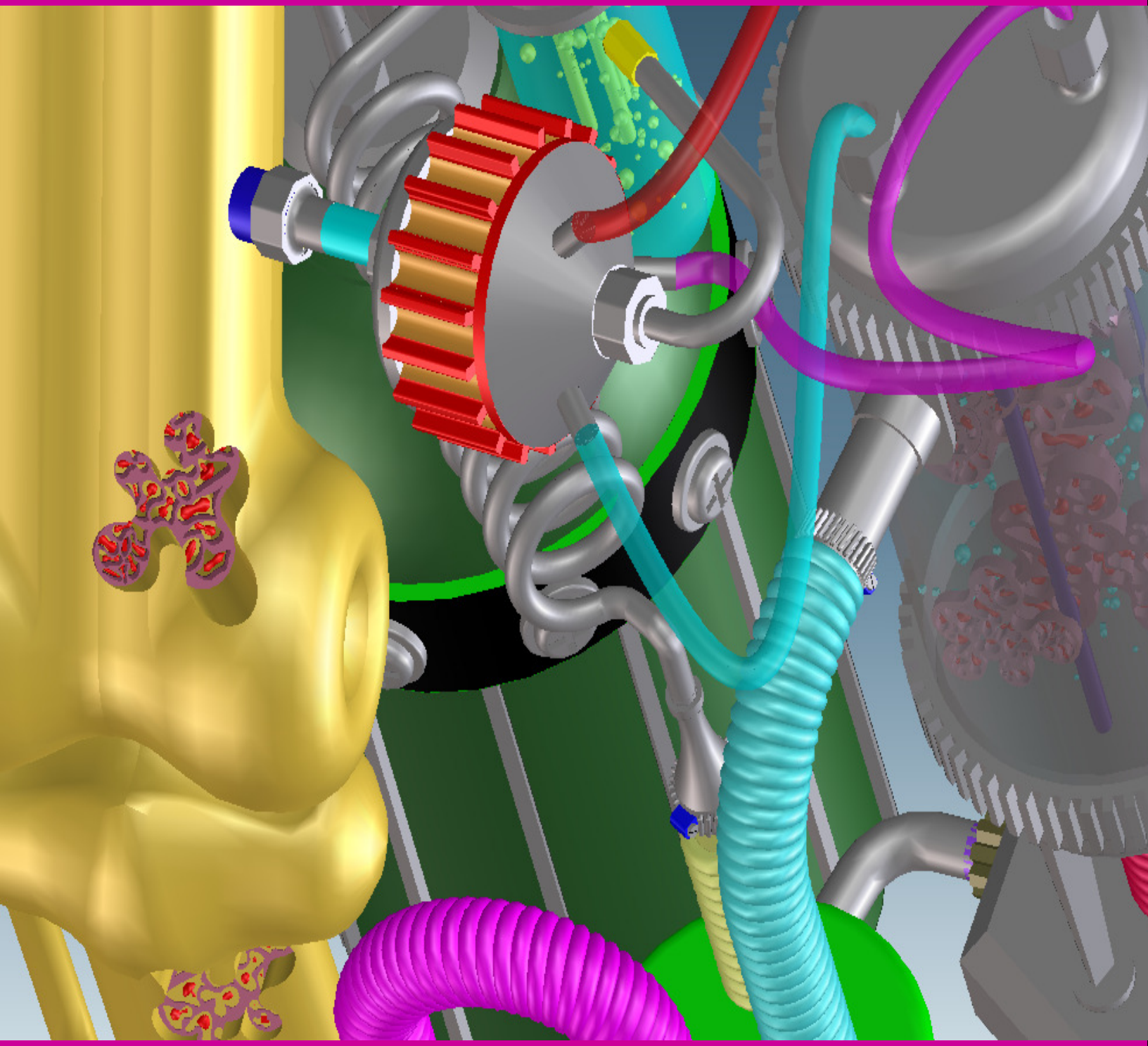
A BIOREACTOR SYSTEM FOR CLINICALLY RELEVANT BONE TISSUE ENGINEERING



FRANK JANSSEN

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A bioreactor system for clinically relevant bone tissue engineering

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CHAPTER 1

“The empty bodies stand at rest
Casualties of their own flesh
Afflicted by their dispossession
But no bodies ever knew
Nobodys
No bodies felt like you
Nobodys
Love is suicide”

Bodies- Smashing Pumpkins.
Picture: Feed Bodies (www.makersofuniverses.com)

CHAPTER 1

GENERAL INTRODUCTION AND AIMS

BONE

The most obvious function of bone is to provide a structural and mechanical support for the human body and the protection of vital organs. It also functions as attachment side for muscles and tendons and it is the major organ for calcium homeostasis and it stores phosphate, magnesium and potassium. Finally, bone also plays an important role in blood production, pH regulation of the body by bicarbonate balancing and sound transduction (1,2).

Bone structure and function

Two types of bone found in the body; *cortical* and *trabecular* bone. *Cortical bone*, also called compact bone, is dense, rigid and compact and it comprises 80% of the total bone mass. It plays a major role in mechanical support and forms the outer shell of the long, flat and small bones. *Trabecular bone* also called cancellous bone makes up the inner layer of the bone and has a spongy, honeycomb-like structure. The spaces between the trabecular meshwork are occupied by bone marrow. It is less dense than cortical bone but has a large surface area and has a higher metabolic activity (3,4).

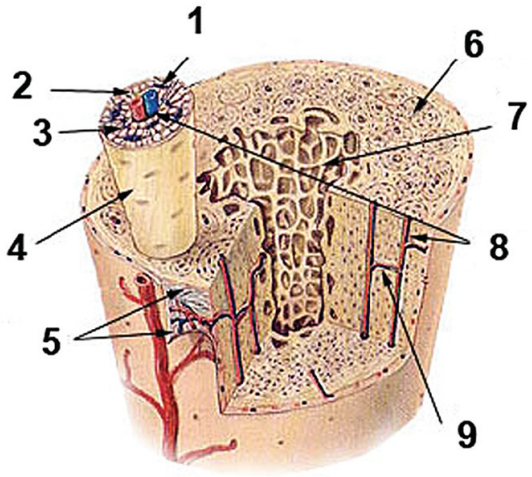

Figure 1*. Anatomy of a long bone showing both cortical and trabecular bone. The osteons of cortical bone are displayed (6), including the Haversian channels (8) that contain blood vessels and nerves. Apart from that, the periosteum (5), which is a highly vascularized membrane that covers the bone surface, can be seen. Other visible structures include the lacunae containing osteocytes (1), lamellae (2), canaliculi (3), osteons (4), trabeculae of spongy bone (7) and Haversian canals (9). Adapted from a figure by the U.S. National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) Program (http://training.seer.cancer.gov/index.html)*

According to the pattern of collagen formation, two types of bone can be identified. *Woven bone* is characterized by a haphazard organization of collagen fibers and is mechanically weak. Woven bone is created when osteoid (a nonmineral matrix of collagen and noncollageneous proteins) is rapidly produced by osteoblasts. This

occurs initially in all fetal bones, but the resulting woven bone is replaced by remodeling and the deposition of more resilient *lamellar bone*. In adults, woven bone is formed when there is very rapid new bone formation, as occurs in the repair of a fracture. Following a fracture, woven bone is remodeled and lamellar bone is deposited. *Lamellar bone* is characterized by a regular parallel alignment of collagen into sheets (lamellae) and is mechanically strong Virtually all bone in the healthy mature adult is lamellar bone (1,7).

On a molecular level, calcified bone contains about 30% organic matrix (2-5% of which are cells), 10% water and 60% inorganic mineral (5). The organic matrix is a well organized network of proteins consisting mainly of collagen type I. It is responsible for the tensile strength of bone. The non collagenous proteins include osteonectin, osteopontin, bone sialoprotein, osteocalcin, decorin and biglycan. The mineral part of bone provides the hardness and rigidity of bone is due to the presence of calcium phosphates, from which hydroxyapatite is the main component (6,7).

There are four different cell types in the organic matrix which are associated with the production, maintenance and (re)modeling of bone. The bone tissue resorbing *osteoclasts*, bone matrix producing *osteoblasts* which can differentiate into the matrix embedded *osteocytes*. The osteoblasts can also differentiate into *bone lining cells* which are resting cells situated on the bone surface (8).

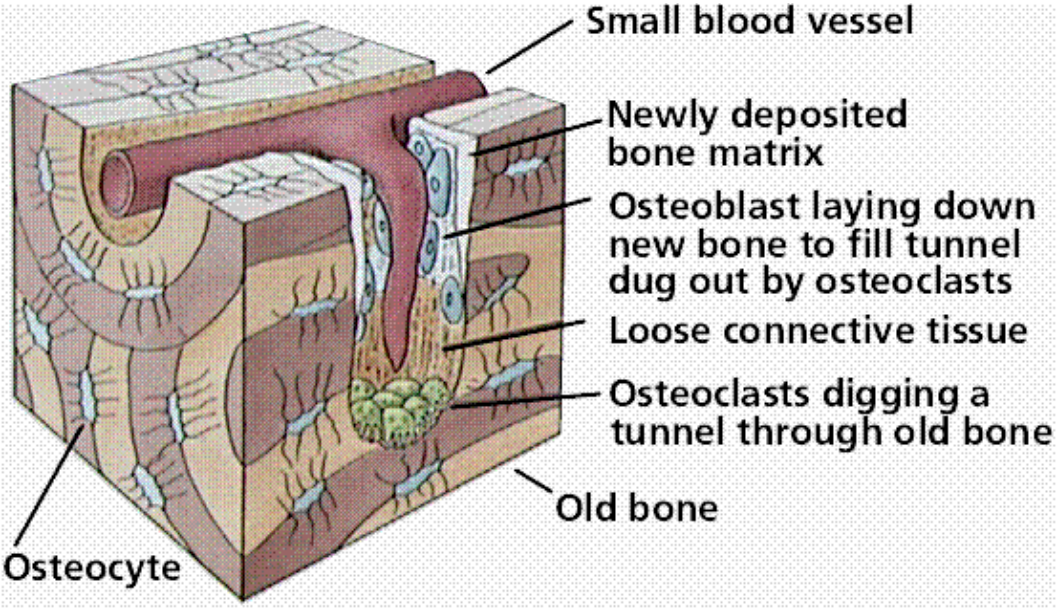

Figure 2. *Cell types present in the organic matrix of bone. Osteoblasts, osteoclasts and osteocytes can be distinguished*

Osteoblasts are cells that are derived from mesenchymal stem cells and are responsible for bone matrix synthesis and its subsequent mineralization. In the adult skeleton, the majority of bone surfaces that are undergoing neither formation nor resorption (i.e., not being remodeled) are lined by bone lining cells. These *bone lining cells* originate from osteoblasts and regulate the calcium balance of the bone (9). They respond to hormones by producing specific proteins that activate osteoclasts. Osteocytes are the most abundant type of cells found in the adult skeleton. They are formed from differentiated osteoblasts that become incorporated within the newly

formed osteoid which eventually becomes calcified bone. *Osteocytes* situated deep in bone matrix maintain contact with newly incorporated osteocytes in osteoid. They also keep in contact with osteoblasts and bone lining cells on the bone surfaces, through an extensive network of cell processes (canaliculi). They are thought to be ideally situated to respond to changes in physical forces upon bone and to transduce messages to the osteoblastic cells on the bone surface, directing them to initiate resorption or formation responses (10). *Osteoclasts* function in resorption of mineralized tissue and are found attached to the bone surface at sites of active bone resorption. These cells are large multinucleated cells, like macrophages, derived from the hematopoietic lineage. Their characteristic morphological feature is a ruffled edge where active resorption takes place with the secretion of bone resorbing enzymes, which digest the bone matrix (11).

Bone (re)modeling and repair

During the development of the skeleton and with maintenance and repair of bone, two different mechanisms can be distinguished. These are *intramembranous* and *endochondral* ossification. I*ntramembranous ossification* involves the replacement of connective tissue membrane sheets with bone tissue and results in the formation of flat bones (e.g., skull, clavicle, mandible). *Endochondral ossification* involves the replacement of a hyaline cartilage model with bone tissue (length increase of long bones e.g., femur, tibia, humerus, radius). Bone is a complex dynamic tissue that is constantly being *modeled* and *remodeled* during our life. *Bone modeling* is when bone resorption and bone formation occur on separate surfaces (i.e., formation and resorption are not coupled). An example of this process is during long bone increases in length and diameter. Bone modeling occurs during birth to adulthood and is responsible for gain in skeletal mass and changes in skeletal form (12). *Bone remodeling* is the replacement of old bone tissue by new bone tissue which mainly occurs in the adult skeleton to maintain bone mass. This process consists of an activation phase, bone resorption, a short reversal phase and finally bone formation (fig 3). During resorption, old bone tissue is broken down and removed by osteoclasts. During bone formation, new bone tissue is laid down to replace the old. This task is performed by osteoblasts. Osteoclast and osteoblast functions are regulated by several hormones including calcitonin, parathyroid hormone, vitamin D, estrogen (in women) and testosterone (in men).

With respect to bone repair, both intramembranous as well as endochondral bone formation play an important role. When for instance a fracture occurs, an area of cell death (necrosis) will be formed. Before any repair can take place, this area has to be cleaned. After this inflammatory phase is over, initial stabilization is realized by cartilage (soft callus) production. Then, this cartilage is replaced by bone as in encochondral bone formation. Simultaneously, (direct) intramembranous bone formation can be found depending on the local oxygen supply. Only when a fracture is stable and with unchanged anatomy intramembranous repair alone will be sufficient. After the repair phase, the remodeling phase follows, comparable to nonfractured bone (13).

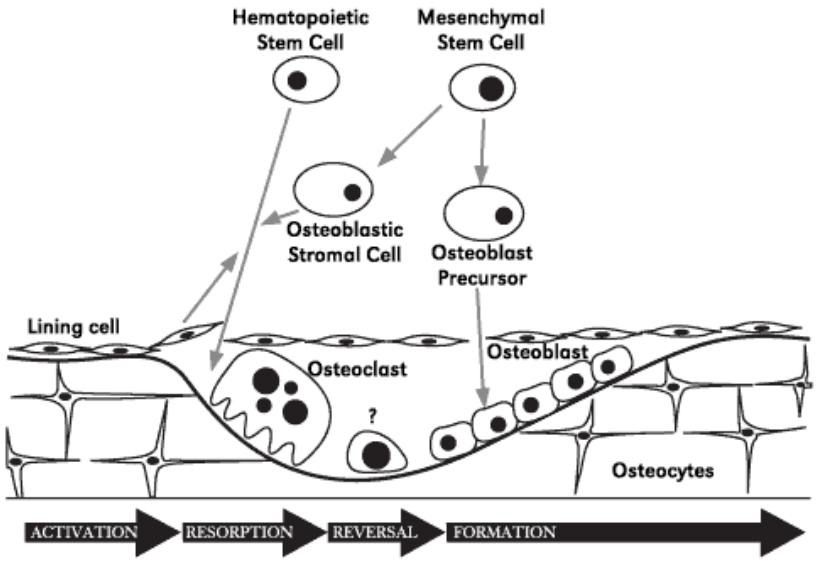

Figure 3. *Schematic representation of the Bone remodeling process. The sequence of activation, resorption reversal and formation is illustrated in figure 3. The activation step depends on cells of the osteoblast lineage, either on the surface of the bone or in the marrow, acting on hematopoietic cells to form bone-resorbing osteoclasts. The resorption phase may take place under a layer of lining cells as shown here. After a brief reversal phase, the osteoblasts begin to lay down new bone. Some of the osteoblasts remain inside the bone and are converted to osteocytes, which are connected to each other and to the surface osteoblasts.*

Need for grafting material

As described before, bone has the intrinsic ability to heal itself when it has been damaged. However, there is still a large group of patients that need surgical interventions, in which additional bone is required for optimal recovery. Patients suffering from extensive bone trauma (i.e. accidents or removal of a bone tumor), infection or congenital disease belong to this group. All these patients can potentially suffer from a critical size defect, which is defined as the smallest size intra-osseous wound that will not heal spontaneously during a life time (14). In this case, the bone is not able to bridge the existing lesion by natural repair. When this bridging does not occur the defect will be filled with fibrous tissue which impairs the structural stability of natural bone.

Other clinical indications requiring additional bone tissue for optimal recovery are spinal fusion and hip revision surgery. Spinal fusion is a surgical procedure which is performed with increasing frequency for many orthopedic and neurological indications. In this procedure, two or more of the vertebrae in the spine are united together so that motion no longer occurs between them. Examples of medical indications are degenerative disc disease, spinal stenosis, spondylolisthesis, fractures and tumors. With regard to bone grafts, it can be calculated that in 2004 about 500,000 bone graft procedures (in the US and EU) are related to the spine (15). The standard technique consists of combining screw instrumentation or fixation with bone grafting between transverse processes and laminae if available (PosteroLateral Fusion, PLF,). The fusion process typically takes 6-12 months after surgery and a successful fusion is depicted in figure 4.

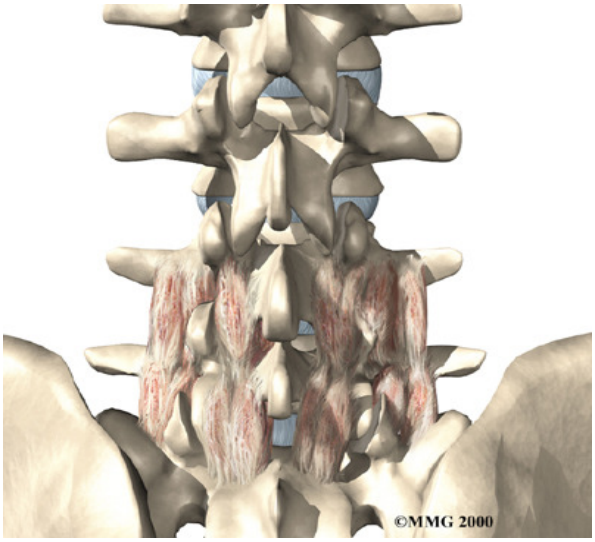

Figure 4. *Schematic representation of a successful PLF of vertebrae L4 and 5 (http://www.eorthopod.com/public)*

Hip revision surgery is another frequently occurring clinical situation where extra bone tissue is needed upon loosening of hip implants. This occurs due to bone resorption (osteolysis) at the interface between implant and the surrounding bone tissue (Figure 5). In order to relieve the patients' pain, surgery is required and ideally the lost bone is replaced with new bone.

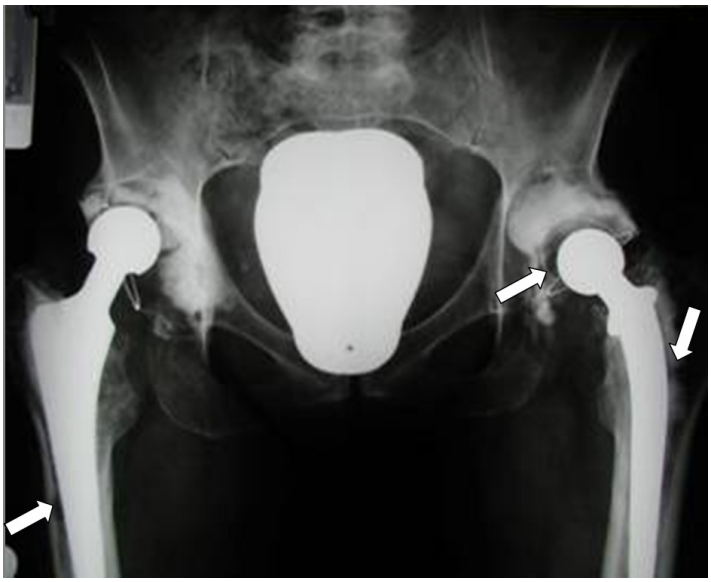

Figure 5. *X-ray of a patient with loose implant. Patients with loose cups and stems. Dark lines around the interface between the cement and bone (arrows) indicate resorption and osteolysis which can occur because of the low bonding strength between titanium (alloys) and bone (16-19).*

All the procedures mentioned above have in common that a considerable amount of grafting material is needed. The autologous bone transplant (autograft) is until today the golden standard in many orthopedic interventions. There are however considerable drawbacks with respect to the use of autograft. In order to acquire the bone graft, an additional surgical site for harvesting has to be created. This invokes the risk of donor site morbidity (20-23), post operative pain (22, 24-26) and infection (23, 26, 27). Furthermore, the availability of autograft is limited which makes spine multi-segments or revision hip arthroplasties untreatable with this source (20,22,23).

An alternative source is the *allogeneic* and *xenogeneic* bone graft (allograft and xenograft). The availability of these sources is generally much higher when compared to autograft. However, immunogenic reactions, poor osteogenic potential and possible disease transfer are related to these sources (28). Current freezing, defatting and lyophilization techniques reduce these risks (29,30), but negatively affect the bone resorption rate and the formation of new bone tissue (22,31). Another approach in order to fill small bone defects is pursued by using *demineralized bone matrix* (DBM) which is widely used in the clinic. DBM is made of cortical bone from which the mineral and cellular components are extracted and consists mainly of collagen. DBM from several species have shown to induce ectopic bone formation, which is mainly caused by bone morphogenetic proteins (BMPs) (32). The readily availability, cost-effectiveness, decreased immunogenicity and relatively low safety risks make DBM an attractive bone graft substitute. However, also DBM has its disadvantages like a preparation and batch dependant osteogenicity. Furthermore, the osteoinductive capacity can be affected by the carrier material mixed with it, since DBM itself provides no structural or mechanical stability (33,34).

Alternatives for human bone grafts

Because of the disadvantages of (human) bone grafts, several alternatives are currently under investigation. The past decades, several natural and synthetic bone graft substitutes have been developed varying from materials like *steel, titanium, coral, bamboo* (35-41). The materials vary in chemical composition and thereby in mechanical and bone bonding properties. Limitations of these materials include poor tissue integration, inability to adapt to the (dynamic) bone environment and the potential need for implant retrieval and/or revision. For load bearing applications, *titanium and titanium alloys* are often used. They are biocompatible and have excellent mechanical properties which make them suitable for these applications (42). One of the drawbacks of these materials is the mismatching between the stiffness or Young's moduli of the biomaterials and the surrounding bone tissue. This can result in insufficient loading of the surrounding bone which can become stress shielded. Eventually, this mechanical mismatch can result in bone resorption and implant loosening as described before (16-19). Therefore, metallic implant designs are focusing on adapting the mechanical properties of metals to those of bone, e.g. by introducing a porous structure and thus reducing the problems associated with stress shielding (43) Although they are widely used in load bearing applications, their ability to bond with bone and their bone conductivity is considerably smaller than *ceramic biomaterials*. These biomaterials, of which glass ceramics and calcium-phosphate ceramics are well known, have in common that they are all bioactive. This means that these materials are capable of forming a very tight bond with the existing bone which typically occurs when an apatite layer can precipitate on the material surface (44,45)

Calcium phosphate biomaterials, with a chemical composition similar to that of bone and teeth mineral are widely used in clinical practice (22). Hydroxyapatite of natural and synthetic origin have been used in applications where a low resorption rate is required for example in spinal fusion. When a high resorption rate is required, tricalciumphosphate (TCP) can be an appropriate biomaterial. It is used in dental applications like filling the gap of periodontal loss as well as repairing cleft pallets. Biphasic calcium phosphate (BCP), which contains both hydroxyapatite and

tricalcium phosphate, combines both physicochemical properties. By varying the content of both compounds, tailor made resorption rates can be obtained. BCP is used clinically for the treatment of patients with scoliosis and for filling defects after tumor resection. In some cases, it has been shown that calcium phosphate biomaterials have the ability to induce bone formation ectopically in vivo (osteoinduction) (46-48). The clinical application of these biomaterials is however limited because of their low mechanical strength and is therefore mostly used in non-load bearing sites.

Another group of bone graft substitutes are represented by *polymers*. A wide variety of these polymers (natural e.g. hydrogels, such as gelatin, agar, fibrin or collagen as well as synthetic bioresorbable polymers e.g. poly lactide/glycolide (PGLA) and polycaprolactone (PCL)) are currently being investigated either as a bone graft substitue or as a scaffold for bone tissue engineering. The mechanical and degradation properties of these polymers can be tailor-made by changing the chemical composition or the fabrication technique (49). Furthermore, some of these polymers are suitable to incorporate bioactive molecules like growth factors, which make them suitable candidates for bone tissue engineering (50). Again, the mechanical properties of these biodegradable biomaterials are generally not suitable in order to use them in load bearing applications. Another disadvantage of these materials is that the osteoconductive and osteoinductive properties are generally less when compared to ceramics.

A group of materials that aims to improve mechanical strength, while retaining osteoconductivity are *composites*. Composites consist of two or more different biomaterials which are combined. For example, the stiffness of calcium phosphate scaffolds can be decreased by combining them with collagen or synthetic polymers while retaining osteoconductive properties (51-53). Another example of hybrid materials is the addition of a calcium phosphate coating to metal implants. This enhances the osteo-integration of the metal, while retaining the favourable mechanical properties (54).

Another important issue, besides the chemical composition, is the three dimensional structure of the scaffold. Interconnected porous structures for example are necessary for bone ingrowth and vascularisation. When using polymers, different 3 dimensional structures can be obtained by using by different processing techniques like solvent casting, salt leaching, 3D printing, rapid prototyping and electrospinning (55). Macro and micro porous calcium phosphate scaffolds can be produced by several different techniques as reviewed by Hertz (56). In addition to the macro structure, the micro structure of biomaterials is also an essential element for osteoinduction (57). In order to overcome the disadvantages of the classical biomaterials, bone tissue engineering has emerged as an alternative approach towards bone regeneration.

BONE TISSUE ENGINEERING (BTE)

New breakthroughs can only be expected from a novel hybrid approach that will reduce the shortcomings of the current material technology. Such a combined, biology driven approach is collectively referred to as “tissue engineering”. The concept of “tissue engineering” was defined by Langer and Vacanti as "an

interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" (58). In practice this term is used for materials or cells replacing or repairing whole or portions of tissues, but the term is also used for substitutes in which cells and materials are combined to create artificial support systems.

Hybrid constructs

A common approach in tissue engineering is the assembly of a hybrid construct consisting of a porous biodegradable matrix or scaffold to which cells can physically adhere. This *in vitro* tissue precursor is often combined with bioactive molecules to stimulate proliferation and/or osteogenic differentiation during the in vitro culture period. Finally, the hybrid construct is implanted into the defect site to induce and direct the growth of new bone as the scaffold material degrades (figure 6).

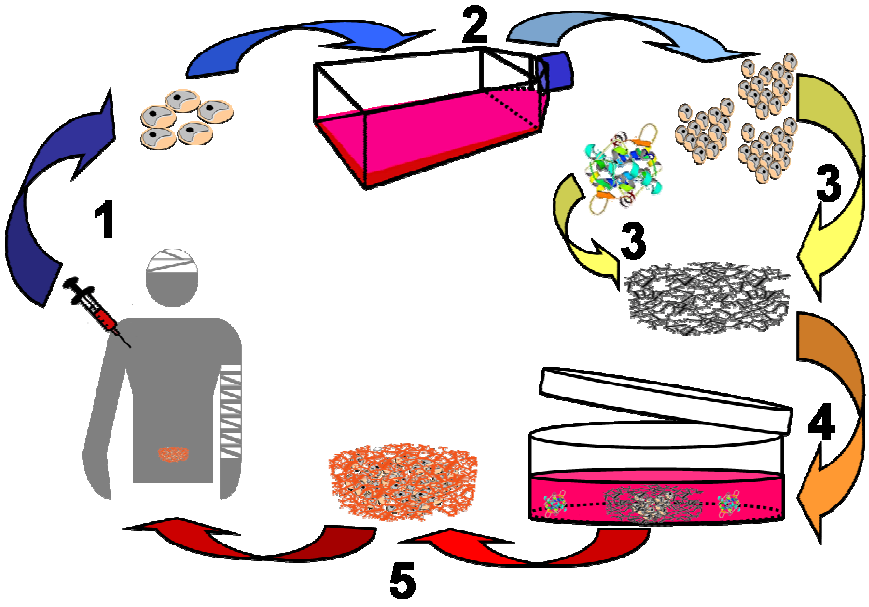

Figure 6. *Cell based bone tissue engineering. 1. A bone marrow biopsy of a patient is harvested and BMSCs are selected by adhesion in tissue culture flasks. 2. Cells are expanded in vitro in tissue culture flasks until a suitable amount is obtained. 3. Cells are combined with a suitable scaffold material, often in the presence of bioactive molecules. 4. Cells are cultured on the scaffold material for a designated period in vitro. 5. The hybrid construct is implanted back into the defect of the patient.*

In the field of bone tissue engineering, biomaterials like ceramics are generally combined with osteogenic cells or osteoprogenitor cells. HA and other calcium phosphate based ceramics are the ones of major interest given their osteoconductivity and their ability to ‘‘integrate’’ with the host bone (59-65). Goshima et al. were the first to demonstrate new bone deposition in porous bioceramic scaffolds seeded with cells, once the constructs were implanted subcutaneously into immunocompromised mice (66-67).

Following that study, several other groups have obtained equivalent results in similar models using BMSC from different species and have shown bone formation both ectopically (68,72) and orthotopically in rodent studies (69-72). Few studies demonstrate this technique in large animal models ectopically (73), orthotopically (74-

76), and even fewer studies compared the functioning ectopically and orthotopically (77). Taking advantage of the immunodeficient mouse model and utilizing the X-ray synchrotron radiation computed microtomography (microCT) and microdiffraction, it was possible to make a qualitative and quantitative evaluation of the performance of different ceramic scaffolds engineered with BMSC, including kinetics of bone formation and scaffold resorption (78-80). The mechanism of bone formation in the tissue engineering approach is not yet fully understood. The new bone could be formed by the implanted cells, or by host cells that are stimulated by the implanted construct, or both as proposed by Goshima et al (81). There is evidence that the implantation of osteoprogenitor cells only has an effect on bone formation if the cells are viable indicating that the implanted cells play an active role in the formation of new bone (73). In order to allow implanted cells to survive at the site of implantation, a suitable nutrient supply and waste disposal needs to be established. Therefore, many attempts have been made to (pre) vascularise the hybrid constructs before implantation. In few cases, the hybrid constructs were implanted to create vascularized bone flaps in an attempt to facilitate vascularization of the newly formed bone (82,83). Whether the active role of cells in hybrid constructs solely comprises the formation of bone by the implanted cells, or also involves the secretion of factors that stimulate bone formation by host cells, remains unknown. In general, positive results have been achieved with osteoprogenitor cells in experimental settings but the effect of the use of these cells in clinical bone defects in humans is still unpredictable (84).

Mesenchymal stem cells

For bone tissue engineering at this moment, the preferred cell source are the mesenchymal stem cells (MSCs). MSCs which are also known as bone marrow stromal cells (BMSCs) or skeletal stem cells (SSCs) were first described by Friedenstein and coworkers who were able to isolate these cells by adhesion selection (85,86).They showed that these MSCs exhibit multipotency and in an impressive series of papers they investigated the in vivo bone forming potential of these MSCs and their potential clinical application (85-89). Many studies show that these cells have the ability to differentiate in vitro into several mesenchymal lineages like adipocytes, osteoblasts, chondrocytes and myoblasts (90-92). Figure 7 shows a graphical representation indicating the multi-potency of these cells.

