulerande faktorn VEGF visade sig hämma stamcellernas förmåga att producera den benstimulerande faktorn BMP2. Detta ger ny insikt i cellers förmåga att utsöndra flera tillväxtfaktorer samtidigt och

understryker vikten av att beakta potentiella interaktioner mellan tillväxtfaktorer i dessa sammanhang.

I det sista delarbetet konstruerades ett benhinneliknande material. Alla kroppens ben omges av en benhinna, som innehåller benbildande celler och utgör en fysisk barriär mot omkringliggande vävnad. Den skapade benhinnan framställdes genom att stamceller, som genetiskt manipulerades till att utsöndra den benstimulerande faktorn BMP2, odlades på ett tunt bärarmaterial. Det cellbärande materialet visade sig kunna inducera bennybildning i muskel på mus och bidrog på ett avgörande sätt till läkning av en skapad bendefekt i käke på råtta.

Tillsammans har dessa studier lett till viktiga insikter om cellers beteende och andra processer som är involverade då man framställer ben med hjälp av vävnadsteknik. Flera av fynden kan leda till nya studier och har potential till utveckling av kliniskt användbara metoder och produkter.

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