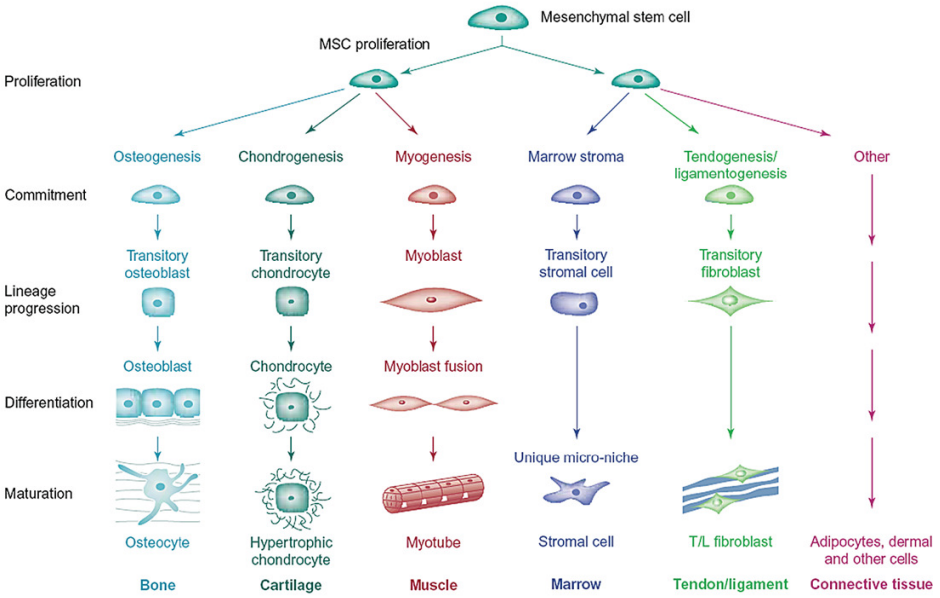

Figure 7. *Mesenchymal stem cells as schematically described by Caplan & Bruder. This figure shows the transitions from the putative mesenchymal stem cell to highly differentiated phenotypes (93).*

Recently, it has been reported that the differentiation capacities of mesenchymal stem cells could be more diverse than the possibilities illustrated in figure 7 (94-96). They report differentiation of mesenchymal stem cells in cells with visceral mesoderm, neuroectoderm and endoderm characteristics in vitro. This would indicate that these cells exhibit pluripotency or plasticity.

Mesenchymal stem cells can be harvested from bone marrow (96), but also e.g from fat (97,98), thymus and spleen (99), peripheral blood (100, 101) , umbical cord blood (102,103), human fetal liver (104), pancreas (105) and other sites (106). Some of these results remain however controversial and it is not clear whether cells of origins other than bone marrow indeed meet all the criteria of MSCs (107, 108). We believe that, at least for the purpose of clinical applications in the near future, the adult bone marrow will remain the source of choice for MSC harvesting. Because of multipotent differentiation capacity, simple adhesion selection on tissue culture flasks and proliferative capacities, the use of MSCs is advocated for many applications in tissue engineering and regenerative medicine.

Drawbacks of the classical BTE protocols

Although tissue engineering is a promising technique, there are still some problems which have to be solved in order to be clinically applicable. Osteogenic constructs are often produced by isolating osteoprogenitor cells from a marrow aspiration biopsy which are multiplied in tissue culture flasks and seeded on and in a three-dimensional scaffold (109,110). For large scale-production, however, this process has some serious drawbacks. The flasks are limited in their productivity by the number of cells that can be supported by a given area, while repeated handling for culture maintenance makes the process labor-intensive and susceptible to human error or initiative. Moreover, the microenvironment of the cells is not readily monitored and controlled which may result in sub-optimal culture conditions (111). Therefore, a

future approach using bioreactors could solve these problems and is depicted in figure 8.

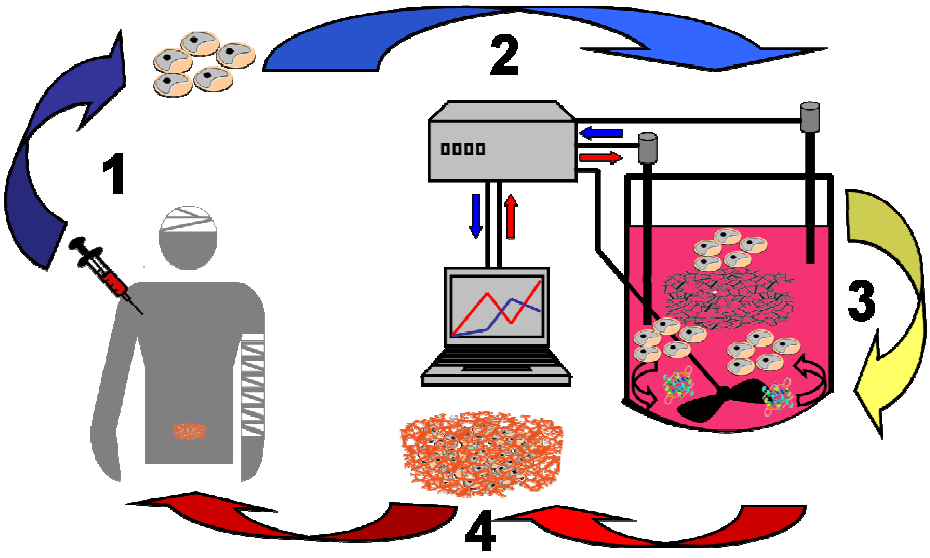

Figure 8. *Cell based bone tissue engineering using a bioreactor approach. 1. A bone marrow biopsy of a patient is harvested. 2. The whole marrow biopsy is directly inoculated in a bioreactor system where BMSCs are seeded on the scaffold material. 3. Cells are expanded in vitro, potentially in the presence of bioactive molecules, on the scaffold material in this system for a designated period. 4. The hybrid construct is implanted back into the defect of the patient. During the seeding and proliferation period, the process is monitored and controlled online with respect to culture parameters.*

Another challenge complicating the clinical application is the available amount of a tissue engineered product. Clinically relevant amounts of hybrid construct (defined as a combination of a biomaterial and bone marrow stem cells) for spinal surgery vary depending the approach from 4-6 cm^3 for an Anterior Interbody fusion (AIF) to 15 cm^3 or more when applying a PosteroLateral fusion (PLF) (112). Production of these amounts of hybrid construct is complicated because of potential mass transfer limitations. Especially diffusion of oxygen is relatively slow and oxygen consumption is high when compared to the transport of other nutrients. It is well known that mass transfer limitations involved during in vitro culturing of 3D constructs result in limited amount of cell growth into the 3D construct under static conditions. Calculations as well as experimental evidence show that few cells tolerate diffusion distances exceeding 0.2 mm (113). For example, rat osteoblasts seeded on porous scaffolds in vitro form a viable tissue that is no greater than 0.2 mm (114). Cardiac myocytes seeded on polyglycolic acid and cultured under static conditions formed tissues of only 0.1 mm thickness (115). To improve cell survival and homogeneity of cell seeding, constructs can be cultivated suspended in culture medium in spinner flasks. Convective flow allows continuous mixing of the medium surrounding the contructs (116). However, only external mass- transfer limitations can be reduced in spinner flasks or stirred tank bioreactors. Bioreactors that perfuse medium through scaffolds allow the reduction of internal mass-transfer limitations and the exertion of mechanical forces by fluid flow (117). Cultivation of osteoblast like cells (118) and rat bone marrow stem cells on 3D constructs in perfusion bioreactors have shown to enhance growth, differentiation and mineralized matrix production in vitro (119-121). However, only few studies have shown in vivo bone formation of animal derived hybrid constructs cultivated in perfusion bioreactors sofar (122, 123), and even fewer using human hybrid constructs (124).

OBJECTIVES AND OUTLINE OF THIS THESIS

For more than fifteen years, scientist and engineers have tried to find a substitute for the autologous bone graft by means of bone tissue engineering. At this point, more than 300 papers about bone tissue engineering in rodents have been published demonstrating the feasibility of the technology, mostly in ectopic sites. Surprisingly, less than 10 studies have reported the orthotopic application of tissue engineered constructs in larger animals as reviewed by Meijer et al (84). For example, successful bone formation has been reported in segmental femur defects in dogs (69) and sheep (75,125) and in iliac wing defects in goats (77). Furthermore, some clinical success has been shown in reconstructed skull (126) and mandibular defects in sheep (127) and dog (128). Although successful tissue engineering in humans has been shown in two studies (129,130), a common problem seems to be that the amount of newly formed bone is insufficient to fully bridge the implant (84,129-132). Human hybrid constructs implanted subcutaneously in immuno-deficient mice resulted in 1-3% newly formed bone of the total pore area available for bone growth depending on the donor used (133). Although much effort is undertaken to understand cellular cues to direct human MSC osteogenic potential, little papers report the increase in osteogenic potential in vivo (134). It is anticipated that at least 15-20% of newly formed bone in an orthotopic site is necessary for successful bone tissue engineering in a clinical application. It is the authors' believe that the success of BTE is ultimately dependant on the success of this technique in clinical applications. In order to apply BTE efficiently and economically in clinical practice, a bioreactor process has to be implemented. Therefore, the overall aim of this thesis is to develop and evaluate the possibility of a bioreactor approach towards controlled and monitored bone tissue engineering.

The objectives of this thesis are:

- To review the current status of bioreactors for bone tissue engineering and discuss strategies to implement bioreactors in science and clinical practice

- Design a bioreactor system for the production of clinically relevant amounts of tissue engineered bone, while monitoring cell growth online

- Validate this bioreactor system by culturing goat MSCs on ceramic scaffolds in vitro and assessing their osteogenic potential in vivo

- Culturing human MSCs on ceramic scaffolds from several donors in this bioreactor system and compare the osteogenic potential of these constructs to statically cultured constructs

- Facilitate the clinical application of bone tissue engineering by drastically reducing the amount of steps involved in the tissue engineering protocol.

- Use a multidisciplinary approach by combining technology and biology to augment the osteogenic potential of human MSCs

In order to reach these objectives, we reviewed the bioreactors currently available for tissue engineering in chapter 2. Furthermore, we designed a disposable, single use perfusion bioreactor system for bone tissue engineering, which can drastically reduce handling, labour and material. In addition this system can produce clinically relevant amounts of tissue engineered product, while monitoring cell growth by oxygen consumption in chapter 3. We validated this bioreactor system by showing the reproducible in vitro cultivation of goat BMSC hybrid constructs and the in vivo bone formation of these constructs in a immuno-deficient mouse model in chapter 4. In chapter 5, we show the feasibility of this system to produce human osteogenic hybrid constructs capable of in vivo bone formation. Chapter 6 presents a protocol to facilitate the clinical application of bone tissue engineering. In our system, we seed and proliferate goat and human BMSCs from crude bone marrow aspirates on calcium phosphate scaffolds thereby avoiding the traditional 2D subculture of these cells. Finally, in chapter 7, we present a multidisciplinary approach in order to augment the in vivo bone formation of human BMSCs by culturing these cells in our bioreactor system in the presence of cAMP. The thesis is closed with a chapter containing a general discussion and conclusion on the performed studies (chapter 8).

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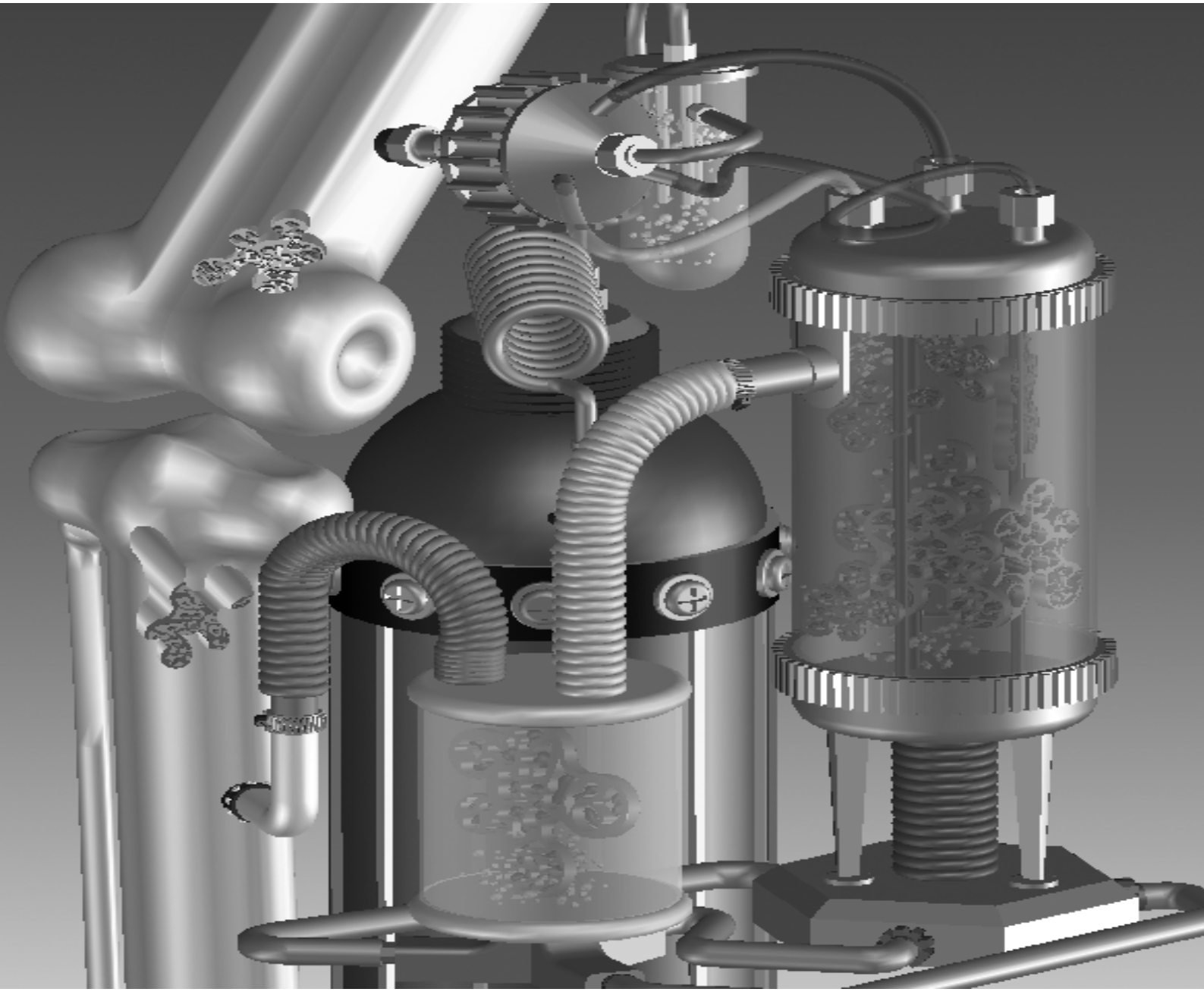
CHAPTER 2



“Scientists investigate that which already is; Engineers create that which has never been”

Albert Einstein

Picture: Maurice Thijssen 2010. An artist impression of the integration of technology and biology in tissue engineering. A tissue engineered construct is implanted from a bioreactor system in a bone defect of a patient.

CHAPTER 2

BIOREACTORS FOR TISSUE ENGINEERING

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SUMMARY

Similar to bioreactors in classical applications, the key functions of bioreactors in tissue engineering are to provide control and standardization of physiochemical culture parameters during cell/tissue culture. Bioreactors can improve the quality (i.e. cell distribution and cell utilization) and reproducibility of the process of seeding cells into 3D porous scaffolds. Mass transport of nutrients and waste products to and from cells within engineered constructs can be enhanced by convective bioreactor systems. Bioreactors which perfuse media directly through the scaffold have the greatest potential to eliminate mass transport limitations and maintain cell viability within large 3D constructs. Mechanical conditioning within controlled bioreactor systems has the potential to improve the structural and functional properties of engineered tissues. However, optimizing the operating parameters (i.e. which specific mechanical force(s) and regimes of application) for a particular tissue will require significant quantitative analysis and computational modeling. By recapitulating aspects of the actual cellular microenvironment that exists in vivo, bioreactors can provide in vitro model systems to investigate cell function and tissue development in 3D environments. Design and development of a tissue engineering bioreactor system should be approached as for classical engineering problems: define the problem, conceptualize the solution, develop a prototype, quantify reactor performance, refine the design, validate reactor performance. Innovative and low-cost bioreactor systems that automate, standardize and scale the production of a tissue-engineered product will be central to future manufacturing strategies, and will play a key role in the successful exploitation of an engineered product for widespread clinical use.

Adapted from Wendt D et al., Chapter 16 Bioreactors for tissue engineering, Tissue engineering 2008; Academic press Elsevier: ISBN 978-0-12-370869-4.

INTRODUCTION

The term ‘ bioreactor’ may initially conjure up images of a fermentation tank mixing a suspension of bacteria, possibly for the production of an antibiotic. In this classical application, the main functions of the bioreactor are to control the environmental conditions (e.g. pH, temperature, pressure) and the nutrient/product concentrations during the bioprocess. The level of control, reproducibility and automation that an optimized bioreactor system enables is essential to manufacture products that must meet specific regulations and criteria regarding efficacy, safety and quality, in addition to being cost-effective. In the context of tissue engineering, the key functions of a bioreactor are essentially the same, i.e. to provide control and standardization (Figure1):

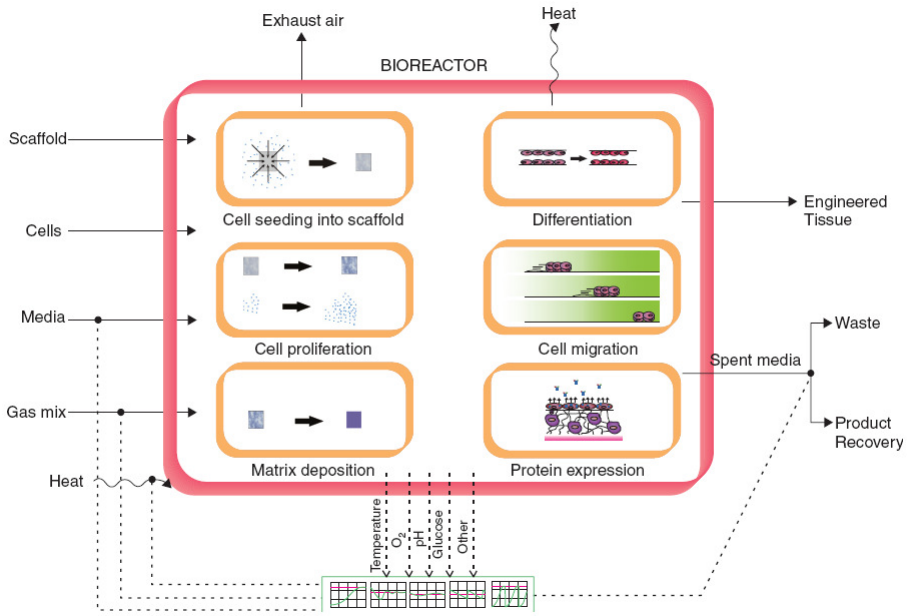

Figure 1. *Tissue engineering bioreactors. Bioreactors can be used to establish control of the physiochemical parameters during cultivation of cells and tissues. In the context of tissue engineering this controlled environment is supportive of processes such cell seeding into 3D scaffolds, cell proliferation, ECM deposition, cellular differentiation, cell migration and protein expression. Control over the environment in which these processes take place, in turn, leads to higher quality, greater reproducibility and improved scalability.*

i) by establishing control over the physicochemical culture parameters during cell/tissue culture, bioreactors offer much potential for improving the quality of engineered tissues; (ii) by standardizing, automating and possibly scaling the manufacture of tissue grafts for clinical applications, bioreactors have a key role to play in facilitating the economically viable and reproducible production of tissue-engineered products. However, in contrast to established industrial fermentation processes, tissue engineering is still an area of ongoing research, and thus bioreactors have the additional role of providing well-defined model systems supporting controlled investigations on cell function and tissue development in threedimensional (3D) environments. Specifically, tissue engineering bioreactors should be designed to enable the application of multiple regulatory signals (e.g. growth factors, hydrodynamic, mechanical or electrical stimuli), to accommodate replicates via modular design and to provide biosensor or imaging compatibility. In this chapter, we will discuss the role of bioreactors in cell-based tissue engineering approaches, focusing primarily on their applications to 3D culture systems. We will

begin by discussing the functions of bioreactors in three key processes of tissue engineering: (i) cell seeding of porous scaffolds, (ii) maintaining adequate mass transport in the seeded constructs and (iii) physical conditioning the developing tissues. We will then present a general strategy for the design and development of a bioreactor system, exemplifying practical considerations in choosing specific bioreactor components. Finally, we will discuss the implementation of bioreactors in the context of *in vitro* 3D model systems and the manufacture of tissue-engineered products for clinical applications.

KEY FUNCTIONS OF BIOREACTORS IN TISSUE ENGINEERING

This section relates to the role of bioreactors in establishing and maintaining a 3D cell culture. Special focus is given to a typical approach in tissue engineering, whereby the development of a tissue is initiated by seeding cells into porous 3D scaffolds.

Bioreactors for cell seeding

Considering that the initial cell density and cell distribution within a 3D scaffold can have a significant impact on the ultimate structure, composition and function of an engineered tissue, cell seeding can be one of the critical steps in the generation of functional tissues. For many applications, including the production of autologous grafts for clinical applications, where the availability of the cell source (e.g. tissue biopsies) is often limited, cells should be seeded with the highest possible efficiency and viability. While cell seeding of hydrogels is a relatively straightforward process, distributing cells into porous 3D scaffolds effectively and reproducibly can be a major challenge, particularly for large scaffolds or those with complex pore architectures. The most commonly used seeding technique, termed ‘ static seeding ’ , consists in simply pipetting a concentrated cell suspension into a porous scaffold. This manual, user-dependent process clearly lacks control and standardization. Stirred-flask ‘ bioreactors can improve the quality and reproducibility of the seeding process, in particular for thin and highly porous scaffolds (1). However, due to insufficient convection of cells into thick or less porous scaffolds, stirred-flask systems can result in low seeding efficiencies and generate nonuniform cell distributions, with a high density of cells lining the scaffold surface (2). Perfusing a cell suspension directly through the pores of a 3D scaffold in a bioreactor can result in a more efficient and effective cell seeding, with more uniformly distributed cells than the above techniques, particularly when seeding thick scaffolds of low porosity (2). The use of a perfusion seeding technique in combination with scaffolds having anisotropic architectures could also allow to control the distribution of cells within large porous scaffolds according to specific, nonuniform patterns (Figure 2).Seeding techniques that involve agitation or convective flow may, however, have adverse consequences on cellular viability and phenotype, necessitating the development of new seeding techniques for particularly shear sensitive cells. For instance, a recently described method to seed cardiac myocytes, which are sensitive to both shear and low oxygen levels, consisted of two steps: cells were first inoculated into the scaffold using a thermally polymerizing gel as a delivery vehicle (for low mechanical stress), then medium perfusion initiated immediately following gelation (for immediate oxygen supply) (3). In the future, the design of bioreactors for cell seeding into scaffolds will

benefit from experimental and/or theoretical analysis of flow parameters within specific scaffold types.

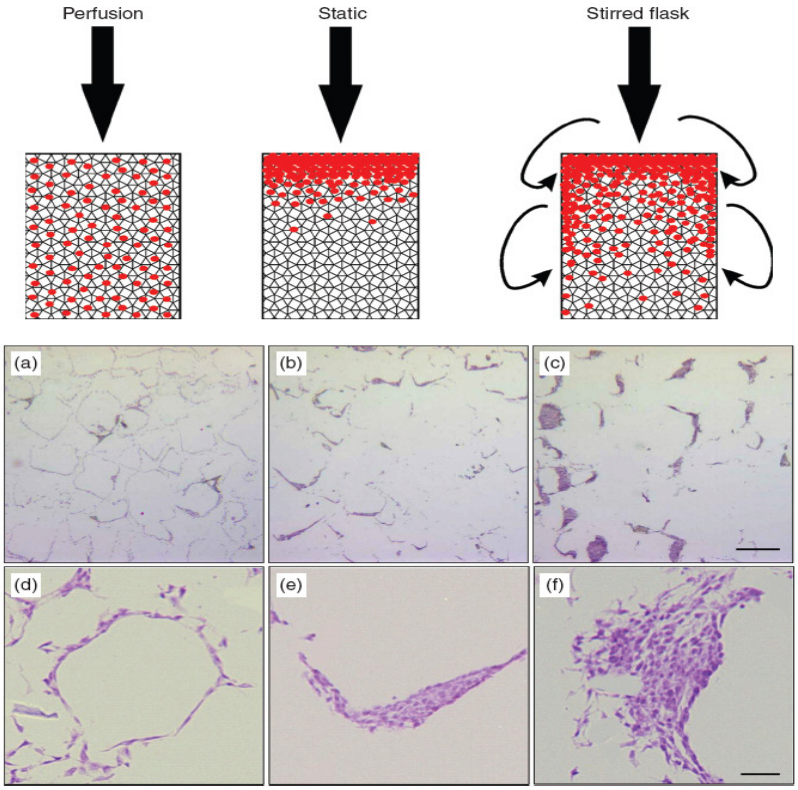

Figure 2. *Cell seeding distributions. Hematoxylin & eosin-stained cross-sections of 4-mm thick foam scaffolds following cell seeding by (a and d) perfusion, (b and e) static loading and (c and f) stirred-flask. Foams seeded by perfusion were uniformly seeded with cells outlining the foam pores. Foams seeded statically contained large cell clusters nonuniformly distributed within the scaffold. Foams seeded by stirred-flask were highly nonuniform and contained very large clusters in the scaffold region directly exposed to fluid flow. Scale bar: a–c = 500 µm; d–f = 100 µm. (2).*

Bioreactors for enhanced mass transport

After distributing cells throughout the volume of a porous scaffold, a key challenge is maintenance of this distribution and cell viability within the interior of the construct during prolonged culture. This requires sufficient mass transport of nutrients and oxygen to the cells, along with adequate removal of their metabolic waste products. The implications of inadequate mass transfer can often be observed following the culture of 3D constructs under conventional static conditions (i.e. with unmixed culture media). Due to diffusional limitations, statically cultured constructs are frequently inhomogeneous in structure and composition, containing a necrotic central region and dense layers of viable cells encapsulating the construct periphery. Although the limiting species are not decisively known, insufficient oxygen transport has been associated with inhomogeneous development of both engineered cardiac (3-5) and cartilage tissues (6,7). Convective transport of media within stirred-flask and rotating-vessel bioreactor systems can enhance mass transport to and from the construct surface and, to a certain extent, within the construct pores. Initially, convection within the pores is dependent upon scaffold geometry and permeability, and may only be effective to a limited distance into the scaffold. Subsequently, as the construct develops, the scaffold pores may occlude with cells and the extracellular

matrix (ECM), further decreasing the efficacy of convection. Bioreactors that perfuse culture medium directly through the pores of the scaffold not only enhance transport at the construct periphery, but also within the internal pores, potentially eliminating mass transport limitations (Figure 3). Perfusion bioreactors have been shown to enhance the growth, differentiation and mineralized matrix deposition by bone cells (8-10), expression of cardiac- specific markers by cardiomyocytes (3,4), and ECM synthesis, accumulation and distribution uniformity by chondrocytes (11,12). Another advantage of perfusion bioreactors over convective systems is the possibility to easily monitor the metabolite consumption of the cells (such as oxygen and glucose) by the use of online biosensors (12,13). This provides an important tool during the development and set-up of bioreactor systems, e.g. allowing us to monitor the cellular proliferation online (Figure 4) (13,14). However, while perfusion bioreactors can offer greater control of mass transport than other convective systems, there still remains the potential for flow to follow a preferential path through the construct (particularly for scaffolds with a wide pore size distribution or if the tissue develops nonuniformly), leaving other regions poorly nourished. Furthermore, optimizing a perfusion system may require a balance between mass transport of nutrients and waste products to and from cells, retention of newly synthesized *ECM* components within the construct, and fluid-induced shear stresses within the scaffold pores. Optimization of the operating conditions should ideally be supported by computational fluid dynamics (CFD) modeling, possibly in conjunction with flow visualization techniques (15). Models developed to date have been valuable for estimation of fluid velocity and shear profiles within the pores of 3D scaffolds. As the fluid dynamics will be dependent on the architecture of a scaffold ' s porous network, highly relevant CFD models could be based on a reconstruction of the actual scaffold microarchitecture, perhaps generated from micro-computed tomography (μ CT) imaging of the porous scaffold (16,17) (Figure 5).A major challenge that still remains is to extend CFD models based on empty scaffolds to later times of construct development when the porous network is filled with cells and ECM.

Bioreactors for physical conditioning

Our body's tissues and organs are subjected to a highly complex biomechanical environment of dynamic stresses, strains, fluid flow and hydrostatic pressure. It is widely accepted that physiological forces not only play an important role in cell physiology *in vivo* , but can also modulate the activity of cells in 3D scaffolds *in vitro .* Innovative bioreactors have been developed to apply one or more regimes of controlled physical stimuli to 3D engineered constructs in an attempt to improve or accelerate the generation of a functional tissue (Figure 6).

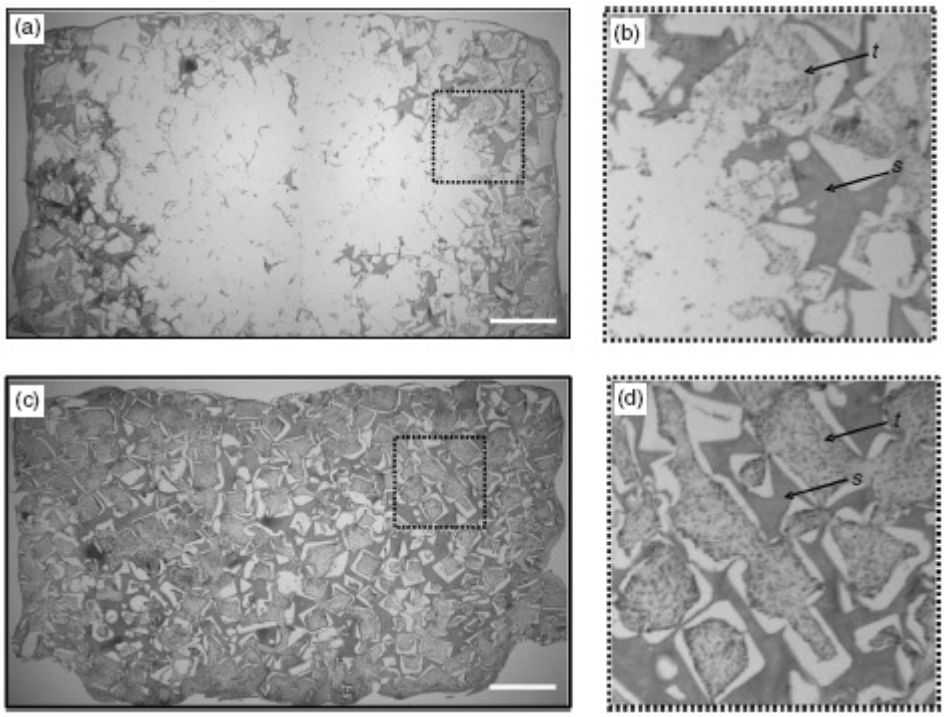

***Figure 3**. Implications of mass transfer on cell and matrix distributions. Human chondrocyte–foam constructs following perfusion cell seeding and 2 weeks of culture. (a and b) Statically cultured constructs; (c and d) perfusion-cultured constructs; (a and c) low magnification images show the tissue distribution throughout the entire cross-section (scale bar = 1 mm); (b and d) higher magnification images identify the tissue ' t ' and scaffold ' s ' within the cross-sections. Statically cultured constructs contained cells and matrix only at the construct surface, reaching a depth of approximately 1 mm into the scaffold (note: the central region of foam scaffold did not adhere to the histology slide due to the absence of cells). In contrast, perfusion-cultured constructs were highly homogeneous, containing a uniform distribution of cells and matrix throughout the cross-section. (12).*

These bioreactor-based model systems have provided compelling evidence that mechanical conditioning of 3D constructs can (i) stimulate ECM production [e.g. dynamic compression to engineered cartilage (18,19) or bone], (ii) improve cell/ tissue structural organization [e.g. fluid flow through engineered blood vessels (20), (iii) direct cell differentiation [e.g. translational and rotational strain to induce mesenchymal progenitor cell differentiation toward the ligament lineage (21) and/or (iv) enhance a specific tissue function[e.g. surface motion to engineered cartilage to enhance lubrication capacity (22).

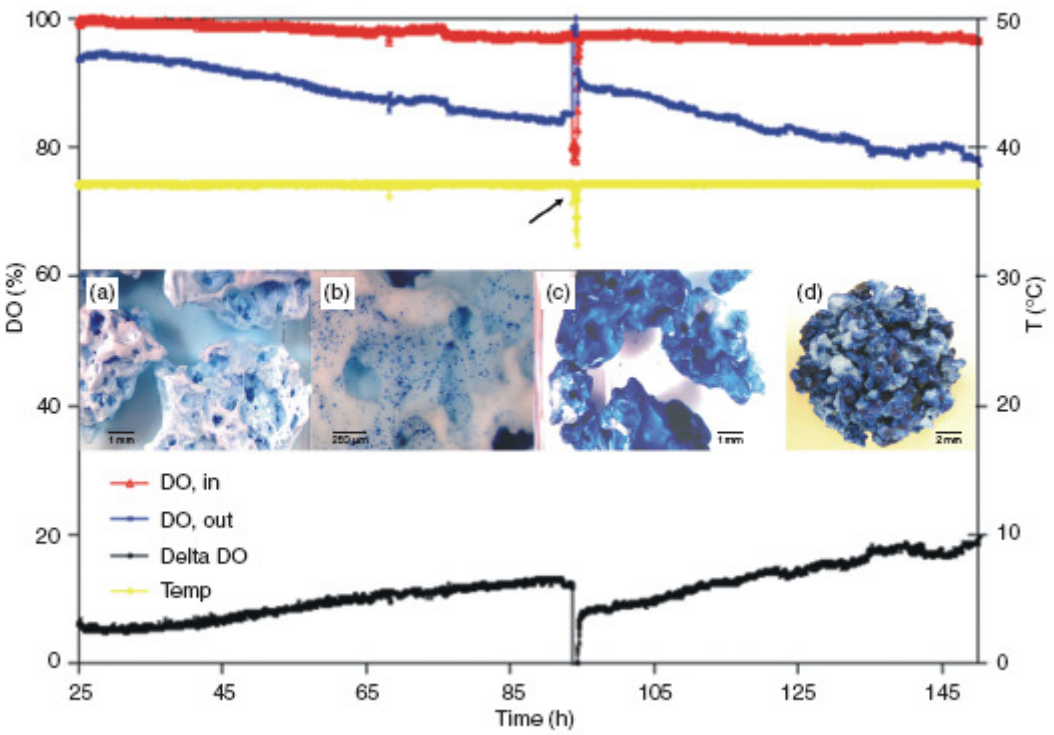

Figure 4. *On-line monitoring of dissolved oxygen concentration in the inlet (DO, in) and outlet (DO, out) medium during dynamic proliferation of BMSCs on 10 cm^3 of ceramic granular scaffolds in a perfusion bioreactor. Oxygen concentrations in the inlet media remained near saturation levels (100% DO) throughout the culture period, whereas oxygen levels measured in the outlet media decreased throughout the 6 days. This increase in oxygen consumption (delta DO) was correlated to the higher number of BMSC observed at day 6 (c and d) as compared to immediately after cell seeding (a and b) (indicated by more intense methylene blue staining). Black arrow indicates temperature and dissolved oxygen disturbance of the system by opening the incubator. Scale bars: b = 250 _ m; a and c = 1 mm; top view of all scaffolds and d = 2 mm. From (13)*

While mechanical conditioning has the potential to improve the structural and functional properties of engineered tissues, little is known about which specific mechanical force(s), or regimes of application (magnitude, frequency, duty cycle), are stimulatory for a particular tissue. At this time, the selection of optimal physical conditioning parameters is greatly complicated by the wide variety of model systems that have been used, such as varying cell types, scaffolds, forces and applied regimes, and culture times. Moreover, since cell–scaffold and cell–ECM interactions play a key role in mechanotransduction (23), engineered tissues at different stages of development, which contain different types and amounts of ECM components, may require different regimes of stimuli. To date, operating parameters for bioreactors applying physical conditioning have generally been determined and refined by a largely trial and error approach. To gain a more comprehensive understanding of how physical factors modulate tissue development, it will be necessary to integrate controlled bioreactor studies with quantitative analyses and computational modeling of mechanically induced fluid flows, changes in mass transport and physical forces experienced by the cells.

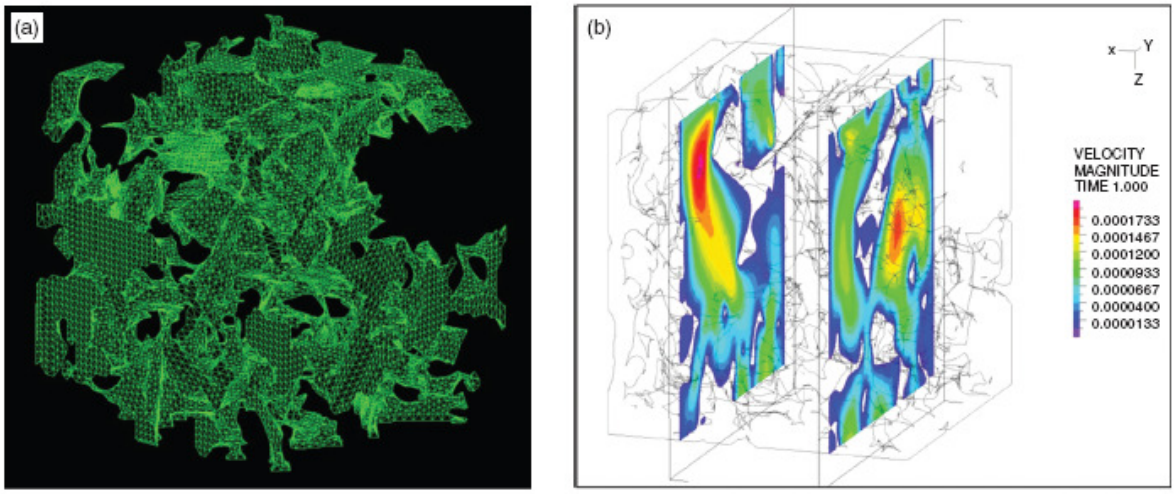

Figure 5. *CFD modeling of perfusion through a foam scaffold. (a) μ CT was used to precisely reconstruct the actual pore microstructure of a foam scaffold. (b) While models based on assumptions with a simplified geometry can provide order-of-magnitude estimates of velocities and shear stresses, the μ CT-based 3D reconstruction provides the basis for a more realistic simulation of the profiles within the tortuous pores. (Images provided by Professor Jürg Küffer, Fachhochschule Beider Basel, Basel, Switzerland.)*

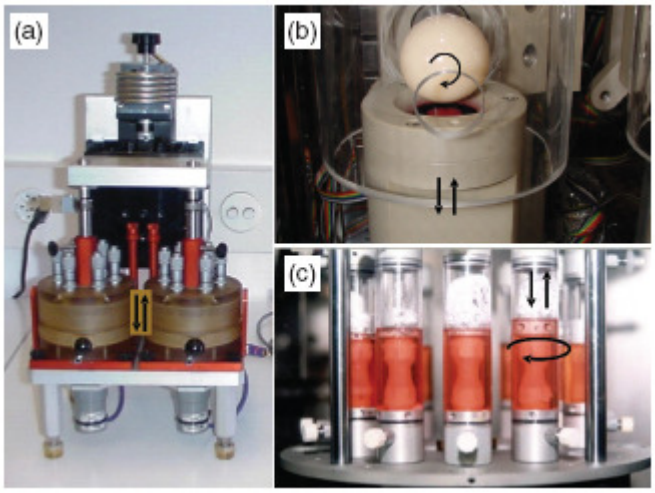

Figure 6. *Bioreactors for mechanical conditioning of engineered constructs. Illustration (examples) of bioreactor systems designed to apply (a) dynamic compression (19), (b) simulated articular motion (24) or (c) torsion and tension (25).*

BIOREACTOR DESIGN AND DEVELOPMENT

As our understanding of the aforementioned aspects of tissue engineering deepens, we are entering a time when the rational design of tissue engineering bioreactor systems is possible and, indeed, essential. Depending on its application, a tissue engineering bioreactor might be as simple as a spinner flask in an incubator or as complex as a fully self-contained, clinically deployed unit for the generation of implantable materials in humans. Regardless of the level of complexity, design of a tissue engineering bioreactor system can be approached as for classical engineering problems, beginning with a definition of the problem.

Problem definition

At its most basic level, the problem definition is a simple statement of the task at hand, e.g. 'to develop a tissue engineering bioreactor for the cultivation of chondrocytes' . However, a more precise definition of the objectives, such as 'to develop a tissue engineering bioreactor for the cultivation and mechanical stimulation of autologous human articular chondrocyte constructs in the clinic, for implantation into humans' , can dramatically influence the design requirements. This detailed definition identifies the basic features of the reactor system (a mechanism for mechanical stimulation is required) and immediately introduces numerous constraints, including regulatory requirements. Additional restrictions regarding operation, performance and economics should also be identified (e.g. the client requires that cuboidal constructs of 1 cm^3 be produced in 6 cm^3 lots, total system cost less than $50,000), as these will directly influence design and construction.

System design and components

With the problem defined, it is possible to conceptualize a solution for the bioreactor design. A basic concept of how to implement the desired seeding, cultivation and conditioning regimes evolves into a more in-depth system design and specification detailing operational parameters such as maximum/minimum temperature, pH, humidity, flow rates and pressure. Based on fundamental engineering principles, the operating parameters can in turn be used to calculate specific component requirements, such as tube diameter, pump speed and heating/cooling requirements. Basic calculations of this type are provided in the following example of bioreactor design. Details of these, and more in-depth calculations applicable to bioreactor design, can be found in any good text on process engineering or transport phenomena (26-28) and it is strongly recommended that for anyone not already familiar with the underlying theory that these be referred to. In many cases, suitable products are available ' off the shelf ' , and can be selected based on the calculated requirements and materials considerations. Key points to consider are that bioreactor materials should be biocompatible (i.e. all materials in contact with the culture environment should be tested for cytotoxicity), nonsupportive of cell adhesion (unless specifically required) and durable (for the required operational life time). For clinical applications the reactor must be designed and developed in compliance with relevant GMP (Good Manufacturing Practice) regulations and materials. Although often overlooked, features such as handling and ease of cleaning should also be given attention throughout the design process. Likewise, integration of various components and unit operations in a possibly modular design deserves major consideration.

Development and implementation

With the basic bioreactor established, ongoing development and design refinements lead towards an optimized system. During this time it is critical that effective means of quantifying reactor performance are implemented. Such measures might include quantification of cell seeding efficiency, extent of cell differentiation or metabolic parameters and may well have been specified within the context of the problem definition (e.g. must achieve a cell seeding efficiency of not less than 70%). Ultimately, at the time of implementation the bioreactor system as a whole must meet the specified design criteria (an optimized component does not always correspond to

an optimized system). The bioreactor system should also provide the advantages identified earlier in the chapter, i.e. to provide control and standardization or to scale and automate tissue manufacture.

BIOREACTORS AS 3D IN VITRO MODEL SYSTEMS

There is an increasing recognition that 3D culture of cells on scaffolds has significantly more relevance for fundamental biological research than standard Petri dish cultures (29). To serve as a biologically sound in vitro system, a 3D culture needs to recapitulate some aspects of the actual cellular microenvironment that exists in vivo . In vivo , the processes of cell differentiation and tissue assembly are directed by multiple factors acting in concert, and according to specific spatial and temporal sequences (Figure 7). It is thought that the cell function in vitro can be mediated by the same factors known to play a role in vivo. The factors of interest for tissue engineering include cytokines, growth and transcription factors, hydrodynamic shear and pressure, and mechanical and electrical signals. Biophysical regulation of cells cultured on scaffolds by combinations of these factors can be achieved by using bioreactors that provide the necessary environmental control and the application of regulatory factors (Figure 8).

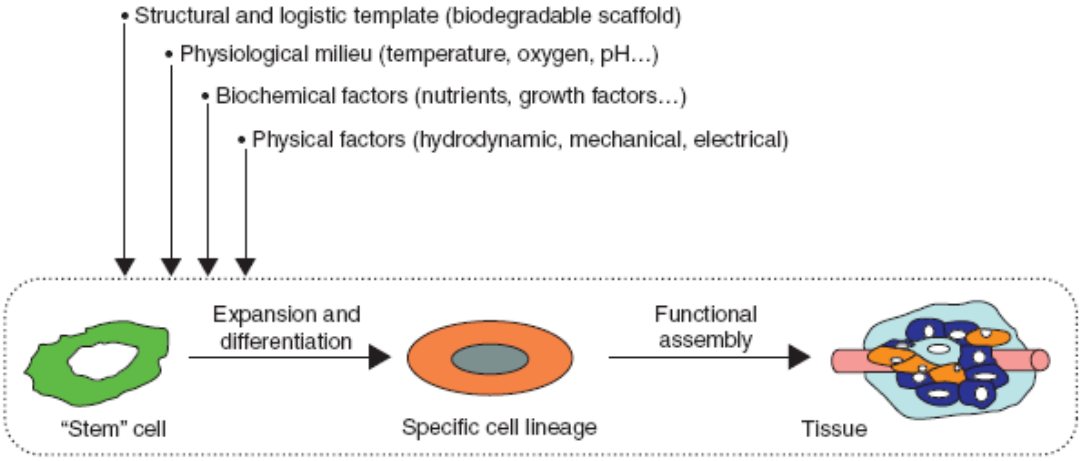

***Figure 7**. Developmental paradigm. Tissue development and remodeling, in vivo and in vitro , involves the proliferation and differentiation of stem/progenitor cells and their subsequent assembly into tissues. Cell function and tissue assembly depend on (a) the availability of a scaffold for cell attachment and tissue formation, (b) the maintenance of physiological conditions in cell/tissue environment, (c) supply of nutrients, oxygen, metabolites and growth factors, and (d) presence of physical regulatory factors.*

We will now discuss two examples in which the use of bioreactors was key to establish biologically sound yet controllable *in vitro* models and to obtain quantitative experimental data, which were then rationalized by mathematical models: (i) the progression of cartilage development and (ii) oxygen transport in engineered cardiac muscle.

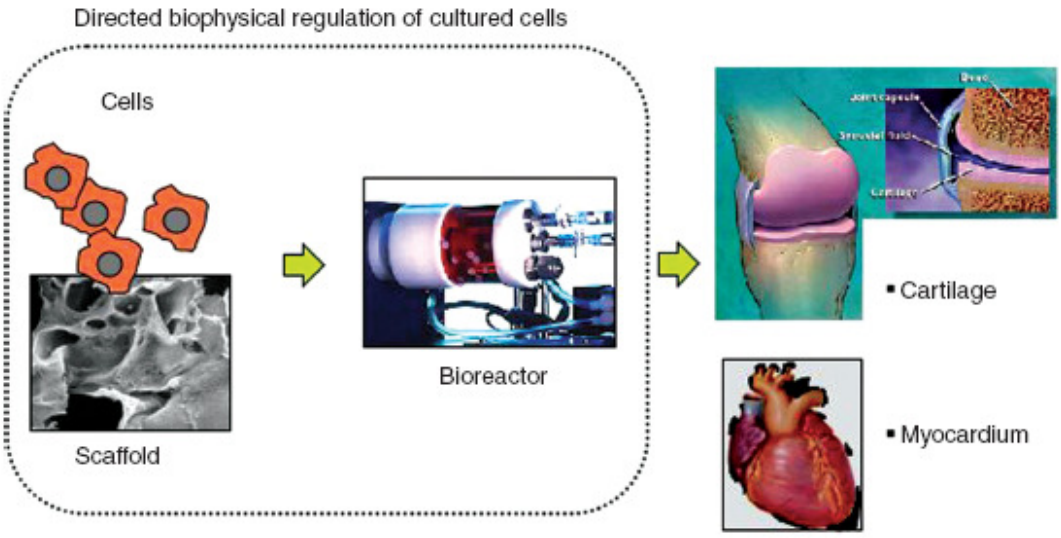

Figure 8. *Tissue engineering paradigm. The regulatory factors of cell function and tissue assembly depicted in Figure 7 can be utilized in vitro to engineer functional tissues. The cells themselves (either differentiated or progenitor/stem cells seeded onto a scaffold and cultured in a bioreactor) carry out the process of tissue formation in response to regulatory signals. The scaffold provides a structural, mechanical and logistic template for cell attachment and tissue formation. Bioreactors provide the environmental conditions necessary for cell growth and differentiation, via control of flow patterns and mass transport, and the application of regulatory signals (biochemical and physical) according to specific spatial and temporal regimes. Clearly, the regulatory cascades associated with specific cell functions are still largely unknown. In this respect, bioreactors help determine the individual and interactive effects of specific factors and the underlying mechanisms of their action.*

Bioreactor studies of tissue engineered cartilage

Engineered cartilages cultured in bioreactors with hydrodynamically active environments (involving mixing of culture medium) are generally structurally and functionally superior to those grown in static cultures. A system that gave particularly good results is the rotating bioreactor. One configuration comprises two concentric cylinders (the inner one serving as a gas exchange membrane) that are rotated around their horizontal axis at the rate of 15–40 r.p.m. The annular space between the cylinders is 110 ml in volume, and can hold up to 12 tissue constructs that are 1 cm in diameter and 5 mm thick. The rotating rate is adjusted such that the settling constructs are suspended in culture medium without external fixation. Importantly, settling of tissue constructs in rotating flow is associated with dynamic changes in the velocity, shear and pressure at construct surfaces. Although the mechanisms underlying the observed enhancement of cartilage development (chondrogenesis) under these conditions are yet to be determined, the effects were attributed to the convective flow at construct surfaces and enhanced mass transport at tissue surfaces. Based on the high quality of tissue-engineered cartilage, rotating bioreactors were used to study the spatial and temporal patterns of cartilage development by bovine calf chondrocytes cultured on fibrous polyglycolic acid scaffolds. The distributions of cells and cartilaginous tissue matrix are shown in Figure 9. Cells at the construct periphery proliferated more rapidly during the first 4 days of culture and initiated matrix deposition in this same region (Figure 9d). Over time, chondrogenesis progressed both inward towards the construct center and outward from its surface. Cell density gradually decreased and became more uniform as the cells separated themselves by newly synthesized matrix and the construct size increased (Figure 9d–f). After 10 days of culture, cartilaginous tissue was formed at the construct periphery (Figure 9b). By 6 weeks of culture, self-regulated cell

proliferation and deposition of cartilaginous matrix yielded constructs that had physiological cell density and spatially uniform distributions of matrix components (Figure 9c). The development of engineered cartilage was analyzed using a spatially varying, deterministic continuum model (30). The model accounted for the diffusion of oxygen and its utilization by the cells, and diffusion of newly synthesized glycosaminoglycan (GAG) and its deposition within the tissue constructs. Model predictions of GAG concentration profiles were consistent with those measured via high-resolution (40 μm) image processing of histological sections of tissue samples harvested at timed intervals (31). Mathematical models of this kind helped rationalize experimental observations, identify some of the mechanisms affecting tissue regeneration and design advanced systems for cartilage tissue engineering. For example, the model confirmed the first-order dependence of GAG synthesis on local concentration of oxygen. The model also helped identify that there is product inhibition of GAG synthesis via a feedback mechanism by which the cells control their immediate environment. Together, these two effects helped explain the experimentally measured gradients in GAG concentrations within cultured tissues. Notably, these relationships could not be identified without the mathematical model by simply correlating the measured data. Model development and verification would not be possible without the utilization of bioreactors capable of providing controlled conditions for tissue growth. Further studies are needed to extend the experimentation and modeling to functional parameters of engineered cartilage and to the bioreactors that more closely mimic the environment of a joint, and include perfusion and mechanical loading during cultivation.

Bioreactor studies of engineered cardiac muscle

In native cardiac tissue, a high cell density (around 10^8 cells/cm^3) is supported by the flow of oxygen-rich blood through a dense capillary network. Oxygen diffuses from the blood into the tissue space surrounding each capillary. Since the solubility of oxygen in plasma at 37°C is very low (130 μM in arterial blood), the presence of a natural oxygen carrier, hemoglobin, increases the total oxygen content of blood by carrying 65 times more oxygen than the blood plasma alone (8630 μM) (32). Under physiologic conditions, only a fraction of oxygen is depleted from the blood hemoglobin in a single pass through a capillary network. Some aspects of the native environment in cardiac muscle were recapitulated using bioreactors, with the goal to study factors that determine the development of thick, synchronously contracting cardiac constructs consisting of functionally coupled viable cells. To mimic the capillary network, cardiomyocytes and fibroblasts isolated from neonatal rat hearts were cultured on elastomer scaffolds with a parallel array of channels that were perfused with culture medium (Figure 10). To mimic oxygen supply by hemoglobin, culture medium was supplemented with a perfluorocarbon (PFC) emulsion; constructs perfused with unsupplemented culture medium served as controls.

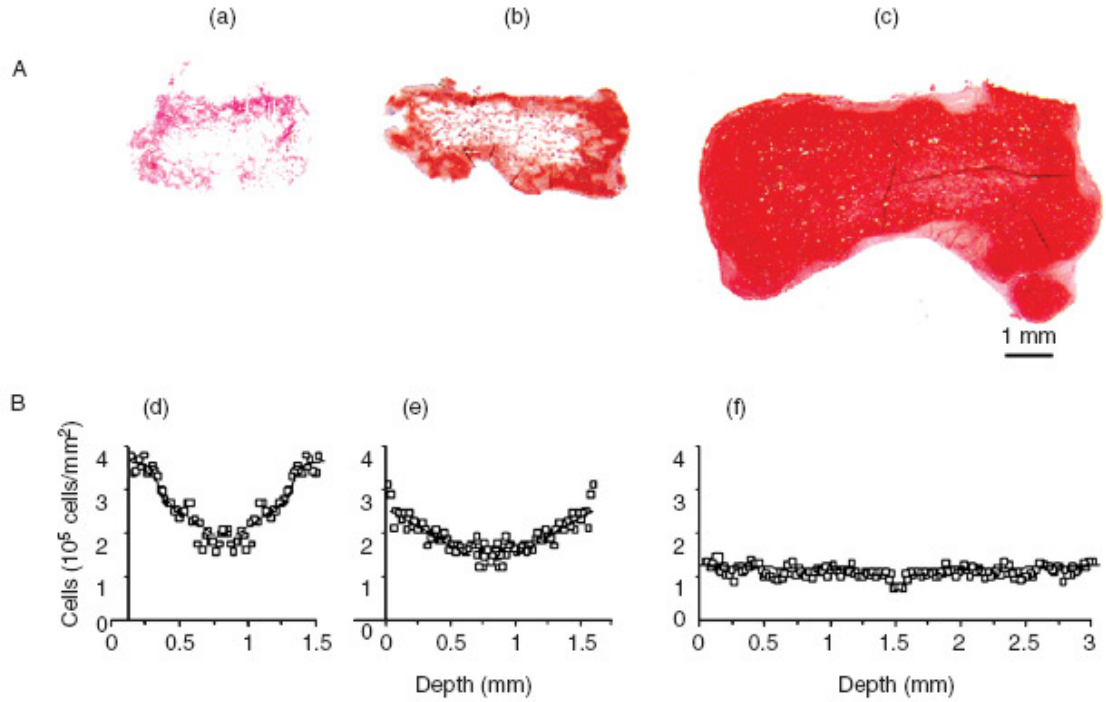

Figure 9. *Bioreactor studies of in vitro chondrogenesis. Bovine chondrocytes were seeded into fibrous scaffolds made of polyglycolic acid and transferred into rotating bioreactors. At timed intervals, tissue constructs were sampled and analyzed to assess the amounts and distributions of cells and tissue matrix. (A) Full crosssections of tissue constructs after (a) 3 days, (b) 10 days and (c) 6 weeks of culture. Stain: safranin-O/fast green. Scale bar: 1 mm. The intensity of stain correlates with the concentration of glycosaminoglycan (GAG), one of the two main components of cartilaginous tissue matrix. (B) Spatial profiles of cell distribution after (d) 3 days, (e) 10 days and (f) 6 weeks of culture (measured by image processing) (30).*

In PFC-supplemented medium, the decrease in the partial pressure of oxygen in the aqueous phase was only 50% of that in control medium (28 versus 45 mmHg between the construct inlet and outlet at the flow rate of 0.1 ml/min). Consistently, constructs cultivated in the presence of PFC had higher amounts of DNA, troponin I and Cx-43, and significantly better contractile properties as compared to control constructs. Improved construct properties were correlated with the enhanced supply of oxygen to the cells within constructs. A mathematical model of oxygen distribution in cardiac constructs with an array of channels was developed. Concentration profiles of oxygen and cells within the constructs were obtained by numerical simulation of the diffusive-convective oxygen transport and its utilization by the cells. The model was used to evaluate the effects of medium perfusion rate, oxygen carrier and scaffold geometry on viable cell density. The model was first implemented for the set of parameters relevant for the work proposed in this application: construct thickness 2 mm, channel diameter 330 μm, channel wall-to-wall spacing 370 μm and medium perfusion velocity of 0.049 cm/s. Subsequently, the model was used to define scaffold geometry and flow conditions necessary to cultivate cardiac constructs with clinically relevant thicknesses (5 mm). In future, the model can be used as a tool for optimization of scaffold geometry and flow conditions.

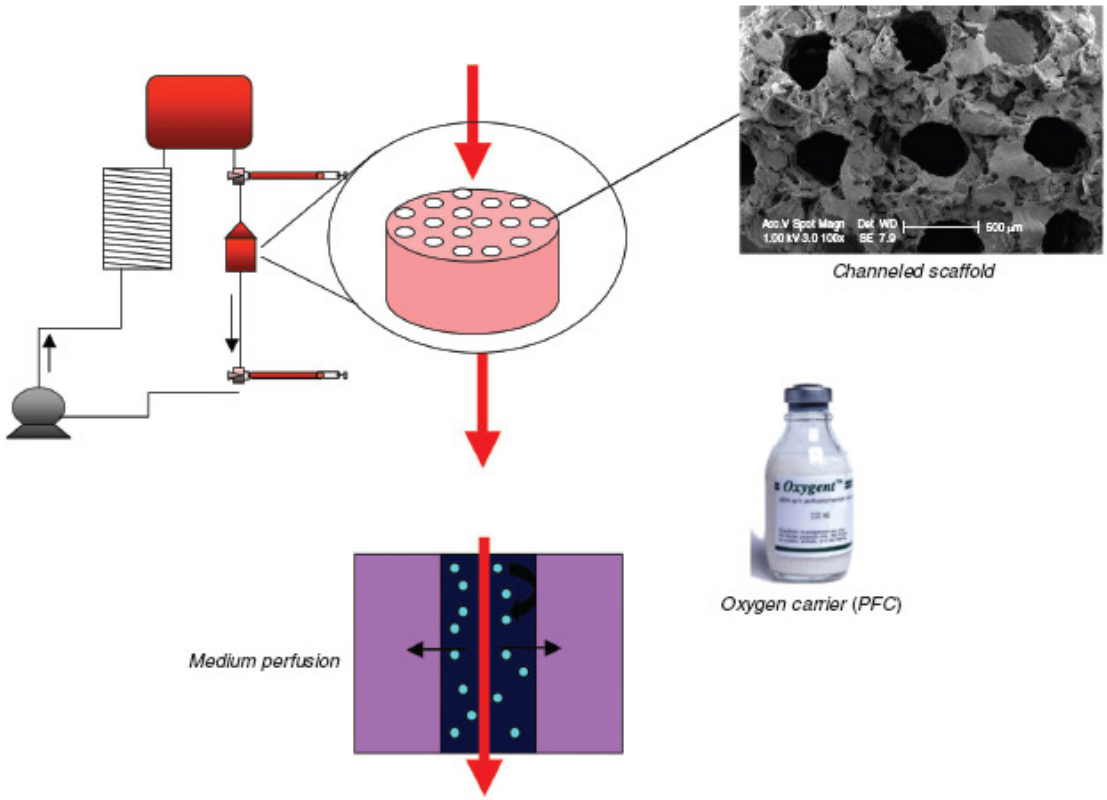

Figure 10. *Biomimetic system for studies of oxygen transport in engineered cardiac tissue. Cell populations obtained from neonatal rat hearts were seeded into highly porous elastomer scaffolds with an array of channels (to mimic the capillary network) and perfused with culture medium containing an oxygen carrier (to mimic to role of hemoglobin) (5).*

Taken together, these studies suggest that bioreactor based 3D model systems, designed to mimic specific aspects of the native cell/tissue milieu, can be a powerful *in vitro* tool for quantitative biological research. Optimization and standardization of these culture systems offers the additional possibility for controlled studies of cell–cell interactions, enzyme induction and cell metabolism, with the potential for automation and high-throughput drug screening (33).

BIOREACTORS IN CLINICAL APPLICATIONS

A major challenge to bring a tissue-engineered product into routine clinical practice is to translate research-scale production models into clinically applicable manufacturing designs that are reproducible, clinically effective, economically acceptable and compliant with GMP requirements. While production processes for tissue engineering products currently rely on unautomated manual techniques, it appears inevitable that innovative and lowcost bioreactor systems which automate, standardize and scale the production process will be *central* to future manufacturing strategies, and will play a *key* role in the successful exploitation of a tissue engineered product for widespread clinical use (Figure 11). Design of an effective manufacturing process, which fits the long-term goals and expectations of the company, must be initiated early in the product development strategy. Altering an established process which down the road proves inefficient or cannot meet product demand will require considerable resources and incur substantial costs, requiring revalidation, and new

regulatory review and approval. While a simple manufacturing design may minimize initial development costs, it may compromise the capacity for scale-up and increase product cost at later stages when a company is attempting to move to profitability. Alternatively, a manufacturing design that addresses scale-up and efficiency early

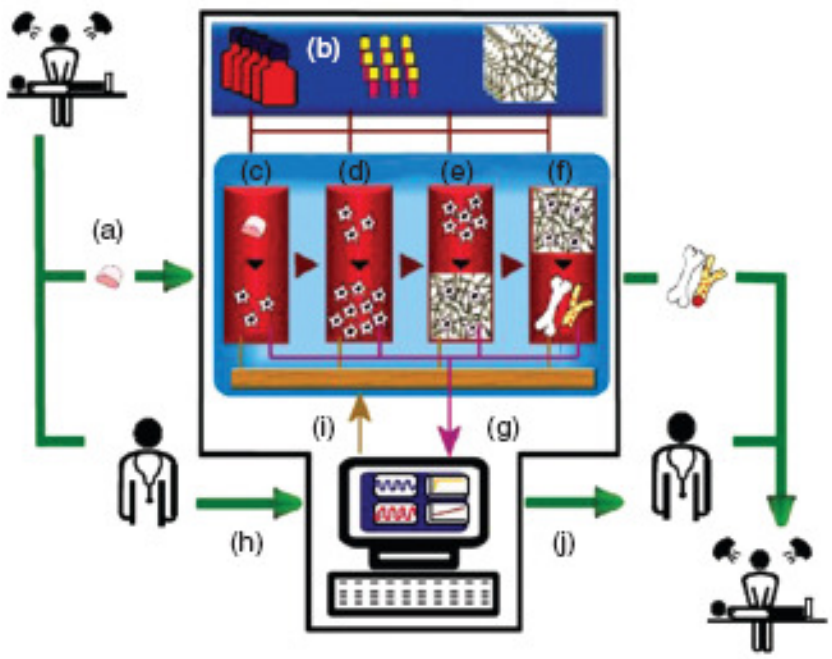

Figure 11. *Vision for a closed-system bioreactor for the automated production of tissue engineered grafts. (a) The surgeon would take a biopsy from the patient and introduce it into the bioreactor located on-site at the hospital. (b) All reagents (e.g. culture medium, medium supplements and scaffolds) would be stored in compartments under appropriate conditions (i.e. temperature, humidity). The bioreactor system could then (c) automatically isolate the cells, (d) expand the cells, (e) seed the cells onto a scaffold and (f) culture the construct until a suitably developed graft is produced. (g) Environmental culture parameters and tissue development would be monitored and inputs fed into a microprocessor unit for analysis. In conjunction with data derived from clinical records of the patient (h), the inputs would be used to control culture parameters at predefined optimum levels automatically (i) and provide the surgical team with data on the development of the tissue, enabling timely planning of the implantation(j). Figure generated by M. Moretti.*

in the product development process maximizes the potential to manufacture large numbers in a uniform and reproducible manner in the long-term, but incurs high initial costs, possibly at a time when a company may be trying to conserve funds and minimize costs. Below we describe several manufacturing strategies for engineering tissue grafts, ranging from current labor-intensive approaches through the concept of a fully automated and controlled closed bioreactor system. Carticel®, produced by Genzyme Tissue Repair, is an autologous cell transplantation product for the repair of articular cartilage defects currently used in the clinic. To produce the Carticel® product, a cartilage biopsy is harvested by a surgeon and sent to a centralized facility where the chondrocytes are isolated and expanded to generate a sufficient number of cells (34) using routine culture systems (i.e. manually by a lab technician, inside a biological safety cabinet, housed in a bio-class 10,000 clean room). Hyalograft C™, marketed by Fidia Advanced Biopolymers, is an alternative autologous cell-based product for the treatment of articular cartilage defects. Chondrocytes, obtained from a biopsy and expanded at a centralized facility, are seeded onto a 3D scaffold and cultured for 14 days using routine tissue culture techniques (35). For both of these products, the simple production systems kept initial product development costs down as the products were established within the marketplace. However, considering that

these manufacturing designs are processes requiring a large number of manual and labor- intensive manipulations, in addition to the autologous nature of the product (each cell preparation is treated individually), the costs of the products are rather high and the ability to serve an increasing number of patients per year may be challenging. Dermagraft, developed by Advanced Tissue Sciences and currently manufactured by Smith & Nephew, is an allogeneic product manufactured with dermal fibroblasts grown on a scaffold for the treatment of chronic wounds such as diabetic foot ulcers (36). Dermagraft is produced using a closed manufacturing system (Figure 12), consisting of a set of cultivation bags with eight cavities, in which cells are seeded onto a scaffold, the growth process occurs, cryopreservation is performed and the cells are finally transported to the clinic. Twelve sets of eight-cavity bags are manifolded together to result in a lot of 96. This simple system allows for incremental scale-up, multiple lots to be manufactured together and the uniform generation of large numbers of units at any one time.

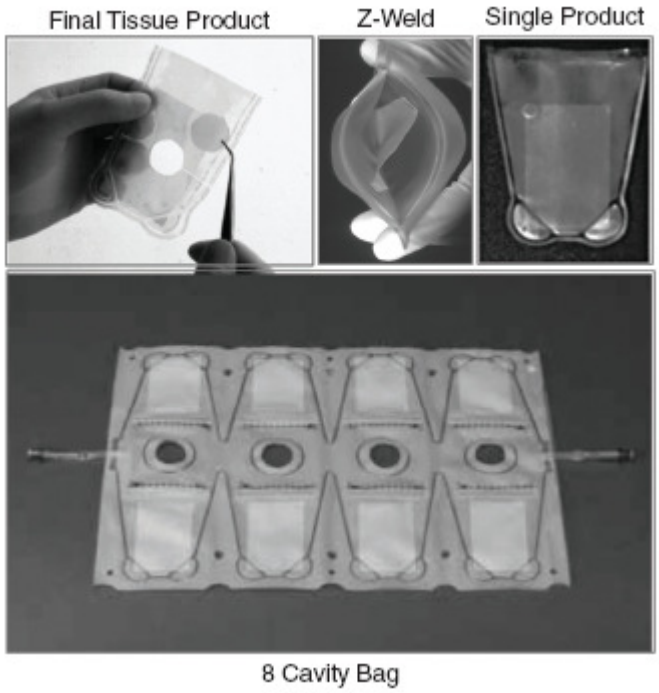

Figure 12. *Dermagraft production system and final product unit. One 2 x 3-inch piece of Vicryl scaffold is Z-welded into an ethylene vinyl acetate (EVA) compartment into which cells are seeded to grow the dermal tissue. The scaffold is held in place along the top and the bottom with welds. The single product unit is manufactured from an eight-cavity multiple processing bag, which is grown vertically in a corrugated manifold system (manifolding 12 of the eight-cavity bags, resulting in a lot of 96 units), designed for even mass transfer and exposure to environmental conditions. The EVA bioreactor is designed to be translucent, enabling placement on the wound, tracing of the wound and cutting of a piece of tissue to the desired size for implantation. (37).*

Although not applicable to autologous cellbased tissue engineering approaches, this scaleable manufacturing system has significant implications on costs of production for allogeneic products. Drawbacks to this strategy include the high level of technical difficulty in product development, long product development time, resultant high product development costs and high initial costs of manufacture (until large enough numbers of units are manufactured). As an alternative to manufacturing engineered products within *centralized* production facilities (Figure 13a) as in the examples described above, a *decentralized* production system, such as a fully automated closed-bioreactor system, could be located onsite within the confines of a hospital. This strategy is exemplified by the concept of ACTES™ (Autologous Clinical

Tissue Engineering System), currently under development by Millenium Biologix (Figure 13b). As a fully automated, closed bioreactor system, ACTES™ would digest a patient ' s cartilage biopsy, expand the chondrocytes, and provide (i) an autologous cell suspension or (ii) an osteochondral graft (CartiGraft™), generated by seeding and culturing the cells onto the surface of an osteoconductive porous scaffold. Clearly, a decentralized strategy such as this possesses the greatest risks upfront, requiring an extensive development time, and significant investments in costs in a highly technical and complex bioreactor system. In the long-term, however, systems like ACTES™ would eliminate complicated logistical issues of transferring biopsies and engineered products between locations, eliminate the need for large and expensive GMP tissue engineering facilities, and facilitate scale-up and minimize laborintensive operator handling, likely reducing the cost of the engineered product.
The broad spectrum of manufacturing designs described show a range of technologies, level of difficulty, time for development and expense. Low-level technology allows for more rapid entry to clinical trials and to market with reduced cost, but downstream in the commercialization efforts this can result in major production challenges as demand for product increases. Higher-level technology involves longer development time and increased costs, and prepares the company for a higher level of sales. However, the higher costs may be incurred for a substantial period of time before the level of sales is able to provide a return of the initial substantial investment. Ultimately, the most appropriate strategy should be determined based on the amount of scientific and clinical data already available to support commercialization of the envisaged cell-based product.

FUTURE PERSPECTIVES FOR BIOREACTORS IN TISSUE ENGINEERING

The generation of 3D tissues *ex vivo* not only requires the development of new biological models rather than those already established for traditional monolayer or micromass cell cultures (38), but also poses new technical challenges owing to the physicochemical requirements of large cell masses. In this context, bioreactors represent a key tool for all processes involved in the engineering of 3D tissues based on cells and scaffolds, including monitoring and control of the relevant culture parameters. These features are essential for the streamlined and possibly more cost-effective manufacture of engineered grafts, and could thus facilitate a broader commercialization and clinical implementation of tissue engineered products. The possibility of reproducible and controlled 3D cultures would also support fundamental studies aiming at investigating mechanisms of cell differentiation and tissue development in biologically relevant models. Understanding the role of specific biochemical or physical factors in tissue development will in turn not only support a more efficient engineering of grafts, but also set the stage for the application of those same factors directly at the site where tissue has to be regenerated. In this regard, bioreactors for *in vitro* culture might be instrumental to identify the requirements for ' *in vivo* bioreactors ' (39), thus promoting the shift from tissue engineering approaches to the more challenging field of regenerative medicine.

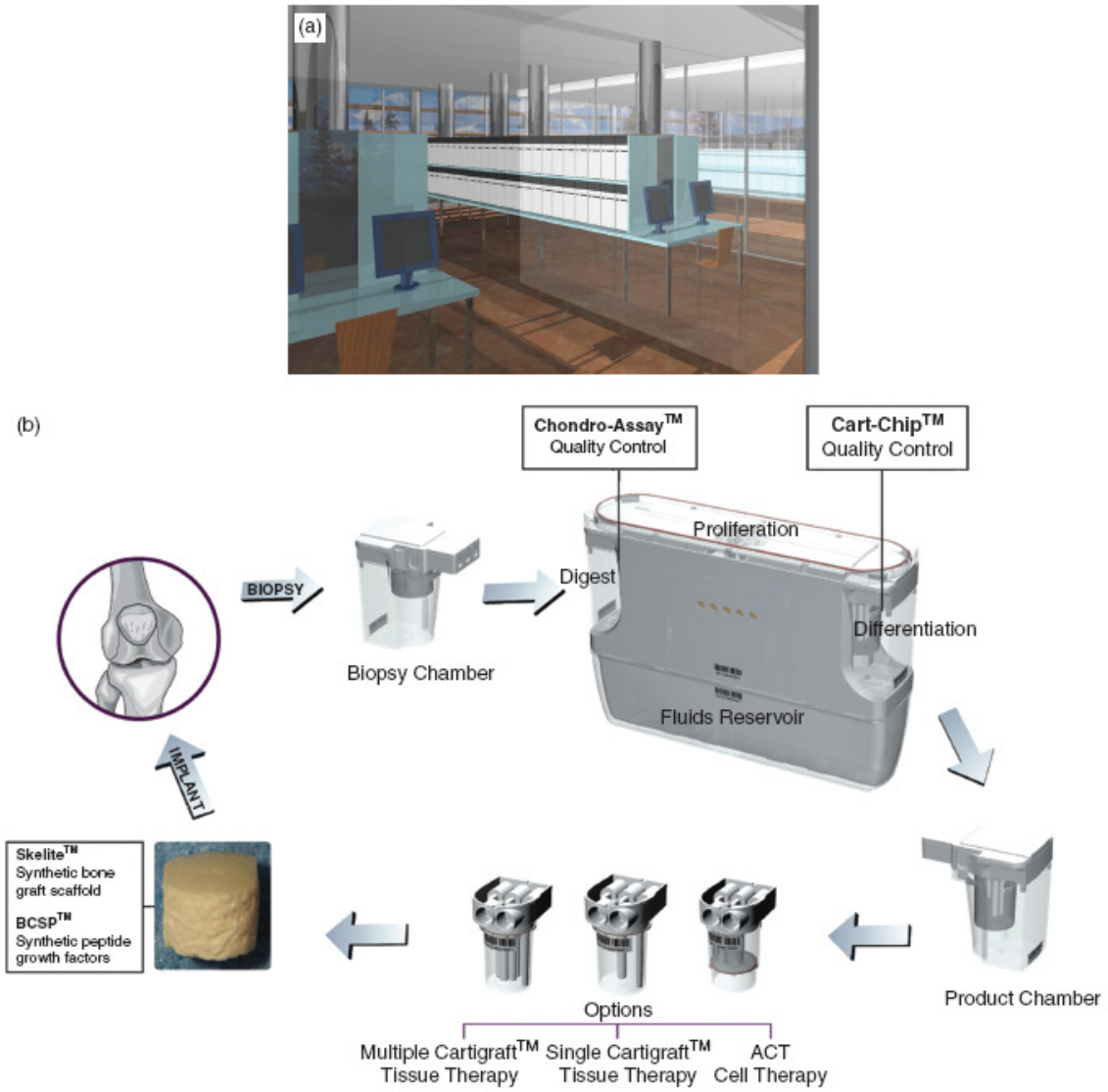

Figure 13. *Centralized and decentralized production units. (a) Computer controlled culture system in a clean room for the centralized production of autologous tissue-engineered grafts (EGM Architects/IsoTis Orthobiologics). (b) A single unit of the decentralized ACTES™ production system (Millenium Biologix).*

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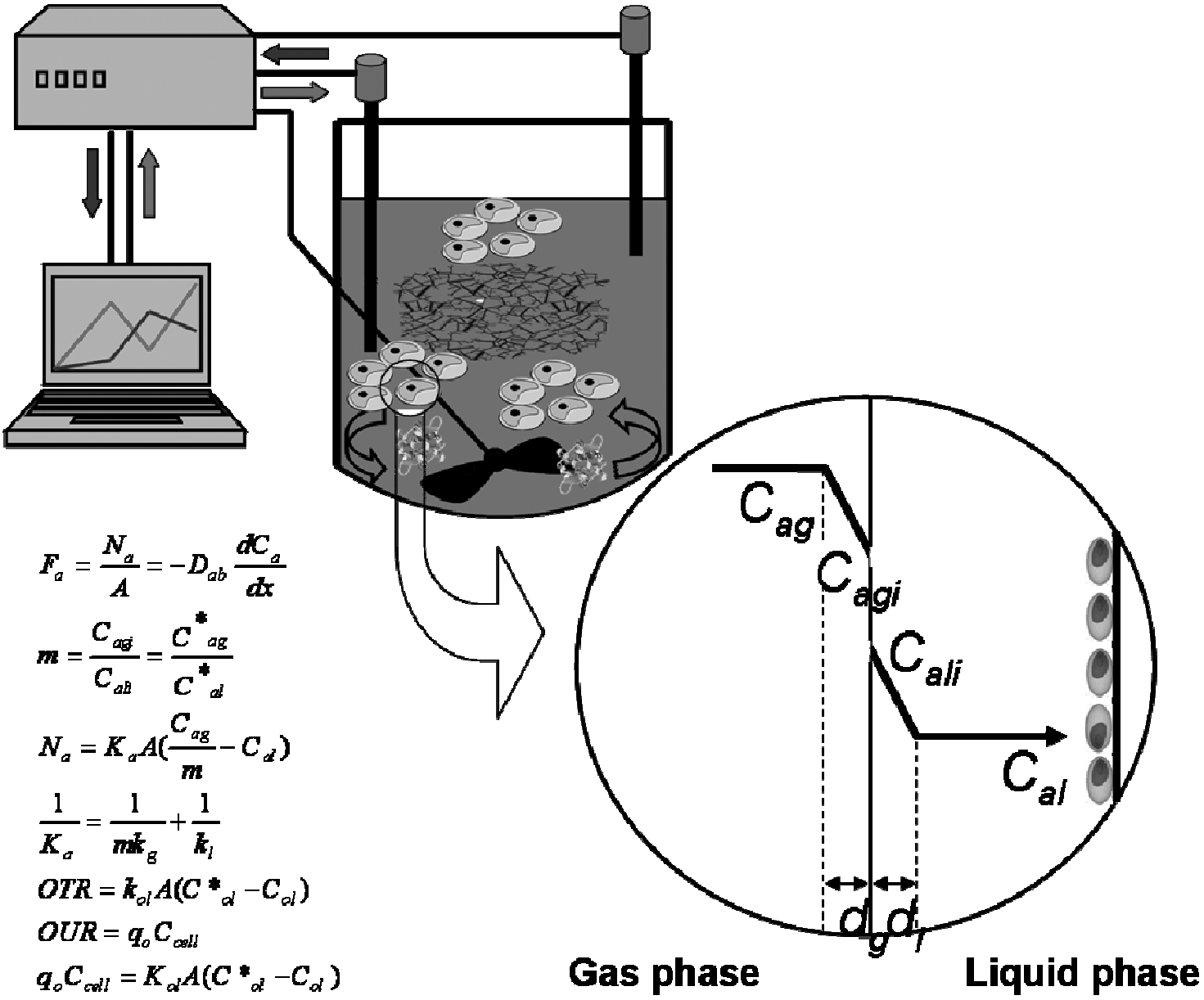
CHAPTER 3



$$F_a = \frac{N_a}{A} = -D_{ab}\frac{dC_a}{dx}$$

$$m = \frac{C_{agi}}{C_{ali}} = \frac{C^*_{ag}}{C^*_{al}}$$

$$N_a = K_a A\left(\frac{C_{ag}}{m} - C_{al}\right)$$

$$\frac{1}{K_a} = \frac{1}{mk_g} + \frac{1}{k_l}$$

$$OTR = k_{ol}A(C^*_{ol} - C_{ol})$$

$$OUR = q_o C_{cell}$$

$$q_o C_{cell} = K_{ol}A(C^*_{ol} - C_{ol})$$

"And so he's brooding and alluding on a perfect design
He thinks that working on behalf of himself is a crime
I'm gonna go home and shut up for a year
And when the year is over I'll reappear
And have a solution

Cause I'm the architect"

The architect- Deus

Picture: A glimpse at mass transport in a bioreactor
(Adapted from: Tissue engineering 2008, Elsevier)

CHAPTER 3

ON LINE MEASUREMENT OF OXYGEN CONSUMPTION BY GOAT BONE MARROW STROMAL CELLS IN A COMBINED CELL-SEEDING AND PROLIFERATION PERFUSION BIOREACTOR

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ABSTRACT

In an effort to produce clinically useful volumes of tissue engineered bone products, a direct perfusion bioreactor system was developed. Perfusion flow rate, flow direction and the position of the bioreactor are factors which influenced the amounts and homogeneity of the cells seeded on the scaffold surface. Goat bone marrow stromal cells (GBMSCs) were dynamically seeded and proliferated in this system in relevant volumes (10 cc) of small-sized macroporous biphasic calcium phosphate scaffolds (BCP, 2-6 mm). Cell load and cell distribution were shown using Methylene Blue Block staining and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining was used to demonstrate the viability of the cells. Although cells were not distributed homogenously after cell seeding, the scaffolds were covered with a viable, homogeneous cell layer after 25 days of cultivation. The hybrid structures became interconnected, and a dense layer of extracellular matrix formed on and in the scaffolds. On line oxygen measurements during cultivation were correlated with proliferating GBMSCs. It was shown that the oxygen consumption could possibly be used to estimate GBMSC population doubling times during growth in this bioreactor system. On the basis of our results, we conclude that a direct perfusion bioreactor system is capable of seeding and proliferating GBMSCs on BCP ceramic scaffolds which can be monitored on-line during cultivation.

INTRODUCTION

At present, the autologous bone graft is the gold standard for reinforcing or replacing bone in many orthopedic interventions. Unfortunately, complications of the harvest procedure during harvest operation are well known (1). Other disadvantages are the elaborate surgical procedure and the limited availability of autologous bone. Tissue engineering of bone by combining bone marrow stromal cells (BMSCs) with a suitable ceramic carrier could provide a potential alternative to autologous bone grafts. Although this technique is promising, there are still some problems which have to be solved for the technique to be clinically applicable.

Currently, in bone tissue engineering, osteoprogenitor cells are commonly isolated from a marrow aspiration biopsy, multiplied in tissue culture flasks (T-flasks) and seeded on and into a three-dimensional scaffold. Subsequently, these cells are induced to differentiate to form an osteogenic construct (2,11). However, for large scale-production this process has some serious drawbacks. One of the problems is that, in order to use a cell therapy approach, large cell numbers will be required. Clinically useful volumes of hybrid construct for orthopedic applications are in the range of 20 to 60 cm^3 (3), whereas these amounts vary from 4 to more than 15 cm^3 for spinal surgery, depending the approach taken (4). T-flasks are limited in their productivity by the number of cells that can be supported by a given area. Many flasks are needed to produce sufficient amounts of cells, which makes the process very bulky. Repeated handling for culture maintenance makes the process labor-intensive and susceptible to human error or initiative. As a result, the manufacturing costs of these conventional processes are limiting the clinical use of tissue-engineered products (5). A bioreactor system could drastically reduce the amount of space and handling steps involved. Furthermore, a considerable cost reduction could be achieved by combining cell seeding, proliferation and differentiation of bone marrow stromal cells on scaffolds in one single closed bioreactor system.

Another issue complicating the production of 3D constructs is mass transfer of nutrients and oxygen. Diffusion is the predominant mass transfer mechanism used in T-flasks. In 3-D culture, however, diffusional rates of nutrients into the scaffolds and metabolites out of the scaffold may not satisfy the requirement of the cells and result in suppression of cell growth. For example, deposition of mineralized matrix by stromal osteoblasts cultured into PLGA constructs reached a maximum penetration depth of 240 μm from the top surface (6). Various bioreactors are used in order to culture tissue-engineered bone. One of the most straightforward bioreactor designs is the spinner flask. Scaffolds seeded with cells are attached to needles hanging from the cover of the flask in the culture medium (7,8). However, only external mass-transfer limitations can be reduced in spinner flasks or stirred tank bioreactors. Therefore, it would be desirable to design a bioreactor in which mass transfer would be increased compared to conventional bioreactors. Bioreactors that perfuse medium directly through the pores of a cell seeded three-dimensional construct reduce mass transfer limitations both at the construct periphery and within its internal pores (9).

In a tissue culture flask and in most bioreactors, the environment of the cells is not readily monitored and controlled. A suitable on-line readout system for the monitoring of cell proliferation on scaffolds is essential in a tissue-engineered production process for large numbers of patients. On-line read out systems provide the facility to optimize culture conditions and adjust them to the patients' specific needs. On-line read out systems can be based on any medium or gas compound the concentration of which changes as a result of cell activity. The limited solubility of oxygen in media combined with the high consumption rate of oxygen by mammalian cells, especially at high cell densities, makes the dissolved oxygen concentration a critical parameter to monitor and control. We report a direct perfusion bioreactor system in which goat bone marrow stromal cells (GBMSCs) can be seeded and proliferated on clinically useful amounts of ceramic scaffolds. During seeding and cultivation, the nutrient and dissolved oxygen flow supplied to the cells can be controlled by adjusting the medium flow perfused through the bioreactor. Cell seeding of scaffolds is the first step in establishing a 3D culture, and therefore different methods of cell seeding in this perfusion system are investigated. Small sized macroporous biphasic calcium phosphate scaffolds are chosen which can potentially fill every shape of defect. These scaffolds, without the presence of cells, showed osteoinductive potential in goats (10) but not in nude mice (11). Furthermore, this system allows the on-line monitoring of oxygen consumption during seeding and cultivation of GBMSCs on ceramic scaffolds. We hypothesize that oxygen consumption can be used to monitor GBMSC proliferation on-line and therefore is a valuable tool in order to help produce bone tissue engineered constructs.

MATERIAL AND METHODS

Bioreactor design

Figure 1 shows the perfusion bioreactor, which has been developed in co-operation with Applikon dependable instruments bv. The bioreactor comprises an inner and outer housing, which are configured as coaxially disposed, nested cylinders. The outer housing has a tubular body made from semi- transparent polycarbonate which consisted of one or two tubes (1,2) of different lengths. When using two tubes, those are clamped together by using a silicone ring between the two parts that are mounted by an outer housing clamp (4). In our studies however, only one tubular body was used. By choosing the length of the tubes, the reactor volume could be varied. The tubular body was closed at both ends with outer housing lids (3), which taper into a medium inlet and outlet port respectively. The outer housing lids consisted of stainless steel. The tubular body and the outer housing lids can be attached to each other by using a silicone ring between those two parts and are mounted by an outer housing clamp (4). The inner housing (5) was designed as a rigid basket from polycarbonate (diameter 2.8 cm, height 2.6 cm) in which the scaffolds were kept press-fit during cultivation.

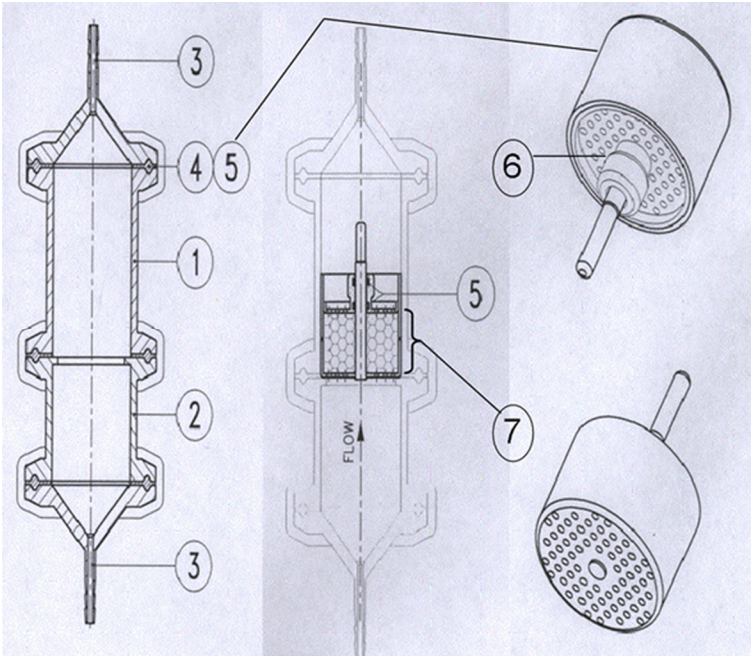

Figure 1. *The perfusion bioreactor, comprising an outer housing (1,2), outer housing lids (3), silicone O- rings, outer housing clamps (4), an inner housing with a gripping surface (5) and a perforated basket (6). The inner housing (basket) contains 10 cc of scaffold material (7).*

The basket had a perforated lid and a perforated bottom, and was placed in the medium flow path to allow axial flow through the basket. Because the basket is kept press-fit in the outer housing, no fluid flow is possible between the outer housing and the basket. The fluid flow is therefore forced through the basket and over and into the scaffold material in the basket. The basket was equipped with a gripping surface so that it could be removed easily from the outer housing.

Bioreactor system

The bioreactor system comprised a bioreactor, a sterile fluid pathway (comprising autoclavable or γ sterilizable tubing) that includes a medium vessel, a pump, an oxygenator and a waste vessel. Autoclavable tubing was purchased from Cole Parmer (Masterflex Pharmed L/S 16) and γ sterilizable PVC tubing was purchased from Rubber B.V. (Rehau raumedic PVC, medical grade, 3.0x4.5 mm). The bioreactor system is shown in figure 2. The fluid pathway contained a temperature sensor and two dissolved oxygen sensors (ApplisenS, Applikon dependable instruments BV, The Netherlands, low drift sterilizable dissolved oxygen sensor, type Z010023520) which were placed at the medium inlet and outlet of the bioreactor. The whole seeding and perfusion system was placed in a temperature controlled box (incubation unit). These incubation units lack a gas-controlled atmosphere, and in order to supply the cells with oxygen and carbon dioxide an oxygenator was developed. The oxygenator comprised a closed chamber containing a gas-permeable inner tube (Fisher, silicon, 3.0x4.5 mm) and a non-permeable outer tube (Fisher, PVC, Rauclair® tubing 6x9 mm). The gas environment in the chamber is kept at a constant level by continuously sparging a mixture of 5% CO_2 and 95% air through the chamber (100 ml/min) and medium is pumped through the gas-

permeable tube. Both oxygen and carbon dioxide diffuse from the gas phase to the medium via the silicon tubing until equilibrium is reached between the gas and liquid phase. This system enables a flow through the bioreactor of a constant pH and a constant oxygen concentration.

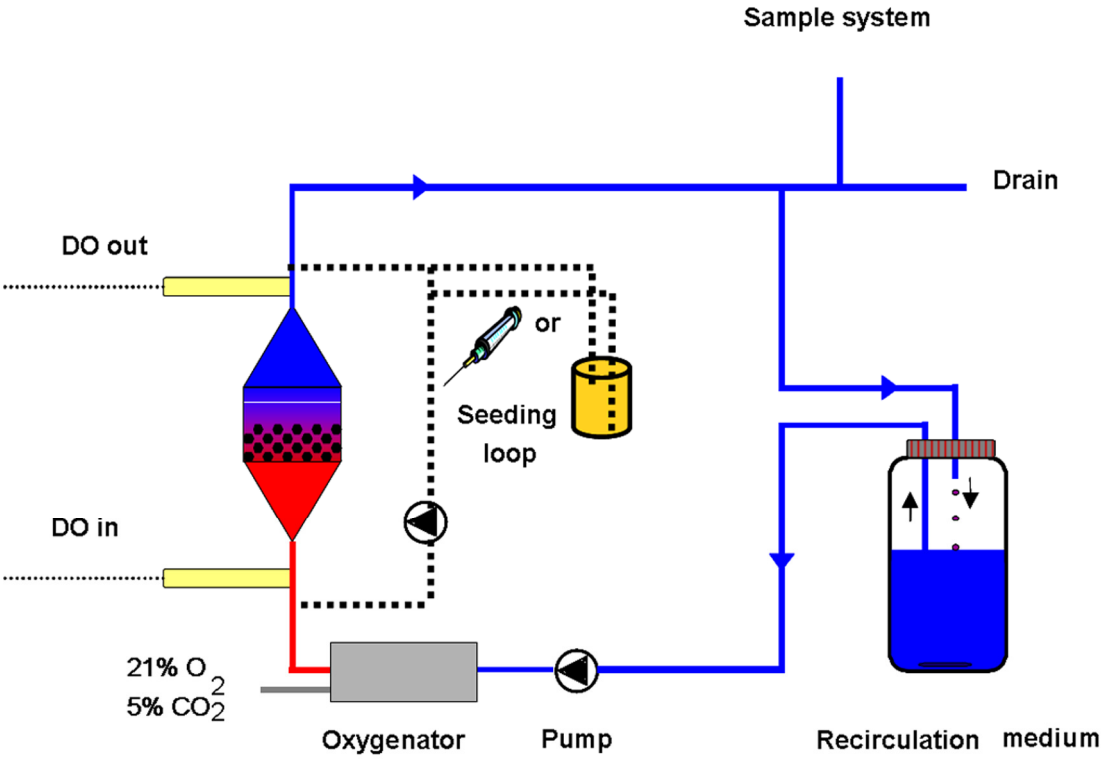

Figure 2. *Process scheme bioreactor system. Two loops can be distinguished a seeding loop (dashed line) and a proliferation loop (solid line). Red lines indicate oxygen rich and blue lines oxygen depleted nutrient flows.*

After assembly, the bioreactor system was heat sterilized (20 min, 121°C) when using pharmed tubing. When using PVC tubing, the bioreactor and sensors were heat sterilized separately and subsequently sterile-coupled to the γ sterilized PVC tubing. After cooling down to room temperature, the bioreactor was opened in a sterile laminar flow cabinet and the inner housing was filled press fit with 10 cc granules of ceramic scaffolds and the medium reservoir was filled with cultivation medium. The system was placed in the cultivation unit, medium circulation was started and the entire system was pre-washed with cultivation medium in order to pre-wet the scaffolds and allow serum proteins to attach.

Initial cell culturing of GBMSCs

After anaesthesia, the pelvic area of a goat was shaved, disinfected and a skin incision was made over the long axis of the iliac crest. The iliac was freed from overlying muscle and fat tissue by sharp and blunt dissection and the Os Ilium was exposed. After rinsing a syringe and attached biopsy needle with 5000 I.E/ml heparin, a bone marrow biopsy of approximately 10 ml was taken from the Cresta Iliaca. The marrow was collected in 30 ml medium to which 1500 I.E heparin was added and transported to the laboratory on ice. Clumps of cells were gently broken

up by resuspending the bone marrow in a syringe with a 20 G needle attached. The numbers of mono nuclear cells were counted in a small sample of the bone marrow suspension to which red cell lysing buffer had been added, and after centrifugation and resuspension, the bone marrow population was plated at a density of $5*10^5$ mononuclear cells/cm^3 in tissue culture flasks (12). At the end of the first passage (P1), the cells were cryopreserved in 10% of Dimethyl sulphoxide (DMSO). Cryopreserved cells were thawed and replated at a density of 5000 cells per cm^2. When cells were near confluence, the cells were washed with phosphate-buffered saline (PBS), enzymatically released by means of a 0.25% trypsin-EDTA solution and replated at a density of 5000 cells/cm^2. After one additional passage, cells were enzymatically released as described before, resuspended in culture medium and transported in a seeding vessel which was attached to the seeding loop of the bioreactor system shown in figure 1. Culture medium comprised of α-MEM supplemented with 15% FBS, antibiotics, 0.1 mM L-ascorbic acid-2-phosphate, 2 mM L-glutamine and 1 ng/ml basic fibroblast growth factor (Instruchemie, The Netherlands). Cells were cultured at 37°C in a humid atmosphere with 5% CO_2

Scaffolds

Biphasic calcium phosphate scaffolds (BCP, OsSaturata, IsoTis, The Netherlands) were made of 36% macroporous (pores > 100 µm) biphasic calcium phosphate. The total porosity of these scaffolds was 59% (average interconnected pore size = 388 µm of all the pores > 100 µm) as measured by Hg porosity measurement. BCP scaffolds were produced according to the H_2O_2 method including naphthalene as described before (10). The material was sintered at 1200°C. The ceramic consisted of 80 ± 5% hydroxyapatite (HA) and 20 ± 5% tricalcium phosphate (TCP) as confirmed by X-ray diffraction and Fourier Transformed Infrared spectroscopy (FITR), no additional impurities were detected. Granules of ∅ 2-6 mm were γ- irradiated at a minimal dose of 25 Kgray. 10 cc of scaffold material consisted of 230 scaffolds ± 10%.

Combined cell seeding and proliferation of GBMSC

16 $*10^6$ GBMSCs were suspended in 25 ml proliferation medium and aspirated into a syringe. Cells were injected with a 20 G needle through a sterile septum into the fluid pathway of the seeding loop in a laminar flow cabinet. Cell seeding took place in a bioreactor system as described before by closing the seeding loop and recirculating a cell suspension through the seeding loop (fig 2) with a flow rate of 4 ml/min (108 µm/s), direction from bottom to top during 4 hours. During this process, the cell suspension perfuses the bioreactor and cells are allowed to attach to the scaffold surface. After seeding, the seeding loop was closed and 60 ml of fresh medium was flushed through the bioreactor and tubing into the waste vessel to remove non-attached cells. After the flush period, the fluid path towards the drain was closed and medium recirculation was started at 4 ml/min (108 µm/s) in order to promote proliferation of the attached GBMSCs. 400 ml of the cultivation medium was used in the recirculation loop. The cultivation medium in the recirculation loop was refreshed

twice every week, and representative samples of scaffolds were taken from different positions of the bioreactor (top, middle and bottom section) during proliferation. The conditions mentioned above are applied unless stated differently and are referred to as standard conditions. In total, four *in vitro* cultures in separate bioreactor systems were performed. Two of these cultures were maintained for 25 days (no oxygen consumption measurement during seeding and proliferation) whereas the other two cultures were maintained for 7 days (oxygen measurement during seeding and proliferation).

GBMSC seeding during cell seeding optimization on ceramic scaffolds

12-16*10^6 GBMSCs, depending on the specific experiment as described in table 1, were suspended in 25 ml proliferation medium and aspirated into a syringe.

Table 1. *Investigated parameters with respect to the seeding of GBMSCs on OsSatura^{tm} BCP scaffolds in a perfusion bioreactor system.*

Investigated parameter	Total amount of GBMSCs used	Investigated variables
Seeding time	14*10^6	Time points: attached cells after 2,4 and 21 hours of seeding
Flow rate (Superficial velocity)	12*10^6	Variation of perfusion flow rate: 40, 10, 4 and 0.4 ml/min = Superficial velocity of 1082, 270, 108 and 11 µm/s respectively
Seeding method I	16*10^6	Direction of seeding: seeding cells from bottom to top vs. top to bottom
Seeding method II	12*10^6	Injection of cell suspension vs. use of a seeding vessel
Seeding method III	15*10^6	Rotated bioreactor vs. non rotated bioreactor

Unless indicated otherwise, cells were injected with a 20 G needle through a sterile septum into the fluid pathway in a laminar flow cabinet. Different GBMSC seeding methods were investigated, such as seeding time (2, 4 and 21 hours), flow rate (0.4, 4, 10 and 40 ml/min) and seeding method (injection of cell suspension versus seeding vessel). When the seeding vessel was used, the cells were not injected into the fluid pathway but suspended in 50 ml proliferation medium in a sterile 500 ml schott bottle. The gas environment of the schott bottle (head space) is kept at a constant level of 5% CO_2 by sparging a gasmixture of 5% CO_2 and 95% air (100 ml/min) through a sterile filter. The flow direction was also investigated (fig 3a, direction bottom-top and fig 3b direction top-bottom). Furthermore, a different seeding method by varying the position of the bioreactor itself was investigated. The complete bioreactor was repeatedly rotated 180 degrees in the horizontal plane ("upside down") every 30 minutes during the 4 hour seeding period, such that the bioreactor itself was inverted (fig 3c). An overview of all executed experiments is depicted in table 1. After the seeding process, representative samples of scaffolds were taken from different positions of the bioreactor (top, middle and bottom section). These experiments were performed in duplo.

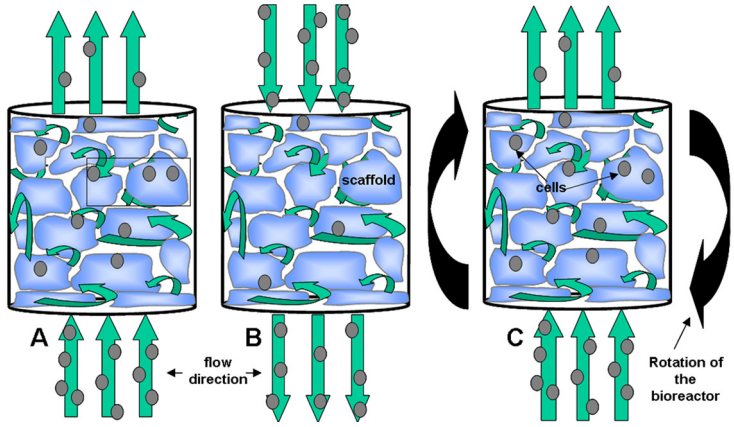

Figure 3. *Schematic representation of the different seeding directions investigated in the bioreactor system. Seeding bottom to top (fig A), top to bottom (fig B) and repeated rotation of the bioreactor in the horizontal plane while the flow direction maintains bottom to top (fig C).*

Cell distribution, load and viability

Cell distribution and cell load on the scaffolds in the bioreactor after the seeding period and proliferation period were qualitatively assessed by using the Methylene Blue (MB) staining method. After sampling, cells on the scaffolds were fixed in 1.5% glutaraldehyde in 0.14 M cacodylic buffer pH 7.4 ± 0.1 adjusted with 1M HCL. After fixation, 1% methylene blue solution was added and incubated for 60 seconds and washed twice with PBS in order to remove non- bound Methylene Blue. Cells on the scaffolds were visualized using light microscopy. For measuring cell viability, MTT staining was used. A solution of 1% MTT was applied on the scaffolds containing cells. After 4 hours of incubation, the MTT solution was removed by flushing the scaffolds with PBS. Scaffolds and cells were visualized using light microscopy.

On-line oxygen measurement during cell seeding

Two oxygen electrodes were placed in the recirculation fluid pathway. One of the electrodes was positioned before the entrance and the other one just after the exit of the bioreactor. Seeding took place by circulating a cellsuspension containing $53*10^6$ cells with a flow rate of 4 ml/min direction alternating from bottom to top and top to bottom over a 4 hour time cycle. The seeding direction was changed every 15 minutes. Oxygen consumption was monitored on-line during the seeding process with sterilizable dissolved oxygen sensors from ApplisenS (Applikon, the Netherlands). This experiment was performed in duplo.

On-line oxygen measurement during combined cell seeding and proliferation

Two oxygen electrodes were placed in the recirculation fluid pathway. One of the electrodes was positioned before the entrance and the other one just after the exit of the bioreactor. Seeding and proliferation took place as described before. Oxygen consumption was monitored on-line during the seeding and proliferation process during 7 days.

In a fixed, ideally mixed, closed system without gas exchange with the environment, the consumption of oxygen by cells can be described by equation 1:

$$\frac{dCol}{dt} = Cx(t) \cdot q_o \qquad (1)$$

in which dCol/dt represents the overall change of the amount of dissolved oxygen in mol O_2 $m^{-3}h^{-1}$ in the medium and Cx(t) the amount of biomass at time t (cell m^{-3}) and q_o the specific oxygen consumption rate (mol $cell^{-1}$ h^{-1})

The oxygen concentration was measured on-line in both the medium inlet and the medium outlet during dynamic proliferation, as can be seen in the process scheme in figure 2. The oxygen electrodes used were sterilizable dissolved oxygen sensors from ApplisenS (Applikon, the Netherlands). This equation is valid when high cell numbers are present. If cell numbers are low, the oxygen consumption of the electrode itself becomes an important factor and cannot be excluded from equation 1. Because the decrease in oxygen concentration in the outlet medium is due to metabolically active cells in the bioreactor, the increase in cell load can be coupled to the increase in difference between inlet and outlet oxygen concentrations. This can be visualized by transforming equation 1 in:

$$\frac{F_l}{V_l} \cdot (C_{o,in} - C_{o,out}) = C_x(t) \cdot q_o \qquad (2)$$

in which F_l is the flow rate of the medium (ml h^{-1}), V_l is the volume of the reactor (ml), $C_{o,in}$ is the oxygen concentration in the inlet medium (mol ml^{-1}), $C_{o,out}$ is the oxygen concentration in the outlet medium (mol ml^{-1}); the flow rate, bioreactor volume and oxygen concentration in the inlet medium are constant.

If the difference between the inlet and outlet oxygen concentrations increases over time, this indicates increasing metabolic volumetric oxygen consumption. An increase in volumetric oxygen consumption is mainly due to an increase in cell numbers because the specific oxygen consumption (that is, the oxygen consumption per cell) is not expected to change dramatically under the operation conditions described. In this case, the average residence time t (= reactor volume/medium flow) = 2.8 minutes (amount of minutes needed to replace the medium content of the bioreactor) is relatively low due to small reactor volume and high medium flow through this reactor and thus decreasing the chance of gradients in the bulk liquid in the bioreactor.

Furthermore, oxygen gradients do not per definition lead to a different value (a non-constant value) of the specific oxygen consumption (qo). Only when the dissolved oxygen concentration is lower than the so called critical oxygen concentration (Ccrit), the value of qo is not constant any more. Unfortunately, no values of Ccrit and qo are known for GBMSCs. Generally, Ccrit values under normal operating conditions are between 5-10% of air saturation (13). The outgoing oxygen concentration as

measured during cultivation in this reactorsystem is always above 75% of air saturation.

Because this process is a “randomly packed bed” reactor process, no exact information is known about the liquid flow around and through the granules. Probably, cells present on the scaffold in so called “dead spots” will die because of lack of oxygen and/or medium components. Nevertheless, although not likely, one cannot exclude the existence of gradients in this reactor set-up. Therefore, it is assumed that the specific oxygen consumption remains constant during cultivation. In this case, equation (2) can be transformed in equation (3):

$$(C_{o,in} - C_{o,out}) = \Delta DO = K_1 \cdot C_x(t) \qquad \text{with} \quad K_1 = q_o . \frac{V_l}{F_l} \tag{3}$$

Based on the oxygen consumption, a first estimation can be made of the cell proliferation rate. Using low seeding densities, exponential growth is assumed as there is no surface limitation at the start of the cultivation. As densities increase, a logistic equation might be more appropriate. For exponential growth we can deduce the following equation:

$$C_x(t) = C_{xo} \cdot e^{\mu t} \qquad \text{and} \quad \frac{C_x(t)}{K2} = e^{\mu t} \quad \text{with} \quad K_2 = C_{xo} \tag{4}$$

in which C_{xo} is the initial cell concentration (cell ml^{-1}), μ is the growth rate (h^{-1}) and t is the cultivation time (h). Substituting equation (3) in (4) results in:

$$\frac{\Delta DO}{K1 \cdot K2} = e^{\mu t} \qquad \text{and} \quad \Delta DO = K_3 \cdot e^{\mu t} \text{ with } K_3 = K_1 \cdot K_2 = q_o . \frac{V_l}{F_l} \cdot C_{xo} \tag{5}$$

Because the Δ DO is directly proportional to the biomass concentration, a plot of Δ DO against the cultivation time results in an estimation of the amount of cells present in the system. Substituting 2*Cx(0) for Cx(t) and td for t (doubling time) in equation (4) gives:

$$t_d = \frac{\ln 2}{\mu} \tag{6}$$

From equation (5) and (6), population doubling times can be calculated.

RESULTS

Defining optimal seeding method on ceramic scaffolds

It was anticipated that various parameters could influence the seeding efficiency of GMBSCs on calcium phosphate scaffold material. Different GBMSC seeding

methods were investigated, such as seeding time, flow rate, flow direction (bottom-top and vice versa) and method (injection of cell suspension versus seeding vessel and rotation of the bioreactor).

The results of different seeding methods after MB staining is shown in figure 4. When seeding cells from the bottom to the top with a flow rate of 4 ml/min (108 µm/s, standard conditions), all scaffolds contained cells. These cells were not homogeneously distributed over the scaffold surface when applying these standard conditions. No difference in cell load on the outside of the scaffold could be observed between scaffolds from the top, middle or bottom section of the bioreactor (results not shown). However, within each fraction variations do exist in cell loads. Furthermore, higher cell loads were always observed on one specific side of the scaffold. This was the side of the scaffold facing the top of the bioreactor, so at the side of the scaffolds which is schematically shown in fig 9a. MB stained particles are shown in fig 4a. Applying a flow direction from top to bottom resulted in poor cell attachment as shown by MB staining in fig 4a. With respect to cell attachment, no visual difference was observed between the two different seeding methods (injection of cell suspension versus seeding vessel) and seeding times (results not shown).

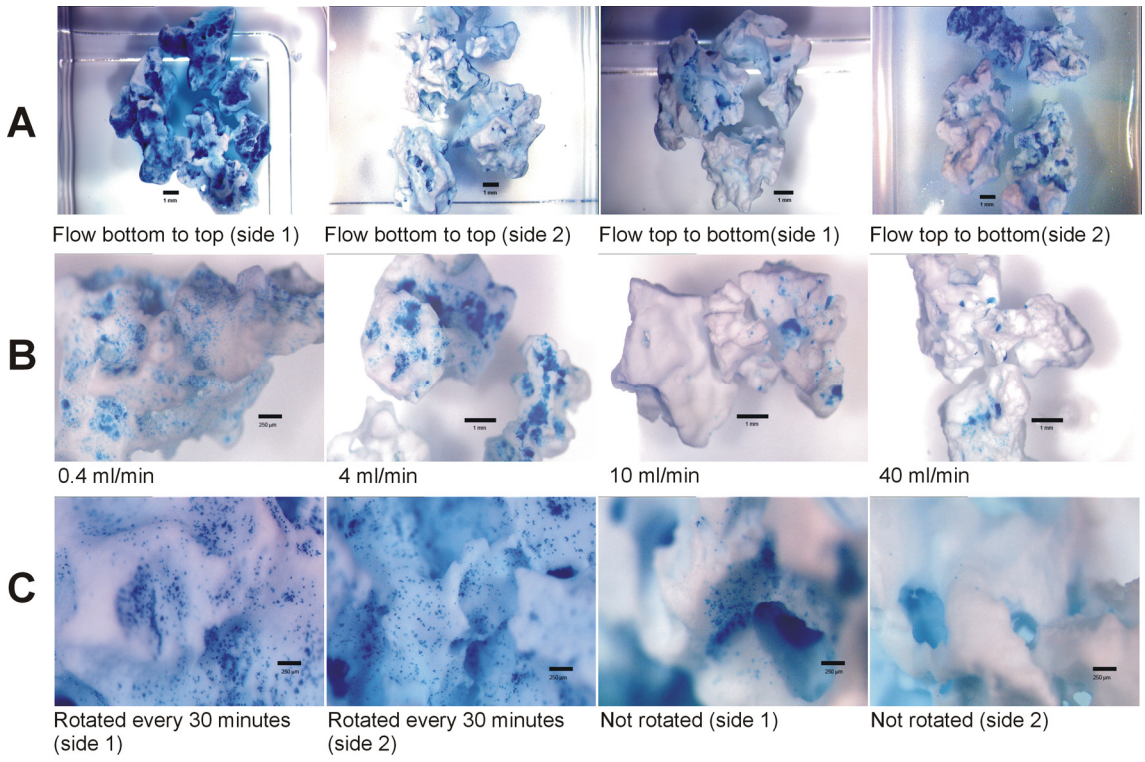

Figure 4. *MB stained GBMSCs on OsSatura[tm] BCP scaffolds after using different methods and conditions of dynamic seeding, showing the influence of the cell seeding direction (fig A), the seeding flow rate (fig B), and the repeated rotation of the bioreactor in the horizontal plane while the flow direction remains bottom to top (fig C). Side 1 refers to the side of the scaffold facing the top of the bioreactor. Side 2 refers to the side of the scaffold facing the bottom of the bioreactor. Scale bars are 1 mm in fig A and B, except for the first picture from the left in fig B (scale bar = 0.25 mm). Scale bars are 0.25 mm in fig C.*

Lower flow rates (0.4 and 4 ml/min) resulted in more attached cells to the outer scaffold surface when compared to higher flow rates (10 and 40 ml/min) as was

demonstrated by MB staining (fig 4b). Using high flow rates, cells were grouped in small clusters on the particle surface which were mainly concentrated around the present pores.

GBMSCs seeded in the bioreactor which had been rotated 180 degrees in the horizontal plane during seeding were more homogenous distributed over the outer scaffold surface as compared to the standard bottom top seeded scaffolds (fig 4c).

Seeding and proliferation of GBMSCs on OsSatura BCP scaffolds

Figure 5 shows an increase in cell load per scaffold in time during the proliferation phase. Cells were seeded according to standard conditions. After 19 days, the scaffolds were covered with a homogeneous cell layer. At this time point, no difference was seen between the between the top, middle and bottom fractions of the bioreactor. Results of the MTT staining showed a large number of viable cells after 25 days of cultivation. After 19 days of cultivation, the hybrid structures appeared to be connected to each other. A very dense layer of extracellular matrix was present on and between OsSatura BCP scaffolds.

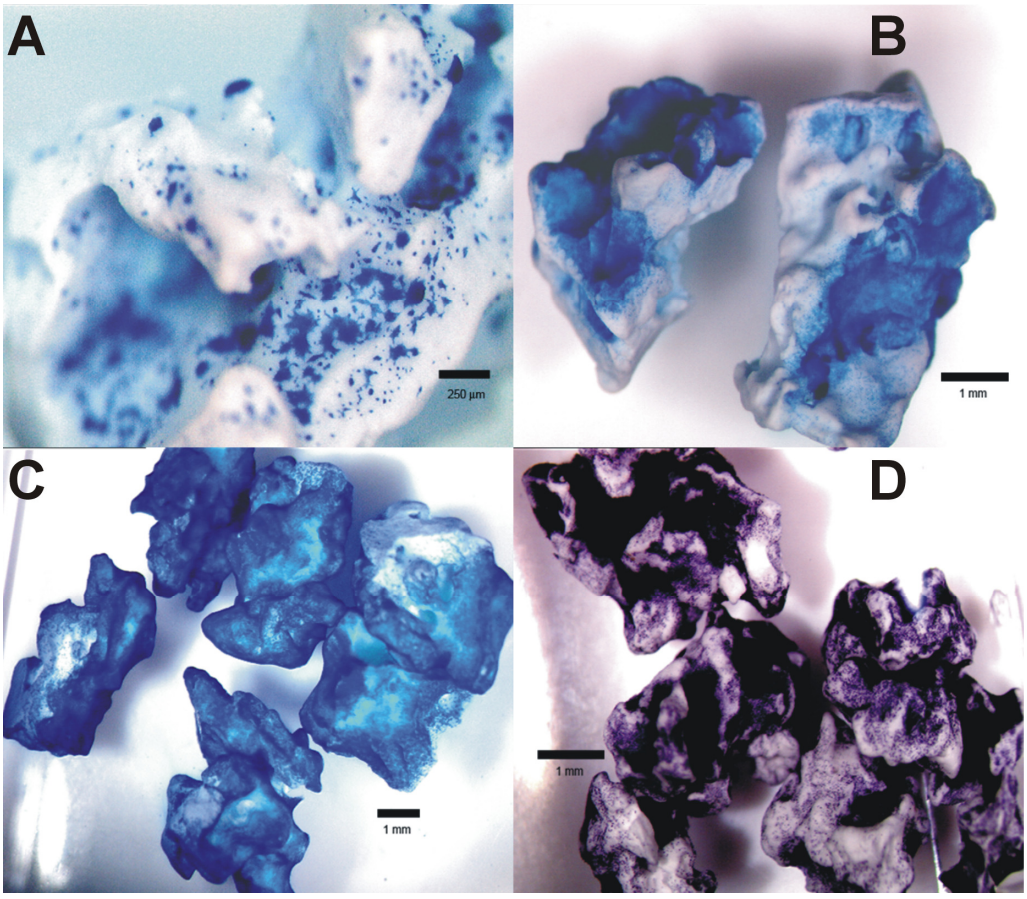

Figure 5. *Methylene Blue (fig A,B and C)and MTT (D) stained GBMSCs on OsSatura™ BCP scaffolds after dynamic seeding (fig A, scale bar is 0.25 mm) and proliferation after 6(B), 19(C) and 25 (D) days. Scale bars in fig B,C and D are 1 mm.*

On-line oxygen measurement during dynamic seeding of GBMSCs

To study oxygen consumption during seeding, the dissolved oxygen concentration was measured in the in and outlet medium of the bioreactor. The difference in oxygen concentration between the in and outlet ports of the bioreactor is shown in figure 6. Because of the alteration in seeding direction, the difference in oxygen concentration switched from negative to positive values and vice versa every 15 minutes. The difference in oxygen concentration gradually increased during the seeding period. Note that in figure 6 there are actually two maximum and two minimum values after changing the alteration in seeding direction after approximately 1.5 hours of seeding. This effect seems to increase in time during seeding, but until now no plausible explanation has been found in order to account for this effect.

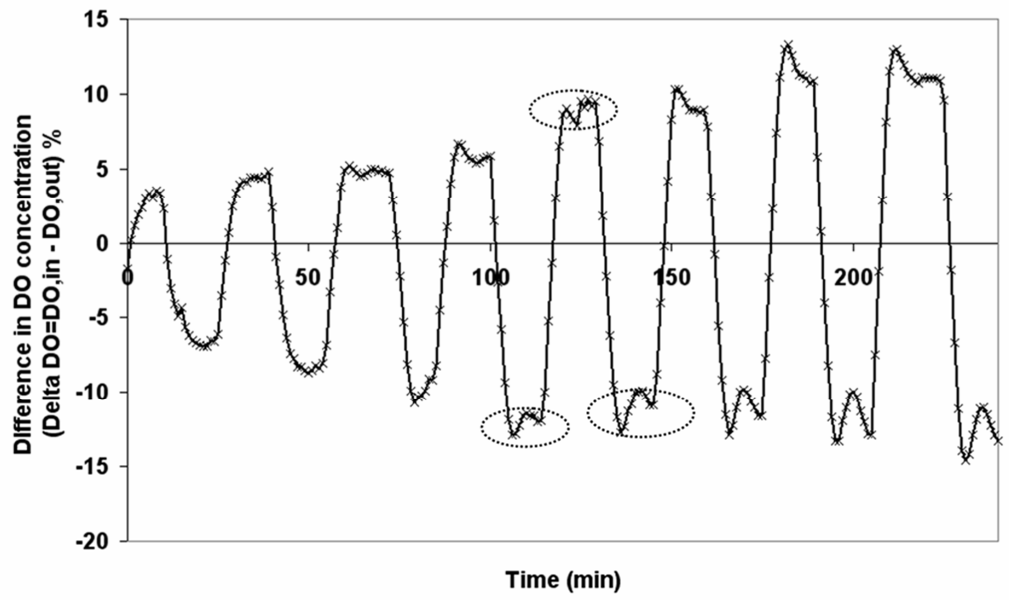

Figure 6. *On-line monitoring of the difference in dissolved oxygen concentration in the in and outlet medium during dynamic seeding of 53*10^6 GBMCS on BCP scaffolds. Seeding direction is alternated every 15 minutes from top to bottom and vice versa. The dotted lines indicate the two maximum and minimum values after changing the alteration in seeding direction. 100% indicates the concentration in medium, which is in equilibrium with the oxygen concentration in the air.*

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On-line oxygen measurement during proliferation of GBMSCs and estimation of cell growth rate

Measurement of ingoing and outgoing oxygen concentrations during proliferation in a bioreactor system is shown in figure 7. During proliferation, the inlet oxygen concentration was kept at a constant level (red line) by saturation of the medium in the oxygenator and the outlet oxygen concentration (blue line) decreased in time. The difference between the ingoing and outgoing oxygen concentration (Δ DO) is also depicted (black line). The Δ DO increases during cultivation from approximately 3% directly after seeding to approximately 22% after approximately 7 days. MB stained scaffold samples taken directly after seeding and after 7 days of proliferation are also shown in figure 7.

Disturbances indicated by an arrow are caused by opening the incubator and taking a sample of the bioreactor content. Opening the incubator resulted in a temperature decrease, influencing the dissolved oxygen measurement. The removal of the biomass (less than 10%) however does not account for the initial disturbance which is about 30%. The effect of temperature disturbance was also seen in blanco runs (no cells present on the scaffolds) and in runs where no biomass was removed (only medium refreshment). There's some experimental evidence that this phenomenon is related to the dissolved oxygen sensor itself in this specific system. Until now, we do not have an exact explanation for the relatively large "recovery" time of the dissolved oxygen concentration measurement.

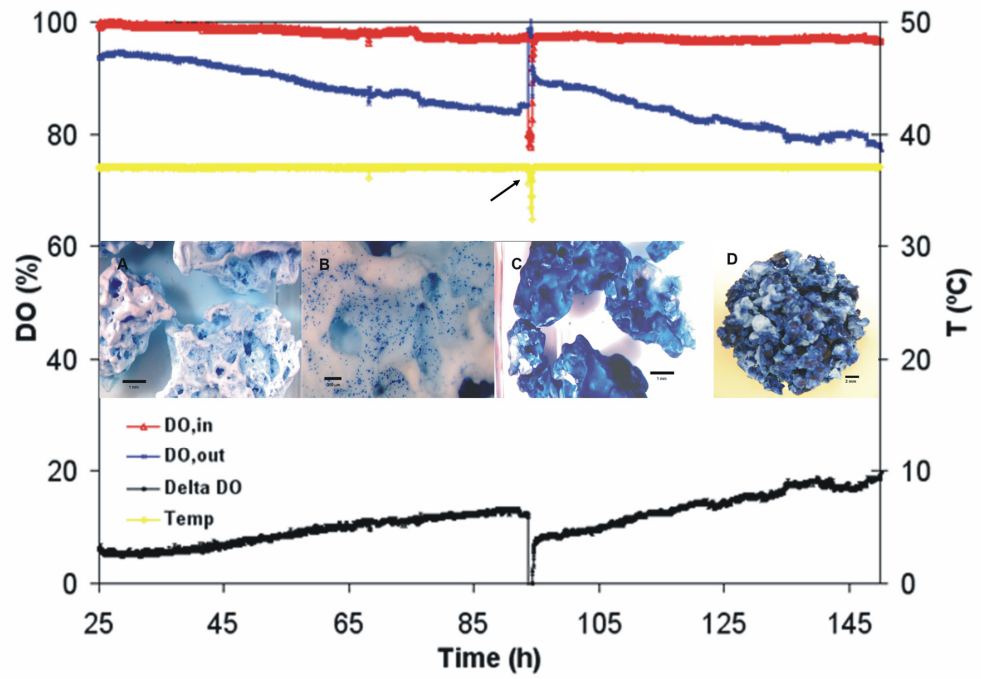

Figure 7. *On-line monitoring of dissolved oxygen concentration in the in and outlet medium and temperature during dynamic proliferation of GBMSCs on BCP scaffolds. Amounts of cells on the scaffolds are shown after seeding (fig A,B) and after 6 days of proliferation (fig C,D) by MB staining. Scale bars are 0.25 mm (fig B), 1 mm (fig A,C) and 2 mm (top view of all scaffolds,fig D). 100% indicates the concentration in medium, which is in equilibrium with the oxygen concentration in the air.*

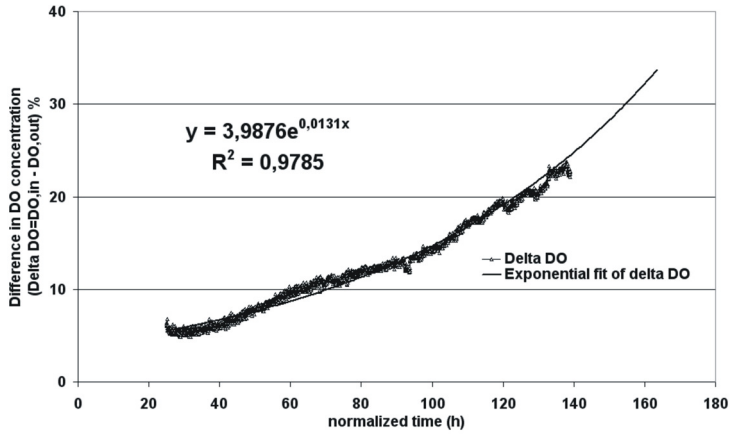

Figure 8. *The drop in oxygen concentration over the bioreactor in time during cultivation of GBMSCs in a perfusion-based bioreactor. The volumetric oxygen consumption rate is fitted to an exponential equation to estimate the cell doubling time on scaffolds under dynamic conditions.*

Assuming exponential growth and no surface limitation within the timeframes of 25 and 168 hours in figure 6, a first estimation of the population doubling time can be calculated. An exponential fit according to the mathematical derivation of equation (5) in the Materials and Methods section was used on the data and is shown in figure 8. In this figure, the Δ DO is corrected for the previously mentioned temperature disturbance. By fitting the Δ DO to an exponential curve, the cell growth rate can be calculated. The obtained cell growth rate is 0.013 h^{-1} (doubling time, td = 53 hours).

DISCUSSION

Cell seeding of scaffolds is the first step in establishing a 3D culture, and might play a crucial role in the progression of tissue formation (14). Although static loading is the most commonly used method, low seeding efficiencies and non-uniform cell distributions are often reported (15,16). Using a multi-pass filtration seeding method, higher initial seeding densities were observed and these cells were distributed more uniformly on the scaffold surface. (17). Only a few direct perfusion bioreactor systems for automated cell seeding have been described. Higher seeding efficiencies and more uniform cell distributions were achieved when compared with static seeding or stirred flask bioreactors (18). Increasing cell seeding efficiency and a more equal distribution of cells on the scaffold material might result in a decreased proliferation time *in vitro*. Furthermore, it has been reported that the average amount of adult stem cells that can be differentiated into the osteogenic lineage from a patient is only about 1-10 per 100.000 cells present in the bone marrow (19), while 200-800*10^6 adult stem cells are required for clinical use to repair e.g. a large bone defect (20). Taken into account that an average bone marrow aspirate of 10 cc will harvest between 500 and 8000 adult stem cells, up to a million-fold multiplication is needed in this case and therefore an efficient seeding process on clinical volumes of scaffolds is required.

Our seeding studies show that perfusion of a cell suspension from bottom to top in this bioreactor system does not result in a homogeneous distribution of cells on all the scaffolds. The prevalent side for cell attachment being the scaffold side facing the top (fig 9a) of the bioreactor suggested that gravity might influence cell attachment. It was hypothesized that the flow conditions at the side of the scaffold facing the top of the bioreactor might be different from the side of the scaffold facing the bottom of the bioreactor. Because of fluid dynamics, it is possible that the apparent flow rate is lower on the top side of the scaffold compared to the average flow rate, favoring cell attachment on this side of the scaffold (fig 9b). Repeated rotation of 180 degrees in a horizontal plane of the bioreactor during cell seeding resulted in a more uniform distribution over the outer scaffold surface as compared to bottom to top seeded scaffolds. The fact that cells were more concentrated around and in the pores when higher flow rates were applied could also be explained by the existence of lower apparent flow conditions at these locations. However, based on these data it is hard to speculate about the total amount of cells present on and in the scaffolds because

no data are available about the amount of cells present in the pores of the scaffolds. Therefore, it is possible that higher seeding flow rates result in higher cell numbers in the pores of the scaffolds compared to the scaffold surface. Until now, no method was found in order to reliably determine viable cell numbers on the BCP scaffold material.

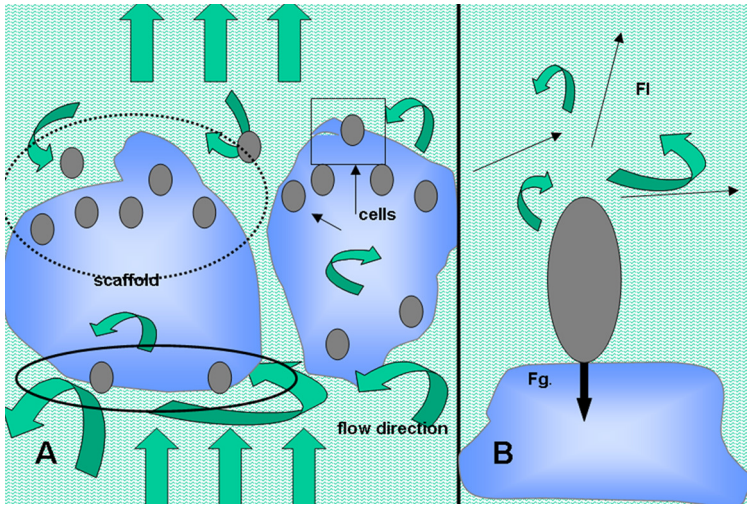

Figure 9. *Magnification of figure 3A: fig 9A: Schematic representation of the observed prevalent side for cell attachment when using a seeding direction from bottom to top: more cells were observed on the upper side of the scaffold (dashed line) compared to the bottom side of the scaffold (solid line). Fig 9B: Several forces are acting on attaching cells ao fluid flow induced forces (Fl) and gravity (Fg).*

Perfusion seeding, as demonstrated in this study, can be integrated in a system which enables seeding and proliferation of cells in a controlled way. Based on this principle, combined seeding and proliferation bioreactor systems have recently been used in engineering vascular grafts (21), cartilage (22) and cardiac (23) tissues. In this study, we show for the first time that clinically useful volumes (10cc) of hybrid (cell-scaffold) constructs can be produced by seeding and culturing GBMSCs on ceramic scaffolds in a direct perfusion bioreactor system. Although after cell seeding not all the cells were distributed homogeneously over the scaffold surface, a homogeneous and viable cell layer could be detected based on MB and MTT staining after 25 days of cultivation. The amount of cultivation steps on tissue culture flasks can be reduced significantly by using this bioreactor system. After isolating the GBMSCs, only two additional cultivation steps were used on tissue culture flasks. When all the cells are cultivated on tissue culture flasks, it is estimated that one would have to use at least five additional cultivation steps in order to obtain more than $200 * 10^6$ cells. Ultimately, we would like to design a bioreactor process in which the bone marrow biopsy could be seeded directly on the scaffold material and therefore exclude all the cultivation steps on tissue culture flasks.

Measurement of oxygen consumption provides us with a tool to study the seeding and proliferation process on-line. The increase of the Δ DO during the cell seeding process indicates increasing cell numbers on the scaffold material. We showed that oxygen consumption is a valuable tool to monitor cell growth during the proliferation period. The on line measurements can be used to estimate the growth rates of the

GBMSCs on the scaffolds. An approximately eightfold increase in Δ DO would correlate with an eightfold increase in biomass concentration according to equation (3) described in the Materials and Methods section. Cell growth rates determined for GBMSCs are comparable to growth rates found in tissue culture flasks. Ultimately, determination of present cell numbers on the scaffold surface would verify the obtained growth rates as estimated by oxygen consumption and also our data obtained in the cell seeding studies.

CONCLUSIONS

Perfusion flow rate, direction and the position of the bioreactor are factors which influence the amounts and homogeneity of the cells seeded on the scaffold surface. Dynamic seeding and culturing of goat bone marrow stromal cells on clinically relevant amounts of ceramic scaffold material is feasible by using a direct perfusion bioreactor system After 25 days, a homogeneous and viable cell layer could be observed based on MB and MTT staining which corresponded with on line measurements of oxygen consumption during the cultivation period. Cell growth rates determined for GBMSCs are comparable to growth rates found in tissue culture flasks. Furthermore, the hybrid structures became interconnected and a dense layer of extracellular matrix was present on and in the scaffold material.

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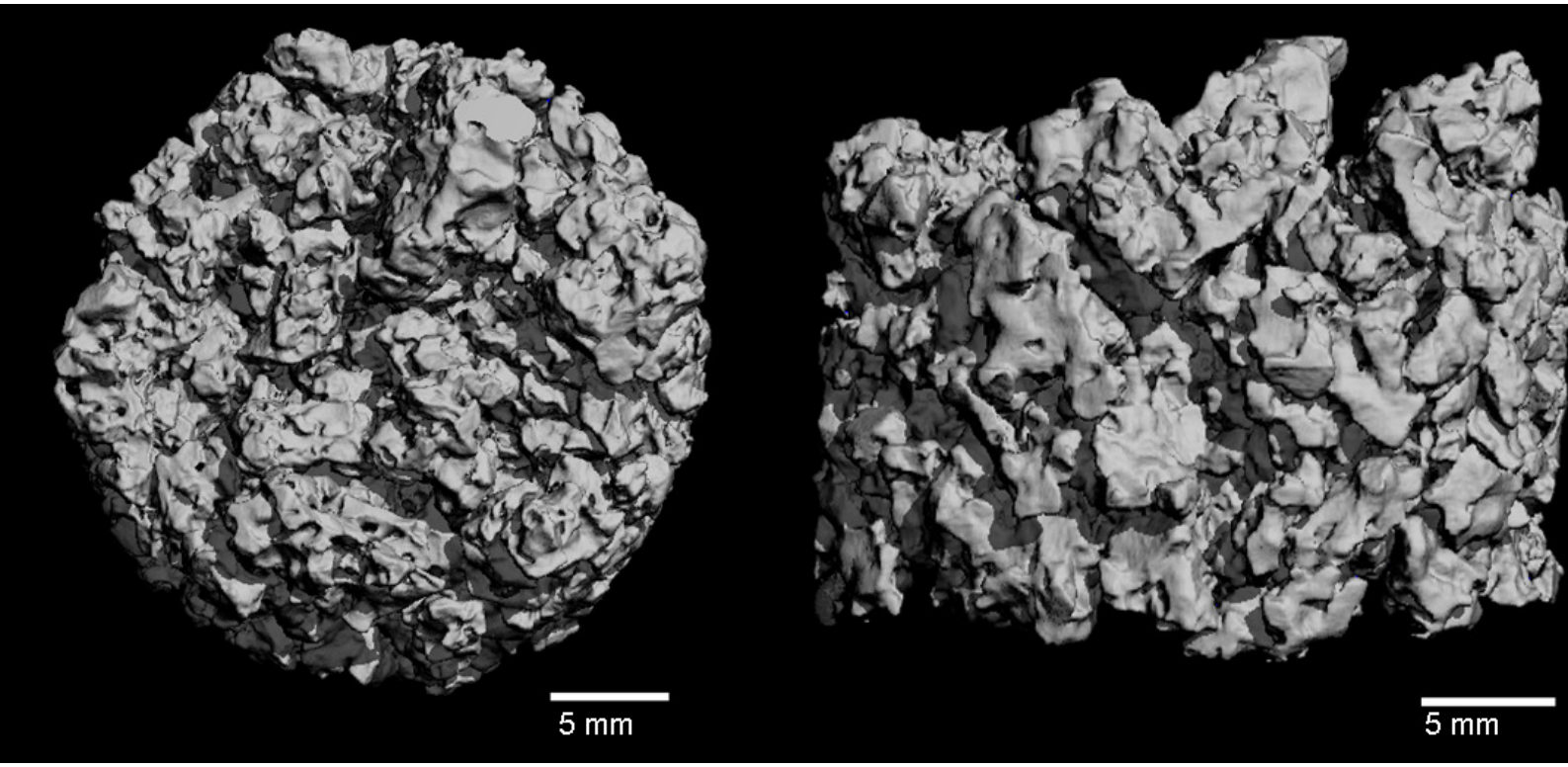
CHAPTER 4



"Your bones got a little machine
You're the bone machine"

Bone Machine- Pixies

Picture: micro CT image of the scaffold bed inside the bioreactor (this thesis)

CHAPTER 4

A PERFUSION BIOREACTOR SYSTEM CAPABLE OF PRODUCING CLINICALLY RELEVANT VOLUMES OF TISSUE ENGINEERED BONE

In vivo bone formation showing proof of concept

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ABSTRACT

In an effort to produce clinically relevant volumes of tissue engineered bone products, we report a direct perfusion bioreactor system. Goat bone marrow stromal cells (GBMSCs) were dynamically seeded and proliferated in this system in relevant volumes (10 cc) of small sized macroporous biphasic calcium phosphate scaffolds (BCP, 2-6 mm). Cell load and cell distribution were shown using Methylene Blue Block staining and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining was used to demonstrate viability of the present cells. After 19 days of cultivation, the scaffolds were covered with a viable, homogeneous cell layer. The hybrid structures became interconnected and a dense layer of extracellular matrix was present as visualized by environmental scanning electron microscopy (ESEM). ESEM images showed within the extra cellular matrix sphere like structures which were identified as calcium phosphate nodules by energy dispersive X-ray analysis (EDX). On line oxygen measurements during cultivation were correlated with proliferating GBMSCs. It was shown that the oxygen consumption can be used to estimate GBMSC population doubling times during growth in this bioreactor system. Implantation of hybrid constructs, which were proliferated dynamically, showed bone formation in nude mice after 6 weeks of implantation. On the basis of our results we conclude that a direct perfusion bioreactor system is capable of producing clinically relevant volumes of tissue engineered bone in a bioreactor system which can be monitored on line during cultivation and show bone formation after implantation in nude mice.

INTRODUCTION

Spinal fusion is considered to be one of the most challenging applications for bone graft substitutes, since even autologous bone has a relatively high rate of failure. All of the current procedures still show nonunion rates varying from 7 to 30% (1). Therefore, tissue engineering of bone by combining bone marrow stromal cells (BMSCs) with a suitable ceramic carrier could provide an interesting alternative, because this technique has the intrinsic potency to overcome the reported disadvantages of autologous bone grafts. The proof of concept of bone tissue engineering has been shown both ectopically (2,3,7) and orthotopically in rodent studies (4,5,6,7). Few studies demonstrate this technique in large animal models ectopically (8), orthotopically (9,10), and even fewer compared the functioning ectopically and orthotopically (11). However, studies comparing tissue engineered bone to autologous bone grafts in a clinically relevant model, or in controlled studies in primates have not been reported. Although this technique is promising there are still some problems which have to be solved in order to be clinically applicable. Osteogenic constructs are often produced by isolating osteoprogenitor cells from a marrow aspiration biopsy which are multiplied in tissue culture flasks and seeded on and in a three-dimensional scaffold (12,13). For large scale-production, however, this process has some serious drawbacks. The flasks are limited in their productivity by the number of cells that can be supported by a given area, while repeated handling for culture maintenance makes the process labor-intensive and susceptible to human error or initiative. Moreover, the microenvironment of the cells is not readily monitored and controlled which might result in sub-optimal culture conditions (14). Another challenge complicating the clinical application is the available amount of a tissue engineered product. Clinically relevant amounts of hybrid construct (defined as a combination of a biomaterial and bone marrow stem cells) for spinal surgery vary depending the approach from 4-6 cm^3 for an Anterior Interbody fusion (AIF) to 15 cm^3 or more when applying a PosteroLateral fusion (PLF) (15). Production of these amounts of hybrid construct is complicated because of potential mass transfer limitations with respect to the supply of oxygen and medium components. It is well known that mass transfer limitations involved during in vitro culturing of 3D constructs result in limited amount of cell growth into the 3D construct (16,17). For example, deposition of mineralized matrix by stromal osteoblasts cultured into PLGA constructs reached a maximum penetration depth of 240 µm from the top surface (18). Rat marrow stromal cells seeded on PLGA scaffolds and cultured in spinner flasks demonstrated enhanced proliferation and expression of osteoblastic markers compared to statically cultured constructs (19). However, only external mass- transfer limitations can be reduced in spinner flasks or stirred tank bioreactors. Bioreactors that perfuse medium through scaffolds allow the reduction of internal mass-transfer limitations and the exertion of mechanical forces by fluid flow (20). Cultivation of osteoblast like cells (21) and rat bone marrow stem cells on 3D constructs in perfusion bioreactors have shown to enhance growth, differentiation and mineralized matrix production in vitro (22, 23, 24). Only few studies show in vivo bone formation of hybrid constructs cultivated in perfusion bioreactors in rodents (25). In an effort to produce clinically relevant volumes of tissue engineered bone products, we report a direct perfusion bioreactor system which can drastically reduce the amount of space and handling steps involved and increase the volumes of tissue engineered product. Furthermore this system allows the on-line monitoring of oxygen consumption during seeding and cultivation of goat BMSCs (GBMSCs) on ceramic scaffolds up to 19

days. We chose a small sized macroporous biphasic calcium phosphate scaffold which could potentially fill every shape of defect and showed osteoinductive potential in goats (26) but not in nude mice (27).

MATERIAL AND METHODS

Bioreactor and bioreactor system

A direct perfusion flow bioreactor was used as described in figure 1. The bioreactor comprised an inner and outer housing, which were configured as coaxially disposed, nested cylinders. The inner housing was designed as a rigid basket from poly carbonate in which the scaffolds were kept press-fit during cultivation. The basket had a perforated lid and a perforated bottom and was placed in the medium flow path for axial flow through the basket. The bioreactor system comprised a bioreactor, a sterile fluid pathway (made of γ sterilized PVC tubing, which had low gas permeability) that includes a medium supply vessel, a pump, an oxygenator and a waste vessel.

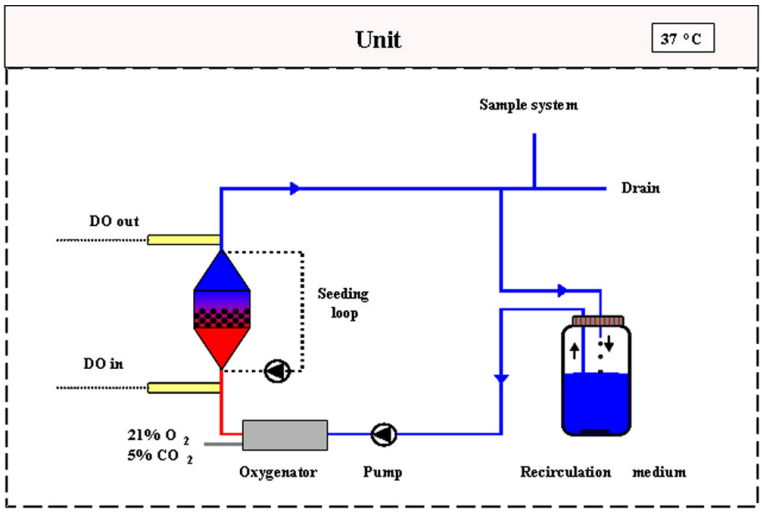

Figure 1. *Process scheme bioreactor system. Two loops can be distinguished: a seeding loop (dashed line) and a proliferation loop (solid line).*

The fluid pathway contained a temperature sensor and two dissolved oxygen sensors, which were placed at the medium inlet and outlet of the bioreactor. The whole bioreactor system was placed in a temperature controlled box (incubation unit), which was kept at 37°C. These incubation units lack a gas-controlled atmosphere and to supply the cells with oxygen and carbon dioxide an oxygenator was developed. The oxygenator comprised a closed chamber containing a gas-permeable silicon tube. The gas environment in the chamber was kept at a constant level of 21% O_2 and 5% CO_2 and medium was pumped through the gas-permeable tube at a flow rate of 4 ml/min. This system enables a medium flow through the bioreactor of a constant pH and a constant oxygen concentration.

Initial cell culturing of GBMSCs in tissue culture flasks

Goat BMSCs aspirates were isolated from the iliac crest and cultured as described in detail (13). Culture medium comprised of α-MEM supplemented with 15% FBS,

antibiotics, 0.1 mM L-ascorbic acid-2-phosphate, 2 mM L-glutamine and 1 ng/ml basic fibroblast growth factor (Instruchemie, The Netherlands). Cells were cultured at 37°C in a humid atmosphere with 5% CO_2. At the end of the first passage (P1), the cells were cryopreserved. Within 12 months, the cryopreserved cells were thawed and replated in tissue culture flasks. When cells were near confluence, the cells were washed with phosphate buffered saline (PBS), enzymatically released by means of a 0.25% trypsin-EDTA solution and replated at a density of 5000 cells/cm^2. After one additional passage, cells were enzymatically released as described before, resuspended in culture medium and transported into a seeding vessel which was attached to the seeding loop of the bioreactor system described in figure 1.

Scaffolds

Biphasic calcium phosphate scaffolds (BCP, OsSatura^{tm}, IsoTis, The Netherlands) were made of 36% macroporous (pores > 100 μm) biphasic calcium phosphate. The total porosity of these scaffolds was 59% (average interconneced pore size = 388 um of all the pores > 100 um) as measured by Hg porosity measurement. BCP scaffolds were produced according to the H_2O_2 method including naphthalene as described before (27). The material was sintered at 1200°C. The ceramic consisted of 80 +/-5% hydroxyapatite (HA) and 20+/-5% tricalcium phosphate (TCP) as confirmed by X-ray diffraction and Fourier Transformed Infrared spectroscopy (FITR), no additional impurities were detected. Granules of ∅ 2-6 mm were γ- irradiated at a minimal dose of 25 Kgray. 10 cc of scaffold material consisted of 230 scaffolds +/- 10%.

Seeding and culturing of GBMSCs

Before cell seeding, 10 cc of scaffold material in the bioreactor was flushed with cultivation medium in order to pre-wet the scaffolds and allow serum proteins to attach to the scaffold surface. $20*10^6$ GBMSCs suspended in 20 ml of cultivation medium were seeded on 10 cc of scaffold material in a bioreactor system as described before. Cell seeding took place by closing the recirculation loop and circulating the cell suspension through the seeding loop for 4 hours at 4 ml/min, flow direction from bottom to top. After seeding, the seeding loop was closed and fresh medium was flushed through the bioreactor and tubing into the waste vessel (connected to the drain) to remove any excess cells. After the flush period, the fluid path towards the drain was closed and medium recirculation was started at 4 ml/min in order to promote proliferation of the attached GBMSCs. The cultivation medium in the recirculation loop was refreshed twice every week. During cultivation at 37°C for 19 days, 3-5 scaffold samples were taken from 3 different positions of the bioreactor (top, middle and bottom section) at several time points. These samples were used for MB and MTT staining. After 12 days of cultivation, random scaffold samples (n=4) were taken for implantation studies in nude mice. After 12 days, the cultivation medium of one bioreactor system was changed by adding 10^{-8} M dexamethasone in the cultivation medium (+ dex condition). The cells in the other bioreactor system were cultured in the original cultivation medium without dexamethasone (- dex condition). Subsequently, the cells in both bioreactor systems were cultured for an additional 7 days. After a total cultivation time of 19 days, scaffold samples (n=4) were taken for implantation studies in nude mice. All experiments described in this study were performed in two separate bioreactor systems. In total, four *in vitro* cultures in separate bioreactor systems were performed (data not shown).

Control scaffold samples were statically seeded with GBMSCs placed in a 24 wells bacteriological grade plate, by applying 100 µl of a cellsuspension with a cell density comparable to the dynamic cell suspension. Cells were allowed to attach for 4 hours, after which an additional 2 ml of culture medium was added to each well. Cells were statically cultured at 37°C in a humid atmosphere with 5% CO_2 and the cultivation medium was changed twice every week. After 12 and 19 days, scaffold samples were taken and used for MB and MTT staining and in vivo implantation studies.

In Vitro studies

Cell distribution, load and viability: Cell distribution and cell load on the scaffolds in the bioreactor after the seeding period and proliferation period were qualitatively assessed by using the methylene blue (MB) staining method. After sampling, cells on the scaffolds were fixed in 1.5% glutaraldehyde in 0.14 M cacodylic buffer pH 7-3-7.4 adjusted with 1M HCl. After fixation 1% MB solution was added and incubated for 60 seconds and washed twice with PBS in order to remove non bound Methylene Blue. Cells on the scaffolds were visualized using light microscopy. For cell viability 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining was used. A solution of 1% MTT was applied on the scaffolds containing cells. After 4 hours of incubation, the MTT solution was removed by flushing the scaffolds with PBS. Scaffolds and cells were visualized using light microscopy.

Extracellular matrix examination

GBMSCs were dynamically seeded and proliferated as described before. After a 19 day in vitro cultivation period, matrix formation was examined by environmental scanning electron microscopy (ESEM) and energy dispersive x-ray analysis (EDX). Samples from cell-scaffold constructs for ESEM analysis were fixed, dehydrated, gold coated and examined in an environmental scanning electron microscope (ESEM; XL30, ESEM-FEG, Philips, The Netherlands). EDX analysis was used to identify the chemical composition of structures present on the scaffolds.

On line oxygen measurement

On line read out systems can be based on any medium or gas compound which concentration changes as a result of cell activity. The limited solubility of oxygen in media combined with the consumption rate of oxygen by mammalian cells especially at high cell densities makes the dissolved oxygen concentration a critical parameter to monitor and control. In the bioreactor process described in figure 1, oxygen was chosen for this purpose. In a fixed, ideally mixed closed system without gas exchange with the environment, the consumption of oxygen by cells can be described by equation 1.

$$\frac{dCol}{dt} = Cx(t) \cdot q_o \qquad (1)$$

In which dCol/dt represents the overall change of the amount of dissolved oxygen in mol O_2 $m^{-3}h^{-1}$ in the medium and Cx(t) the amount of biomass at time t (cell m^{-3}) and q_o the specific oxygen consumption rate (mol $cell^{-1}$ h^{-1})

The oxygen concentration was measured on line in the medium inlet and medium outlet during dynamic proliferation as can be seen in the process scheme in figure 1. The oxygen electrodes used were sterilizable dissolved oxygen sensors from Applisens (Applikon, the Netherlands). This equation is valid when relatively high cell numbers are present. In this model, we consider cell numbers relatively high if oxygen consumption of the electrode is less than 5% compared to the oxygen consumption of the present cells. DO sensors consume +/- $7*10^{-13}$ mol O_2 per second (Applikon bv, the Netherlands). Specific oxygen consumption of bone marrow stem cells were not found in literature, but e.g. hematopoietic cells show specific oxygen consumption rates between $5*10^{-18}$ and $3*10^{-17}$ mol O_2*cell^{-1}s^{-1}(28, 29). If we assume that specific oxygen consumption of mesenchymal stem cells is in the same order of magnitude, we can calculate that an initial oxygen consumption rate of $1.4*10^{-11}$ (20 x higher than the oxygen consumption of the DO electrode) corresponds with 0.46 – $2.8*10^{6}$ cells and a seeding efficiency of 2-14%. Although seeding efficiencies are not quantitatively determined, it is likely that seeding efficiencies are higher than the previously mentioned values and therefore the influence of the oxygen consumption of the electrode is probably lower than 5% from the beginning of the process. Because the decrease in oxygen concentration in the outlet medium is due to metabolically active cells in the bioreactor, the increase in cell load can be coupled to the increase in difference between inlet and outlet oxygen concentration. This can be visualized by transforming equation 1 in:

$$\frac{F_l}{V_l} \cdot (C_{o,in} - C_{o,out}) = C_x(t) \cdot q_o \qquad (2)$$

in which F_l is the flow rate of the medium (ml h^{-1}), V_l the volume of the reactor (ml), $C_{o,in}$ oxygen concentration in the inlet medium (mol ml^{-1}), $C_{o,out}$ the oxygen concentration in the outlet medium (mol ml^{-1}), the flow rate, bioreactor volume and oxygen concentration in the inlet medium are constant.

If the difference between the inlet and outlet oxygen concentration increases in time this indicates increasing metabolic volumetric oxygen consumption. An increase in volumetric oxygen consumption is mainly due to an increase in cell numbers because the specific oxygen consumption (*i.e.* the oxygen consumption per cell) is not expected to change dramatically. Therefore it is assumed that the specific oxygen consumption remains constant during cultivation. In this case, equation (2) can be transformed in equation (3).

$$(C_{o,in} - C_{o,out}) = \Delta DO = K_1 \cdot C_x(t) \qquad \text{with} \quad K_1 = q_o . \frac{V_l}{F_l} \qquad (3)$$

Based on the oxygen consumption a first estimation can be made for the cell proliferation rate. Using low seeding densities, exponential growth is assumed as there is no surface limitation at the start of the cultivation. As densities increase a logistic equation might be more appropriate. For exponential growth we can deduce the following equation:

$$C_x(t) = C_{xo} \cdot e^{\mu t} \qquad \text{and} \qquad \frac{C_x(t)}{K2} = e^{\mu t} \qquad \text{with} \qquad K_2 = C_{xo} \tag{4}$$

In which C_{xo} is the initial cell concentration (cell ml^{-1}), μ is the growth rate (h^{-1}) and t the cultivation time (h), respectively. Substituting equation (3) in (4) results in

$$\frac{\Delta DO}{K1 \cdot K2} = e^{\mu t} \qquad \text{and} \qquad \Delta DO = K_3 \cdot e^{\mu t} \text{ with } K_3 = K_1 \cdot K_2 = q_o . \frac{V_l}{F_l} \cdot C_{xo} \tag{5}$$

Because the Δ DO is directly proportional to the biomass concentration, a plot of Δ DO against the cultivation time results in an estimation of the amount of cells present in the system. Substituting 2*Cx(0) for Cx(t) and td for t (doubling time) in equation (4) gives:

$$t_d = \frac{\ln 2}{\mu} \tag{6}$$

From equation (5) and (6), population doubling times can be calculated

In Vivo studies

Random scaffold samples from both bioreactors were taken after 12 and 19 days of cultivation, as described in the section *Seeding and culturing of GBMSCs,* in a sterile LAF cabinet and were soaked in α-MEM supplemented with antibiotics. Prior to implantation, the samples were washed in PBS. From both reactors at both time points, 4 scaffolds were subcutaneously implanted in 2 nude mice (2 mice for each time point). In total, 8 dynamically cultured hybrid constructs were implanted in 4 nude mice. Control scaffold samples which were statically seeded and cultured with cells for the same period of time were also implanted. After 6 weeks, the mice were sacrificed and the implants were removed. Subsequently, the implants were fixed in 1.5% glutaraldehyde in 0.14 M cacodylic acid buffer, pH 7.3 and used for further histological processing.

Histology

The fixed samples were dehydrated and embedded in methyl methacrylate for sectioning. Approximately 10μm thick, undecalcified sections were processed on a histological diamond saw (Leica Microtome, Nussloch, Germany). The sections were stained with 0.3% basic fuchsin and 1% metylene blue, in order to study bone formation.

RESULTS

In vitro studies: Seeding and proliferation of GBMSCs on OsSatura BCP scaffolds.

Previous research in our group had shown that cells were present on all the scaffolds in the bioreactor and no difference in cell load could be observed between the top, middle and bottom fraction of the bioreactor (results not shown). However, as shown by MB staining, between individual scaffolds variations in cell load do exist and cells were not distributed homogeneously over the scaffold surface (Figure 2) MB staining also shows an increase in cell load per scaffold in time during the proliferation phase. After 19 days, the scaffolds were almost totally covered with a homogeneous cell layer. At this time point, no difference was seen between the between the top, middle and bottom fraction of the bioreactor. Results of the MTT staining showed a large number of viable cells after 19 days of cultivation (figure 2)

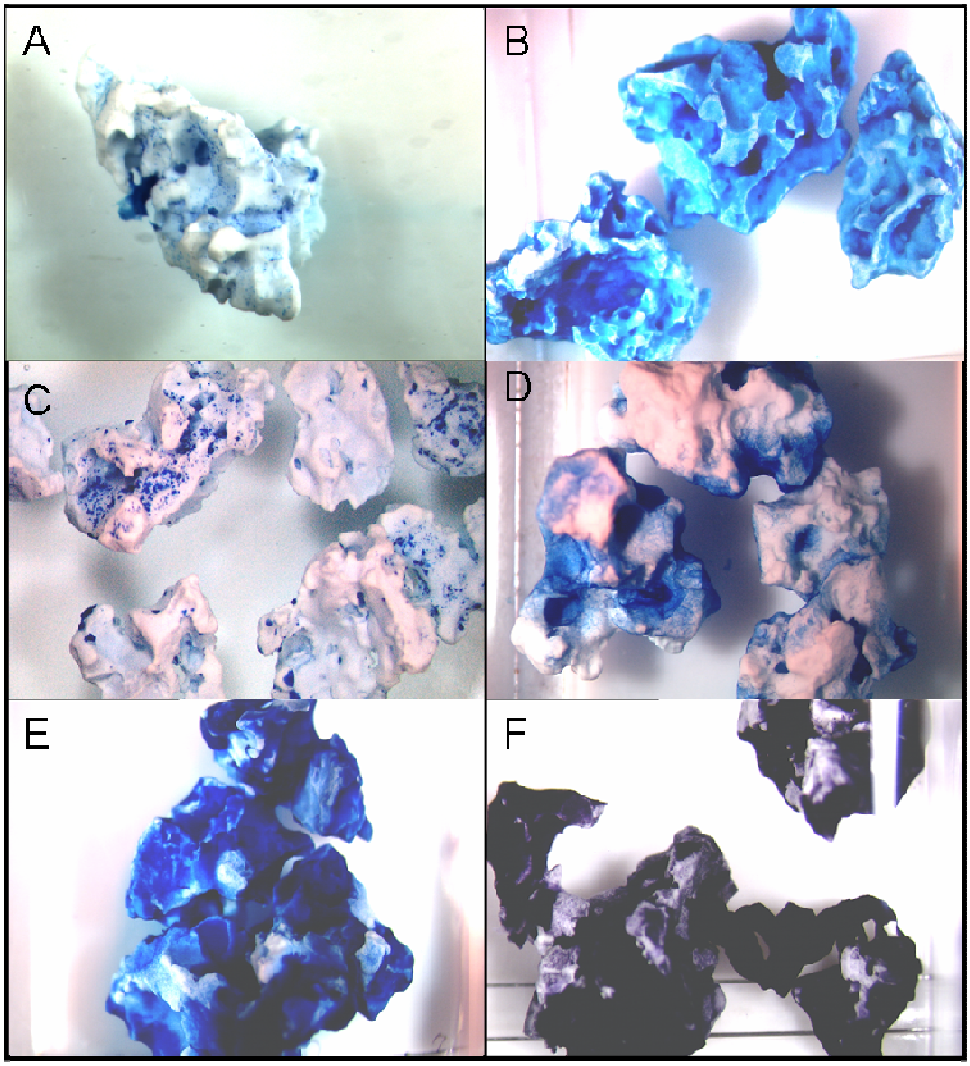

Figure 2. *Methylene Blue (MB) and MTT stained GBMSCs on OsSatura^{tm} BCP scaffolds after static and dynamic seeding and proliferation. A) MB, after static seeding, B) MB, after static proliferation for 19 days, C)MB, after dynamic seeding, D) MB, after dynamic proliferation for 7 days, E) MB, after dynamic proliferation for 19 days, F) MTT, after dynamic proliferation for 19 days.*

After 19 days of cultivation, the hybrid structures became interconnected. A very dense layer of extracellular matrix was present on and between OsSatura BCP scaffolds, presumably of a collageneous nature. On the ESEM images, sphere like structures (+/-1 µm) were observed which are indicated in figure 3a with a white circle. EDX analysis showed that these nodules consisted of calcium phosphate, which is depicted in fig 3b.

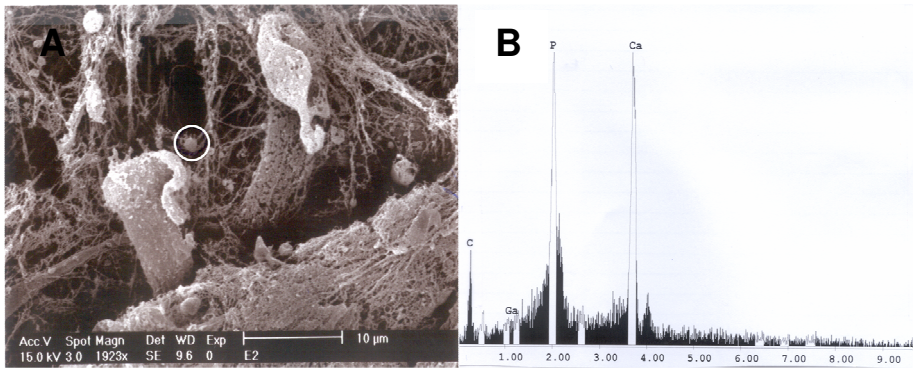

Figure 3. *Scanning electron micrograph (fig A) of GBMSCs grown on OsSatura[tm] BCP scaffolds after 19 days of dynamic proliferation. White circle depicts calcium phosphate nodule as determined by EDX analysis (fig B).*

Measurement of ingoing and outgoing oxygen concentration during proliferation in both bioreactors is depicted in figure 4a and b. During proliferation, the inlet oxygen concentration was kept at a constant level (red line) by saturation of the medium in the oxygenator and the outlet oxygen concentration (blue line) decreased in time. The difference between the ingoing and outgoing oxygen concentration (Δ DO) is also depicted in figure 4a and b. The Δ DO increases during cultivation from approximately 3-4% directly after seeding to a maximum of about 32-34% after approximately 12 days.

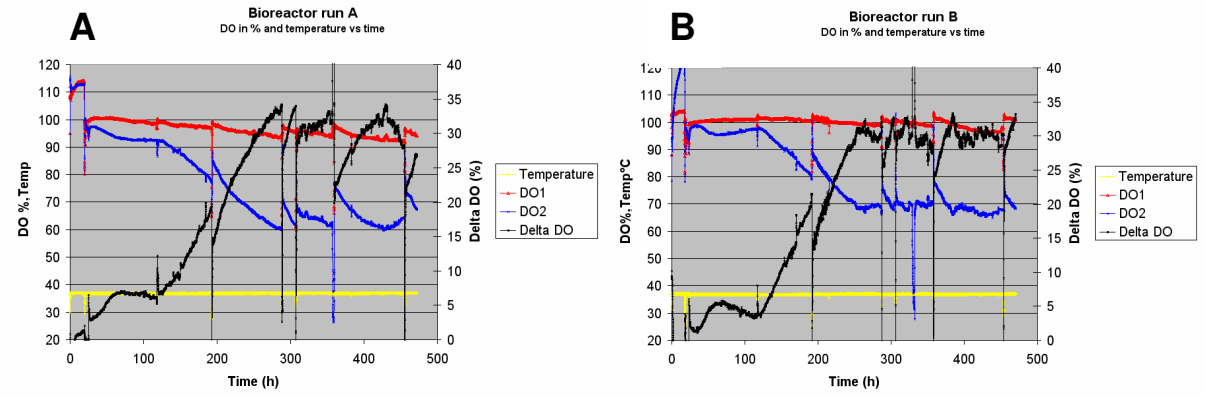

Figure 4*. On-line monitoring of dissolved oxygen concentration in the in (DO,1) and outlet (DO,2) medium and temperature during dynamic proliferation of GBMSCs on BCP scaffolds for two separate bioreactor runs (resp. A and B,). The net dissolved oxygen consumption (Δ DO = DO,in-DO,out) is also depicted. 100% indicates the concentration in cultivation medium, which is in equilibrium with the 20% oxygen in air.*

Disturbances indicated with an arrow are caused by opening the incubator and taking a sample of the bioreactor. Opening the incubator results in a temperature decrease influencing the dissolved oxygen measurement. After approximately 48 hours, the increase of the Δ DO was not developing exponentially. A plateau value of about 6-7% was reached and maintained during the next 72 hours. After 118 hours, medium was refreshed and exponential growth was resumed. This phenomenon, although

consistent, was not observed any more after the second medium refreshment. Therefore, depletion of a medium component is not likely to explain the delay in growth. The delay in growth could possibly be caused by lysing cells which were previously not removed. Exponential growth stopped after 12 days of cultivation. Because at this point the scaffolds were almost completely covered with cells, this could be caused by either diffusion or surface limitation.

Assuming exponential growth and no surface or diffusion limitation within the timeframe of 48 and 240 hours in figure 4a, a first estimation of the population doubling time can be calculated. An exponential fit according to the mathematical derivation of equation (5) in the materials and method section was used on the data. The ∆ DO is corrected for the previously mentioned temperature disturbance and the data in which the first plateau value is reached are omitted in fig 5.

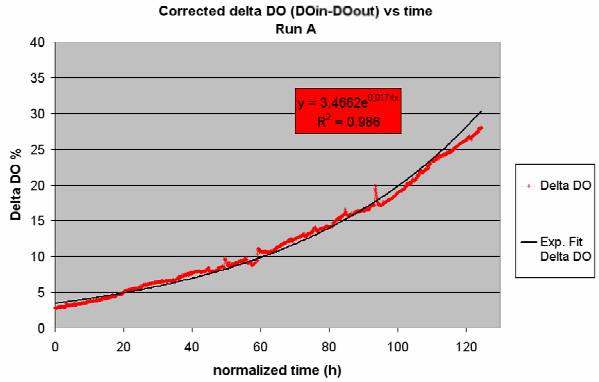

***Figure 5**. Net dissolved oxygen consumption (∆DO = DO,in – DO,out) during dynamic proliferation of run A, combined with an exponential fit in order to estimate the growth rate of GBMSCs on OsSatura^{tm} BCP scaffolds*

By fitting the ∆ DO with an exponential curve, the cell growth rate can be calculated. The obtained cell growth rate is 0.017 h^{-1} (doubling time, td = 40 hours). The same approach for a separate bioreactor run (data not shown) results in a cell growth rate of 0.019 h^{-1} (td = 36 hours) which is comparable to the population doubling time of 1 - 2 days which is generally found for GBMSCs cultured in tissue culture flasks (unpublished results).

In vivo experiments

After subcutaneous implantation, abundant bone formation could be observed in all hybrid constructs (fig 6). *De novo* formed bone was deposited against the walls of the scaffold material. In many samples, areas with mineralized bone (fig 6a, red color) and osteoid (fig 6a, pinkish color) could be identified. Osteocytes are visible within the bone matrix, and osteoblasts are present in a layer on top of the newly formed bone. Blood vessels were often associated with and in close proximity to newly formed bone. No difference was observed with respect to the static and dynamic culturing time of 12 and 19 days and the amount of newly formed bone.

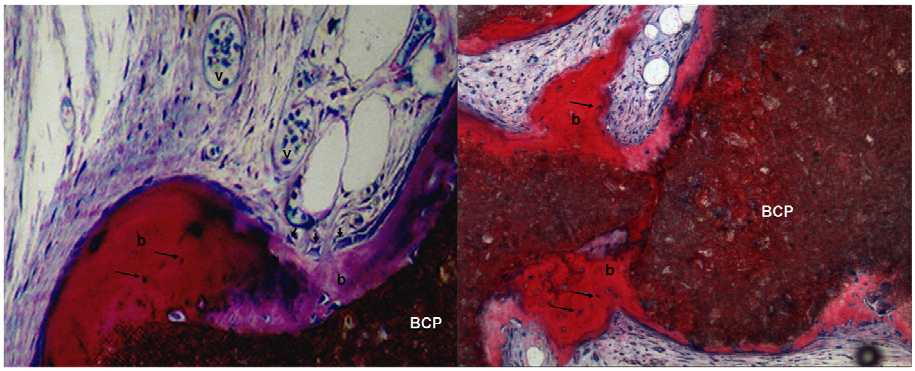

Figure 6. *Bone formation by GBMSCs after subcutaneous implantation of dynamically seeded and cultured hybrid constructs. New bone (b) is formed on the surface of the OsSatura^{tm} BCP scaffolds (BCP), and blood vessel (v) formation is visible in the vicinity of the newly formed bone tissue. Arrows designate embedded osteocytes and arrow head the layer of osteoblasts.*

DISCUSSION

Perfusion seeding, as demonstrated in this study, can be integrated in a system which enables seeding and proliferation of cells in a controlled way. Based on this principle, combined seeding and proliferation bioreactor systems have recently been used in engineering vascular grafts (30), cartilage (31) and cardiac (32) tissues. In this study we show for the first time that clinically relevant volumes (10cc) of tissue engineered bone can be produced by seeding and culturing GBMSCs on ceramic scaffolds in a direct perfusion bioreactor system. Although after cell seeding not all the cells were distributed homogeneously over the scaffold surface, an almost homogeneous and viable cell layer could be detected based on MB and MTT staining after 19 days of cultivation. Increasing cell seeding efficiency and a more equal distribution of cells on the scaffold material might result in a decreased proliferation time. Moreover, the initial distribution of cells is associated with the tissue formed within the construct and therefore implying that a more homogeneous cell seeding should lead to a more uniform tissue generation (17,33). Therefore, we are currently investigating the process of cell seeding and seeding efficiency in perfusion bioreactor systems. Measurement of oxygen consumption provides us with a tool to study the seeding process on line. In this study, we showed that oxygen consumption is a valuable tool to monitor cell growth during the cultivation period. The on line measurements can be used to estimate the growth rates of the GBMSCs on the scaffolds. An approximately tenfold increase in Δ DO would correlate with a tenfold increase in biomass concentration according to equation [3] described in the materials and methods section. Cell growth rates determined for GBMSCs are comparable to growth rates found in tissue culture flasks. Ultimately, determination of present cell numbers on the scaffold surface would verify the obtained growth rates as estimated by oxygen consumption. Until now, we have not been able to reliably determine viable cell numbers on the BCP scaffold material.

Bioreactors that perfuse medium exert a certain degree of fluid induced shear on the cells present on the scaffold material. Increasing evidence suggests that mechanical conditioning influences the activity of cells present on artificial carriers. Cultivation of osteoblast like cells (21) and rat bone marrow stem cells on 3D constructs in perfusion bioreactors have shown to enhance growth, differentiation and mineralized matrix production in vitro (22-24). Although it was not the aim of our study to

investigate the influence of mechanical forces in a perfusion bioreactor, abundant matrix formation containing calcium phosphate nodules was observed on and in the perfused osteogenic constructs which is consistent with the previously mentioned studies. The presence of calcium phosphate nodules might be indicative for differentiation under in vitro conditions.

Subcutaneous implantation of hybrid constructs in nude mice consisting of OsSatura BCP scaffolds and GBMSCs cultivated under dynamic and static conditions resulted in abundant de novo bone formation. These results are in agreement with previous studies of both hydroxy apatite (HA) and BCP ceramics with statically cultured GBMSCs implanted in nude mice. (13, 34). In addition, no distinct difference could be observed with respect to the static and dynamic culturing and the amount of newly formed bone. The overall small number of animals in this study does not allow a quantitative interpretation of the obtained results between these two groups. Eventually, bone formation in a critical size defect of hybrid constructs produced in bioreactors would result in proof of concept in a large animal model. Previous results showed that viable cells on BCP scaffolds resulted in more bone formation when implanted ectopically in goats when compared to the bare BCP scaffold (35). However, vascularity in an ectopic acceptor site is much higher when compared to an orthopic site. Survival of cells in large sized grafts for orthopedic reconstruction will be compromised amongst others due to the absence of vascularisation during the first week after implanting (36). Therefore, the ultimate challenge would be to obtain vascularisation within the osteogenic construct before implanting it in the acceptor site.

CONCLUSIONS

Dynamic seeding and culturing of goat bone marrow stromal cells on clinically relevant amounts of ceramic scaffold material is feasible by using a direct perfusion bioreactor system. After 19 days a homogeneous and viable cell layer could be observed based on MB and MTT staining which corresponded with on line measurements of oxygen consumption during the cultivation period. The hybrid structures became interconnected and a dense layer of extracellular matrix was present as visualized by environmental scanning electron microscopy. Subcutaneous implantation of dynamically cultured hybrid constructs resulted in abundant de novo bone formation in nude mice, which was at least comparable to the amount of bone formed by the static control.

ACKNOWLEDGEMENTS

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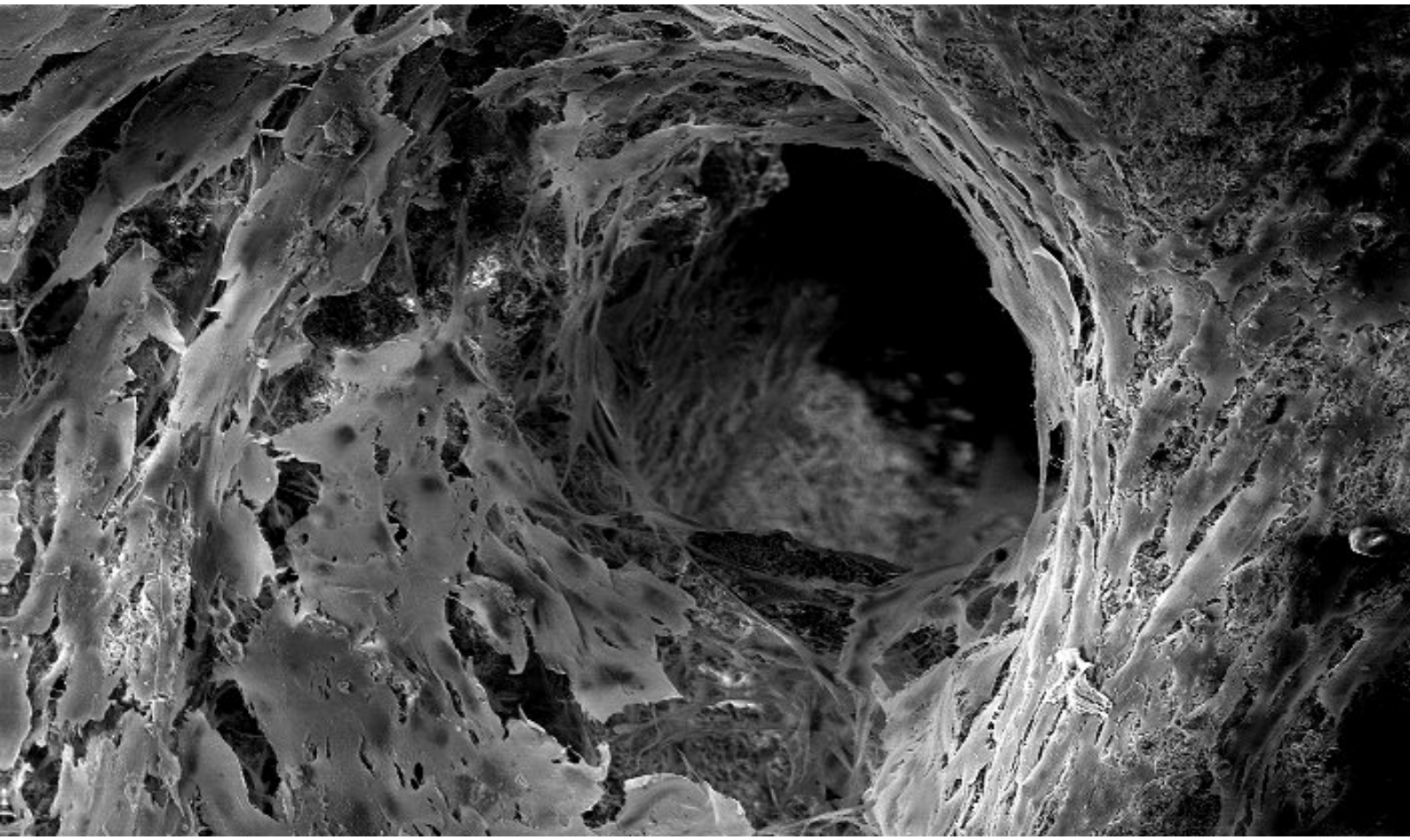
CHAPTER 5



“It could be wrong could be wrong,
but it should have been right”.

Resistance-Muse

Picture: Electron Scanning Micrograph (SEM) of a pore in a scaffold. Resistance is generated by perfusion flow through these pores and positive effects are reported in literature with respect to ostegenic differentiation in vitro and bone formation in vivo(this thesis).

CHAPTER 5

HUMAN TISSUE ENGINEERED BONE PRODUCED IN CLINICALLY RELEVANT AMOUNTS USING A SEMI-AUTOMATED PERFUSION BIOREACTOR SYSTEM: A PRELIMINARY STUDY

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ABSTRACT

The aim of this study was to evaluate a semi automated perfusion bioreactor system for the production of clinically relevant amounts of human tissue engineered bone. Human bone marrow stromal cells (hBMSCs) of 8 donors were dynamically seeded and proliferated in a perfusion bioreactor system in clinically relevant volumes (10 cm^3) of macroporous biphasic calcium phosphate scaffolds (BCP particles, 2-6 mm). Cell load and distribution was shown using Methylene Blue staining. MTT staining was used to demonstrate viability of the present cells. After 20 days of cultivation, the particles were covered with a homogeneous layer of viable cells. Online oxygen measurements confirmed the proliferation of hBMSCs in the bioreactor. After 20 days of cultivation, the hybrid constructs became interconnected and a dense layer of extracellular matrix was present as visualized by scanning electron microscopy (SEM). Furthermore, the hBMSCs showed differentiation towards the osteogenic lineage as was indicated by collagen type I production and alkaline phosphatase (ALP) expression. We observed no significant differences in osteogenic gene expression profiles between static and dynamic conditions like ALP, BMP2, Id1, Id2, Smad6, collagen type I, osteocalcin, osteonectin and S100A4. For the donors that showed bone formation, dynamically cultured hybrid constructs showed the same amount of bone as the statically cultured hybrid constructs. Based on these results, we conclude that a semi automated perfusion bioreactor system is capable of producing clinically relevant and viable amounts of human tissue engineered bone that exhibit bone forming potential after implantation in nude mice.

INTRODUCTION

Bone marrow stromal cells (BMSCs) have been extensively investigated both in experimental and clinical settings. These cells exhibit multi-potency (1-3) which potentially enable them to be used for the treatment of several diseases (4,5). In the field of tissue engineering, BMSCs have been used for bone repair since one of their most important differentiation pathways seems to be osteogenic (6). The proof of concept for bone tissue engineering by combining bone marrow stem cells with suitable biomaterials (hybrid constructs) has been shown both ectopically (7,8,12) and orthotopically in rodent studies (9-12).

Although cell based bone tissue engineering is a promising concept, there are still some problems which have to be solved in order to be clinically applicable (13). Firstly, osteogenic constructs are often produced by isolating osteoprogenitor cells from a marrow aspirate which are multiplied in tissue culture flasks and subsequently seeded on and in a three-dimensional scaffold (14,15). It has been reported that the average amount of adult stem cells that can be differentiated into the osteogenic lineage from a patient is only about 1-10 per 100000 cells present in the bone marrow (16), while 200-800 million adult stem cells are required for clinical use to repair a large bone defect [17]. Taken into account that an average bone marrow aspirate of 10 ml will harvest between 500 and 8000 adult stem cells, up to a million-fold multiplication is needed for clinical treatment. For large scale-production, the current 2D multiplication process in tissue culture flasks has some serious drawbacks. The flasks are limited in their productivity by the number of cells that can be supported by a given area, while repeated handling for culture maintenance makes the process labor-intensive and susceptible to contamination. Moreover, the microenvironment of the cells is not monitored and controlled which results in sub-optimal culture conditions (18). Furthermore, the 2D proliferation of these cells is not comparable with the in vivo situation. It has been shown that 2D expanded BMSCs have a diminished differentiation capacity in comparison with those found in fresh bone marrow (19,20). It is hypothesized that a 3D culture system may represent a physiologically more favourable environment for BMSCs than a tissue culture flask, as shown for several cell types (21).

Another challenge that complicates the clinical application of BMSCs in bone tissue engineering is the available amount of tissue engineered product. Clinically useful volumes of hybrid constructs for spinal surgery vary from 4 to 15 cm^3 (22), whereas these amounts are often more than 20 cm^3 for some other orthopedic applications (23). Production of these amounts of hybrid construct is complicated because of potential mass transfer limitations with respect to the supply of oxygen and medium components. It is well known that mass transfer limitations occur during in vitro culturing of various 3D constructs, resulting in a limited amount of cell growth into the 3D construct (24,25). Bioreactors that perfuse medium through scaffolds allow the reduction of internal mass-transfer limitations and the exertion of mechanical forces by fluid flow (26). Cultivation of osteoblast like cells and rat bone marrow stem cells on 3D constructs in perfusion bioreactors have shown to enhance growth, differentiation and mineralized matrix production in vitro (27-30). Only few studies have shown in vivo bone formation of animal derived hybrid constructs cultivated in perfusion bioreactors (31,32). None of these studies were performed using human

BMSCs. Previously, we have reported a direct perfusion bioreactor system which can drastically reduce the amount of space and handling steps involved and increase the volume of tissue engineered product for bone tissue engineering. Furthermore, this system allowed the online monitoring of oxygen consumption during seeding and cultivation of the hybrid constructs (33). We demonstrated that the produced hybrid constructs (using goat BMSCs as a model system) gave rise to in vivo bone formation after implantation in nude mice (32), whereas the bare scaffold showed no osteoinductive potential in nude mice (34). In this study we evaluated the direct perfusion system for human bone tissue engineering. We report the cultivation of human BMSCs (hBMSCs) from 8 different donors in 13 independent bioreactor runs. The runs varied in seeding density, perfusion rate and cultivation time. The obtained hybrid constructs were evaluated with respect to cell load, viability, in vitro differentiation and in vivo bone formation.

MATERIAL AND METHODS

Production of human hybrid constructs

Initial cell culturing of hBMSCs in tissue culture flasks

Human bone marrow aspirates were obtained from 8 patients that had given written informed consent. The aspirates were isolated from the iliac crest and cultured as described before in detail (20). Culture medium comprised of α-MEM supplemented with 10% FBS, antibiotics, 0.1 mM L-ascorbic acid-2-phosphate, 2 mM L-glutamine, $1*10^{-5}$ mM Dexamethasone and 1 ng/ml basic fibroblast growth factor (bFGF). hBMSCs were cultured at 37°C in a humid atmosphere with 5% CO_2. At the end of the first passage (P1), the cells were cryopreserved. Within 12 months, the cryopreserved cells were thawed and replated in tissue culture flasks. When cells were near confluence, the cells were washed with phosphate buffered saline (PBS), enzymatically released by means of a 0.25% trypsin-EDTA solution and replated at a density of 5000 cells/cm2. Subsequent passages were performed when cells were near confluence, usually 4-5 days later.

Scaffolds

Biphasic calcium phosphate scaffolds (BCP, OsSatura^{tm}, IsoTis, The Netherlands) were made of 36% macroporous (pores > 100 μm) biphasic calcium phosphate. The total porosity of these scaffolds was 59% (average interconnected pore size = 388 μm of all the pores > 100 μm) as measured by Hg porosity measurement. BCP scaffolds were produced according to the H_2O_2 method including naphthalene as described before (34). The material was sintered at 1200°C. The ceramic consisted of 80 +/-5% hydroxyapatite (HA) and 20 +/- 5% tricalcium phosphate (TCP) as confirmed by X-ray diffraction and Fourier Transformed Infrared spectroscopy (FITR), no additional impurities were detected. Granules of ∅ 2-6 mm were γ- irradiated at a minimal dose of 25 Kgray. 10 cm^3 of scaffold material consisted of 230 scaffolds +/- 10%. The packed scaffold bed inside the bioreactor was visualized by Micro CT imaging and is depicted in figure 1.

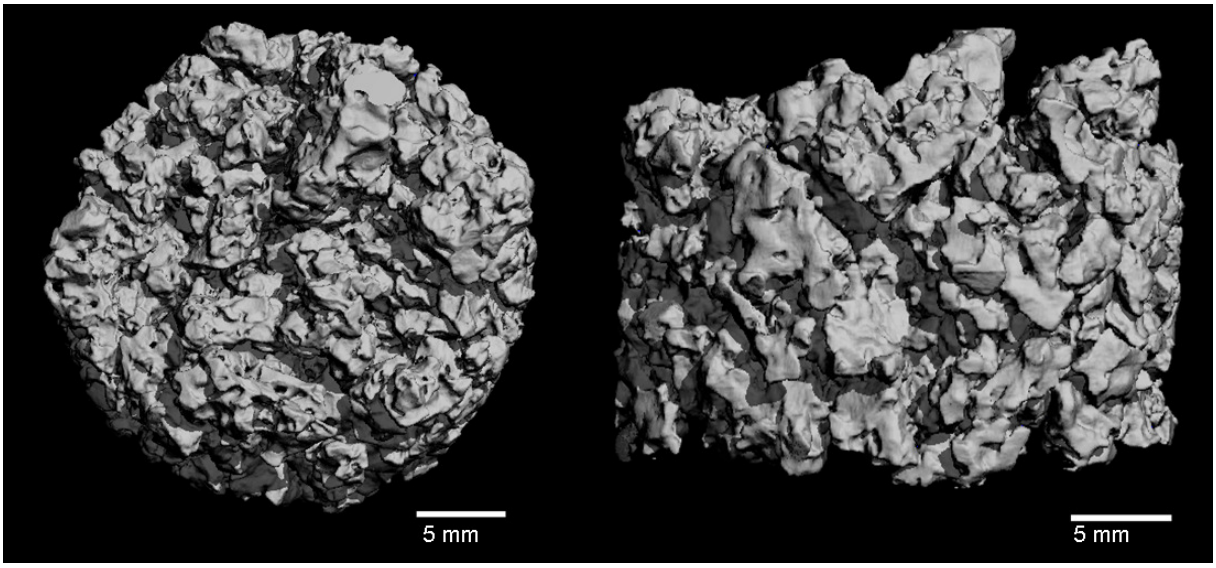

Figure 1. *Micro CT images of the packed scaffold bed inside the bioreactor. View from the top (left) and the side (right)*

Compaction density of the randomly stacked scaffold bed was 0.81 +/- 0.02 as determined for 5 different 10 cm^3 batches.

The semi automated bioreactor and bioreactor system

A direct perfusion flow bioreactor was used as described previously (32,33) and is schematically shown in figure 2.

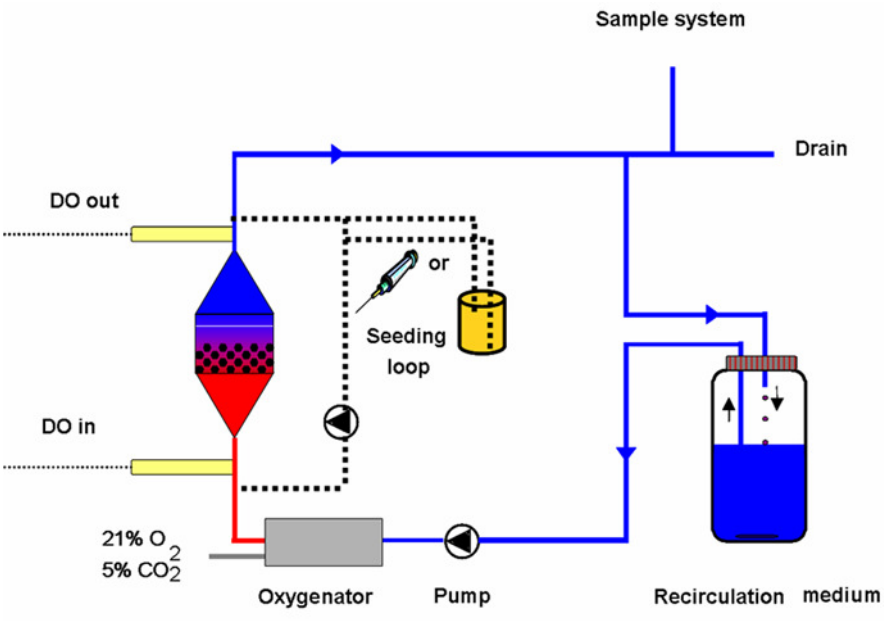

Figure 2. *Process scheme bioreactor system. Medium is perfused from the bottom to the top allowing a mediumflow over and through the scaffold bed (stacked black dots). Two loops can be distinguished: a seeding loop (dashed line) and a proliferation loop (solid line).*

In short, the bioreactor consists of an inner and outer housing, which were configured as coaxially disposed, nested cylinders. The bioreactor system comprised a

bioreactor, a sterile fluid pathway (made of γ sterilized PVC tubing, which had low gas permeability) that includes a medium supply vessel, a pump, an oxygenator and a waste vessel. The individual components of the bioreactor system can be detached in a sterile way by using a tube sealer (Teruseal^{tm}, Terumo). After sampling, these components can be attached again in a sterile way using a tube welder (TSCD^{tm}, Terumo). The fluid pathway contained a temperature sensor and two dissolved oxygen sensors, which were placed at the medium inlet and outlet of the bioreactor.

The whole bioreactor system was placed in a temperature controlled box (incubation unit), which was kept at 37°C. These incubation units lack a gas-controlled atmosphere and to supply the cells with oxygen and carbon dioxide an oxygenator was developed. The oxygenator comprised a closed chamber containing a gas-permeable silicon tube. The gas environment in the chamber was kept at a constant level of 20% O_2 and 5% CO_2 and medium was pumped through the gas-permeable tube. This system enables a medium flow through the bioreactor of a constant pH and a constant oxygen concentration.

Seeding and culturing of hBMSCs in the bioreactor system

hBMSCs, cultured as described before, were suspended in culture medium and transported into a seeding vessel which was attached to the seeding loop of the bioreactor system described in figure 1. Before cell seeding, 10 cm^3 of scaffold material in the bioreactor (dimensions of the bioreactor chamber: diameter 2.8 cm, height 2.6 cm) was flushed with cultivation medium in order to pre-wet the particles and allow serum proteins to attach to the scaffold surface. hBMSCs of various passages and in different concentrations were suspended in 20 ml of cultivation medium and seeded on 10 cm^3 of scaffold material in a bioreactor system. hBMSCs of 8 different human donors were used in a total of 13 separate bioreactor runs. An overview of all the experiments executed is depicted in table 1.

***Table 1**. Schematic overview of the bioreactor runs and process conditions*

Run #	Donor #	Gender/Age	Passage #	Amounts of HBMSC seeded (*10^6)	Cultivation time (days)
1	1	M/72	2	1	20
2	1		2	6	20
3	1		2	12	20
4	2	M/ 44	2	12	20
5	3	F/62	1	1	20
6	3		1	1	20
7	4	M/75	1	1	20
8	4		1	4	20
9	5	F/ 24	5	12	40
10*	5		2	12	7
11*	6	M/ 63	2	12	7
12*	7	F/ 68	2	12	7
13*	8	M/ 21	2	12	7

*Hybrid constructs in run # 10-13 were seeded statically.

Cell seeding took place by closing the recirculation loop and circulating the cell suspension through the seeding loop for 4 hours at 4 ml/min (108 μm/s, unless stated

otherwise), flow direction from bottom to top. After seeding, the seeding loop was closed and fresh medium was flushed through the bioreactor and tubing into the waste vessel (connected to the drain) to remove any non adhered cells. After the flush period, the fluid path towards the drain was closed and, unless stated otherwise, medium recirculation was started at 4 ml/min (108 μm/s), in order to promote proliferation of the attached hBMSCs. The culture medium (with a composition as described before) in the recirculation loop was refreshed twice every week. During cultivation at 37°C, 3-5 scaffold samples were taken from 3 different positions of the bioreactor (top, middle and bottom section) at several time points. These samples were used for MB and MTT staining. At the end of the cultivation period, random scaffold samples were taken for quantitative PCR (run # 11-13) and implantation studies in nude mice (all runs).

Static seeding and culturing

Static control scaffolds (48 particles, approximately 2 cm^3) were statically seeded with hBMSCs placed in a 25 wells bacteriological grade plate. Particles were placed in groups of 3 particles and 100 μl of a cell suspension (with a cell density comparable to the dynamic cell suspension) was applied on top of the particles. Cells were allowed to attach for 4 hours at 37°C, after which an additional 2 ml of culture medium was added to each well. Cells were statically cultured at 37°C in a humid atmosphere with 5% CO_2 and the cultivation medium was changed twice every week. Culture times of the static hybrid constructs were identical to the dynamically cultured constructs (see table1).

Online oxygen measurement

The oxygen concentration was measured online in the medium inlet and medium outlet during dynamic proliferation as can be seen in the process scheme in figure 1. The oxygen electrodes used were sterilizable dissolved oxygen sensors from Applisens (Applikon, the Netherlands). In previous studies we showed that the difference in oxygen concentration between the medium inlet and medium outlet (Δ DO), when assuming a constant specific oxygen consumption (qo), liquid volume of the bioreactor (Vl) and perfusion flow rate (Fl), is directly proportional to the biomass concentration (32,33).

Measurement of metabolites

Metabolites in the cultivation medium (glucose, lactate and ammonia) were measured in time using the vitros DT 60 medium analyzer. Dilutions for ammonia measurements were made in phosphate buffer pH 7.5.

2.2 Characterization of human hybrid constructs

Cell distribution, load and viability

Cell distribution and cell load on the particles in the bioreactor were qualitatively assessed by using MB staining. After sampling, cells on the particles were fixed in 1.5% glutaraldehyde in 0.14 M cacodylic buffer pH 7.4 ± 0.1 adjusted with 1M HCL. After fixation, 1% methylene blue solution was added and incubated for 60 seconds and washed twice with PBS in order to remove non-bound Methylene Blue. Cells on

the particles were visualized using light microscopy. For measuring cell viability, MTT staining was used. A solution of 1% MTT was applied on the particles containing cells. After 4 hours of incubation, the MTT solution was removed by flushing the particles with PBS. Particles and cells were visualized using light microscopy.

Alkaline phosphatase staining

Expression of alkaline phosphatase (ALP) was evaluated by an Azo-dye method. Briefly, hybrid constructs were washed twice with PBS and fixed for 2 hours in 4% paraformaldehyde. After washing the hybrid constructs twice with PBS, the samples were incubated in a Naphtol AS-BI phosphate solution (6-Bromo-2-phosphohydroxy-3-naphthoic acid o-anisidide) containing 0.1% w/w Fast Blue R salt (Sigma, The Netherlands) for 15 minutes at room temperature. Prior to incubation, the samples were incubated; the solution was filtered through a 0.2 μm filter in order to remove non dissolved Fast Blue R salt.

Collagen type I assay

Expression of collagen type I by bone marrow stem cells cultured on particles was determined by immunohistochemistry. Fresh samples of hybrid constructs were hydrated by washing them in 100% PBS at 37°C. PBS was removed and samples were blocked with 100% blocking buffer (BB, X0909, DAKO) for 60 minutes at room temperature. Dilutions of the primary antibody (Mouse monoclonal to Collagen type 1, reacts with human and goat, Abcam Ab23446, CSI 008-01) were made in PBS with 10% BB. Samples were incubated with the primary antibody at 4°C for 16 hours and subsequently washed 3 times with PBS with 10% BB. Samples were incubated with a secondary antibody (Rabbit polyclonal to mouse IgG with a Horse Radish Peroxidase conjugate, Abcam, ab6728) for 60 minutes at room temperature. Samples were washed 3 times with PBS. Subsequently a DAB chromogen solution was prepared by adding 3 drops of DAB solution (3, 3'-diaminobenzidine chromogen solution, DAKO) in 1 ml of DAB buffer (buffersolution pH 7,5 DAKO). Samples were incubated in 100 μl of DAB chromogen solution for 10 minutes at room temperature. Positive (goat bone) and negative (incubations without primary antibody and particles without cells) controls were also included in this experiment.

SEM and EDX analysis

After the in vitro cultivation period, matrix formation was examined by scanning electron microscopy (ESEM) and energy dispersive x-ray analysis (EDX). Samples from cell-scaffold constructs for ESEM analysis were fixed, dehydrated, gold coated and examined in an environmental scanning electron microscope (ESEM; XL30, ESEM-FEG, Philips, The Netherlands). EDX analysis was used to identify the chemical composition of structures present on the particles.

RNA isolation and quantitative PCR

The effect of static and dynamic culture systems on expression of osteogenic marker genes was analyzed by isolating RNA at the end of the culture period for bioreactor run # 11-13. The RNA was isolated by a Trizol RNA kit (Qiagen) and DNase treated with 10U RNase free DNase I (Gibco) at 37°C for 30 minutes. DNAse was inactivated at 72°C for 15 minutes. Two μg of RNA was used for first strand cDNA synthesis

using Superscript II (Invitrogen) according to the manufacturer's protocol. One µl of 100x diluted cDNA was used for collagen type 1 (COL1) and 18s rRNA amplification and 1 µl of undiluted cDNA was used for other genes. PCR was performed on a Light Cycler real time PCR machine (Roche) using a SYBR green I master mix (Invitrogen). Data was analyzed using Light Cycler software version 3.5.3, using fit point method by setting the noise band to the exponential phase of the reaction to exclude background fluorescence. Expression of osteogenic marker genes are calculated relative to 18s rRNA levels by the comparative ΔCT method (35) and statistical significance was found using student's t test ($P<0.05$).

In vivo bone formation

Random scaffold samples from bioreactors were taken after the in vitro cultivation period in a sterile LAF cabinet and were soaked in α-MEM supplemented with 1% Penicilline/Streptomycine. Prior to implantation, the samples were washed in PBS. Control scaffold samples which were statically seeded and cultured with cells for the same period of time were also implanted. Nude male mice (Hsd-cpb:NMRI-nu, Harlan) were anaesthetized by isofluorine inhalation, and subcutaneous pockets were made. The number of implanted dynamically cultured hybrid constructs, the number of mice used and a picture of the subcutaneous implantation are depicted in figure 3.

Run #	Cultivation time in vitro (days)	Number of cultured hybrid constructs implanted per mouse	Number of mice used	Total number of implanted cultured hybrid constructs
1-8	20	6	2	12
9	40	6	6	36
10-13	7	3	10	30

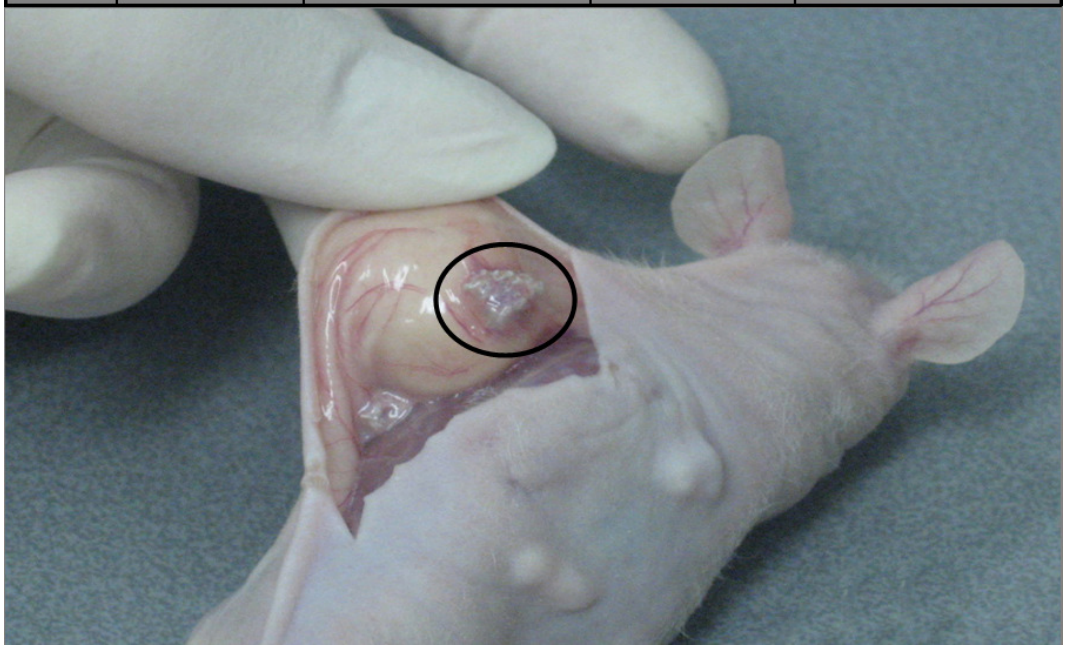

Figure 3. *Number of implanted dynamically cultured hybrid constructs and the numbers of mice used. Subcutaneous implantation of hybrid constructs is visualized. Black circle depicts a pocket containing 3 separate hybrid constructs.*

The incisions were closed using a vicryl 5-0 suture. After 6 weeks the mice were sacrificed using CO_2 and samples were explanted, fixed in 1.5% glutaraldehyde (Merck) in 0.14 M cacodylic acid (Fluka) buffer pH 7.3.

The fixed samples were dehydrated and embedded in methyl methacrylate (Sigma) for sectioning. Approximately 10µm thick, undecalcified sections were processed on a histological diamond saw (Leica Microtome, Nussloch, Germany). The sections were stained with 0.3% basic fuchsin and 1% methylene blue, in order to visualize bone formation. Histomorphometry was performed by scanning histological slides of stained sections of the whole hybrid constructs (run #10-13). At least three sections (from 3 separate hybrid constructs) for all conditions per mouse were made. From these scans, the surface area of the whole scaffold (region of interest, ROI), surface area of the BCP ceramic (MAT) and the surface area of formed bone (BONE) is determined. The ratio of total amount of bone formed as a percentage of the available pore area (BIP) is determined according to equation 1. The obtained results were tested for statistic significance using a two tailed student t-test ($p<0.05$).

$$BIP = BONE/(ROI-MAT)* 100\% \quad (1)$$

RESULTS & DISCUSSION

Production of human hybrid constructs in a perfusion bioreactor system

Because of known patient variability with respect to proliferation and differentiation capacity of hBMSCs (20,40), we cultured cells on BCP particles in the perfusion bioreactor system of 8 different donors in 13 separate runs. The runs varied in seeding density, perfusion rate and cultivation time (see table 1).

Effect of seeding density on hBMSC growth

In bioreactor runs # 1-3 and 7-8, cells were cultured in different seeding densities for 2 donors. In run # 1-3, cells were cultured in three seeding densities (donor # 1) varying from 1 to 12 million cells. Samples were taken after 3, 10 and 17 days and stained with MB to visualize cell load and cell distribution (figure 4.). Initially, more cells were visible on the particles for the highest seeding density (fig.4G). Between individual particles variations in cell load existed and cells were not distributed homogeneously over the scaffold surface at the first time points (fig.4A,D and G). An increase in cell load per scaffold in time was observed during the proliferation phase for all seeding densities. After 17 days, the particles were largely covered with a homogeneous cell layer for all seeding densities. At this time point, no differences were seen between the top, middle and bottom fraction of the bioreactor. Furthermore, there was no visual difference in cell load between the different seeding densities (fig.4C,F and I).

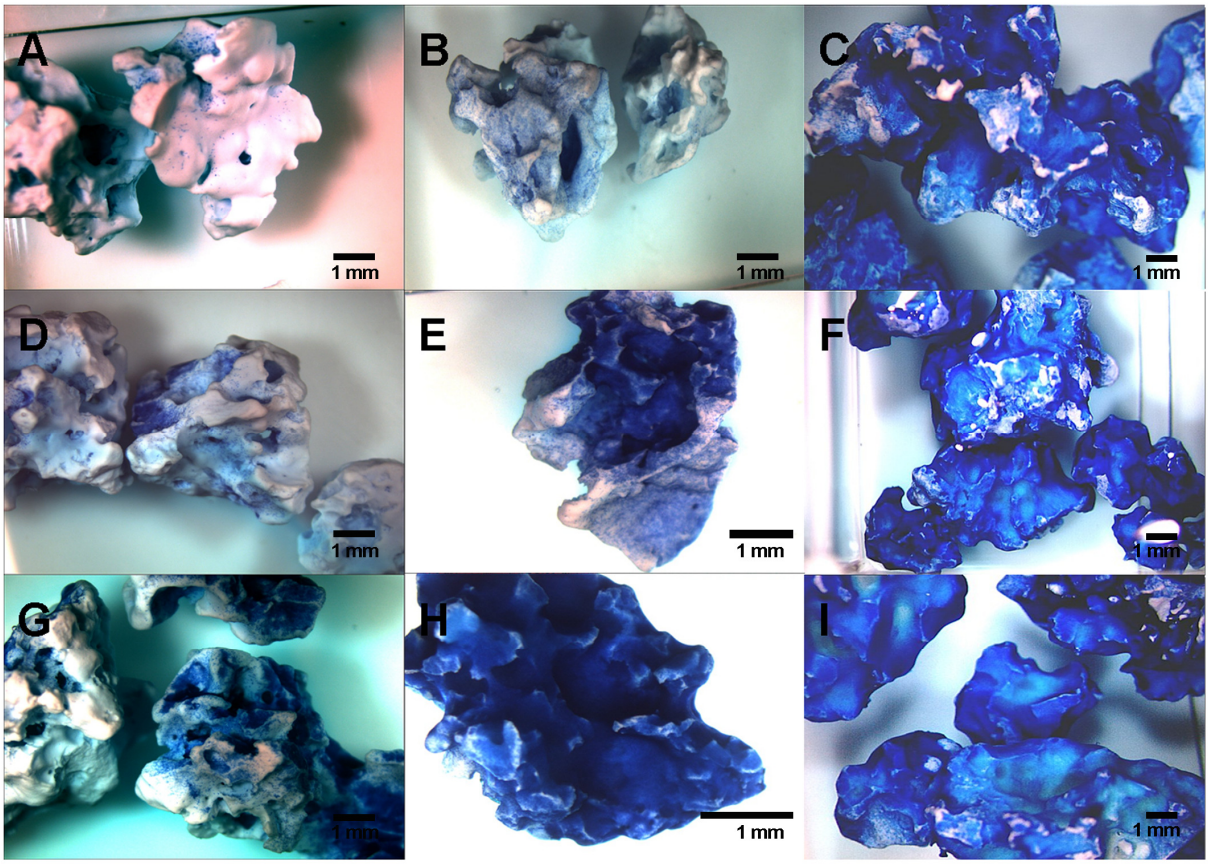

Figure 4. *MB stained hBMSCs on OsSatura^{tm} BCP particles after dynamic proliferation from left to right after 3, 10 and 17 days for three different seeding densities: 1*10^6 (A,B,C), 6*10^6 (D,E,F) and 12*10^6 (G,H,I),*

We observed identical trends for different seeding densities in run # 7 and 8 (data not shown). The oxygen consumption supported the observed cell growth patterns (figure 5). The ingoing and outgoing dissolved oxygen concentrations were measured during proliferation in all three bioreactor runs. During proliferation, the inlet oxygen concentration was kept at a constant level of 100% by saturation of the medium in the oxygenator and the outlet oxygen concentration decreased in time. The difference between the ingoing and outgoing oxygen concentration (Δ DO) for run # 1-3 is depicted in figure 5. The Δ DO increases during cultivation and plateau values of about 18% were reached for all seeding densities. This plateau value is reached after approximately 10, 13 and 19 days when seeding respectively 12, 6 and 1 million cells. During cultivation, the cell load on the particles could be correlated with the difference in oxygen consumption, which was already demonstrated for goat BMSCs (31). The arrows in figure 5 correspond with the time points at which the cell loads are depicted in figure 4. When the plateau values are reached (i.e. the Δ DO concentrations are equal for the 3 runs), the visually observed cell amounts present on the particles are identical. Furthermore, the oxygen consumption data were fitted in order to determine the growth rate of the cells on the particles as described before (32).

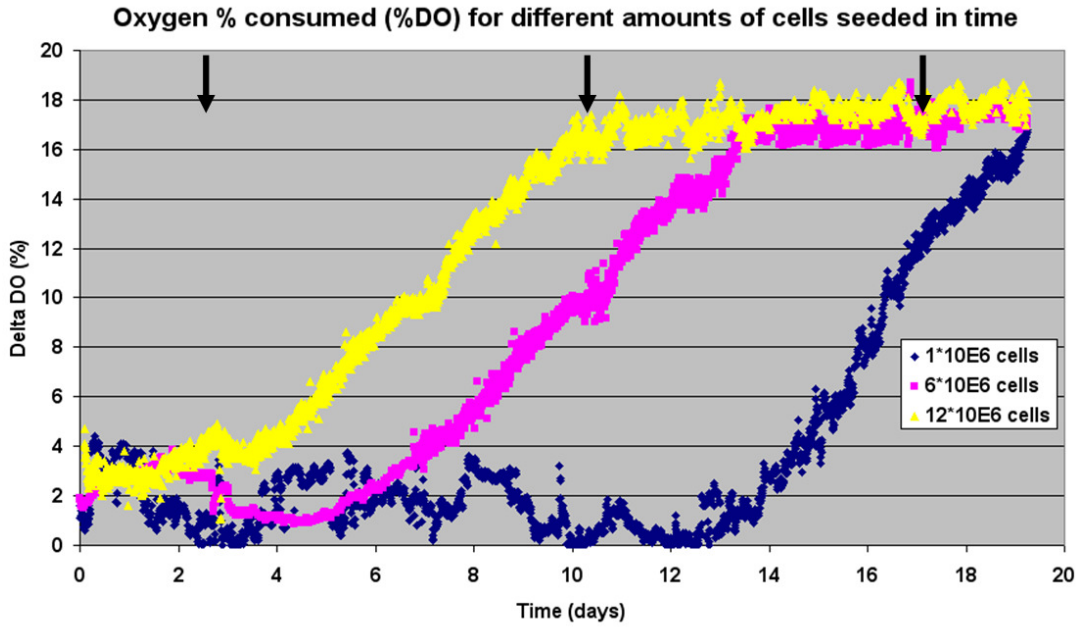

Figure 5. *Net dissolved oxygen consumption (∆DO = DO,in – DO,out) during dynamic proliferation of hBMSCs from donor #1 on OsSatura™ BCP particles for three different seeding densities: 1*10^6^, 6*10^6^ and 12*10^6^ cells. 100% indicates the concentration in cultivation medium, which is in equilibrium with the 20% oxygen in air. Arrows represent different time points (3,10 and 17 days) for which the cell load is visualized by MB staining in figure 4.*

Remarkably, an exponential fit of the data correlated not as well as a linear fit. Under optimal conditions, one would expect exponential cell growth, but apparently unknown factors are inhibiting the cell growth under these conditions. The data for both fits are shown in table 2.

Table 2. *Population doubling times, linear expansion factors and their correlation coefficient R^2 after resp exponential and linear fit of DO data of bioreactor run # 1-3*

Bioreactor run	Seeding density	Td (d) Exp. fit $y=xe^{ut}$ (R^2)	Expansion factor (d^{-1}) Lin. fit $y=ax+b$ (R^2)
#1	$1*10^6$	57 (0.94)	2.79 (0.98)
#2	$6*10^6$	61 (0.74)	1.86 (0.99)
#3	$12*10^6$	87 (0.94)	1.93 (0.98)

Cultures which were seeded at 6 and 12 million cells had a comparable expansion factor, whereas the culture seeded with 1 million cells had a significantly higher growth rate. This could be explained by the fact that cells, at lower seeding densities, experience less cell to cell contact. It has been described before that cell to cell contact can stimulate differentiation and inhibit proliferation. Furthermore, we have already reported higher cell proliferation rates when culturing human mesenchymal stem cells on 2D tissue culture flasks at low cell seeding densities when compared to higher cell seeding densities (36). In conclusion, hBMSCs were successfully seeded in different densities and proliferated in a perfusion bioreactor system.

Effect of perfusion rate on hBMSC growth

In run # 5 and 6 (donor # 3), cells were cultured at 1 and 4 ml/min respectively to evaluate the effect of the perfusion rate (pr) on cell growth. The difference between the ingoing and outgoing oxygen concentration (∆ DO) is depicted in figure 6.

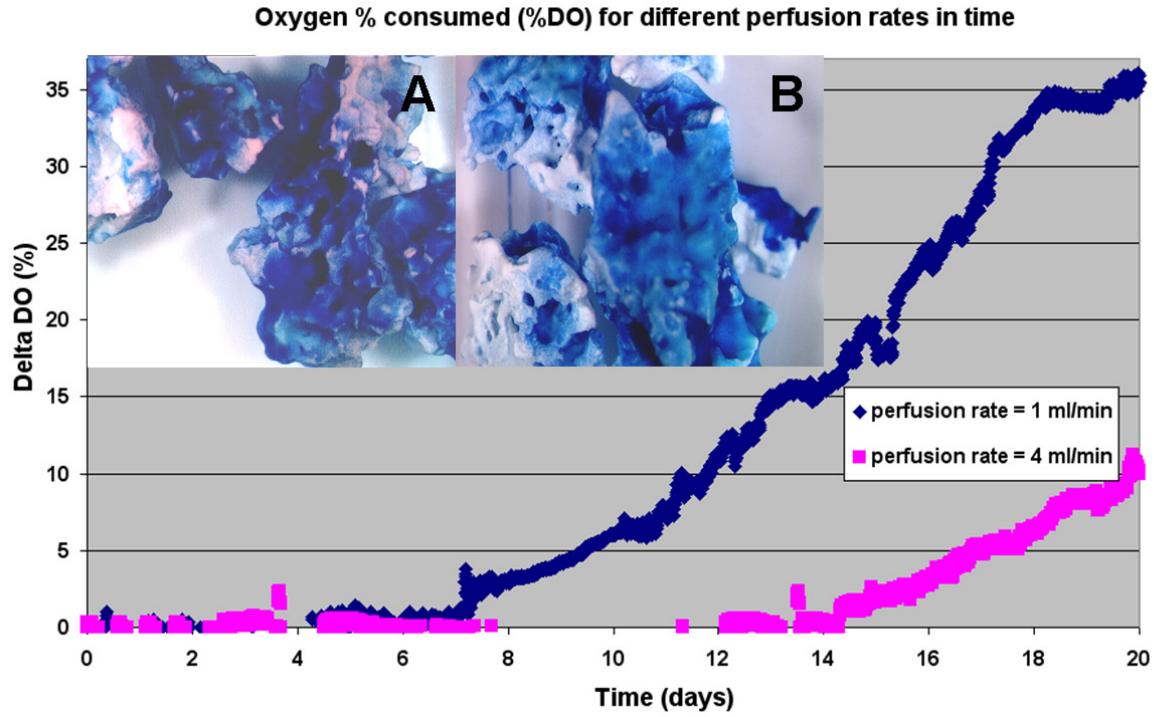

***Figure 6**. Net dissolved oxygen consumption (ΔDO = DO,in – DO,out) during dynamic proliferation of hBMSCs from donor #3 on OsSatura™ BCP particles for two perfusion rates: 1 and 4 ml/min. Picture A (1 ml/min) and B (4 ml/min) show MB stained hBMSCs after 18 days of proliferation. 100% indicates the concentration in cultivation medium, which is in equilibrium with the 20% oxygen in air.*

The Δ DO increases during cultivation for both perfusion rates, but the signal is detected after 7 days when using a perfusion rate of 1 ml/min compared to 15 days for 4 ml/min. Furthermore, a linear reciprocal relationship between the perfusion rate and the Δ DO was observed. The Δ DO reading of culture # 5 (pr 1 ml/min) is approximately 4 times higher than the reading of culture # 6 (pr 4 ml/min) as can be seen in figure 6. After 17 days of cultivation the Δ DO of culture # 5 is about 26% whereas the Δ DO of culture # 5 is about 6%. The difference in Δ DO equals the difference in perfusion rate between the two cultures. This can be explained in terms of the fluid residence time. A decrease in perfusion rate resulted in an increase of the average residence time of a fluid package in the bioreactor, allowing the present cells to consume more oxygen. After 20 days of cultivation, no difference in cell load could be detected between the two perfusion conditions. The particles were largely covered with cells which are depicted in figure 6a and b. In conclusion, no effect could be observed for the evaluated perfusion rates with respect to the cell load on the particles after cultivation.

Cell metabolism during cultivation

Cells were cultured in a bioreactor system for 40 days in run # 9 (donor # 5). Medium samples were analyzed for glucose, lactate and ammonia concentrations. During the cultivation, the glucose consumption increased as well as lactate and ammonia production (figure 7). Glucose is used as a carbon and energy source, and lactate and ammonia are waste products of cell metabolism. This indicated cell growth and correlated with an increase in Δ DO during cultivation. A thick layer of living cells was present on the particles even after 40 days of cultivation as was shown by MTT staining in figure 7.

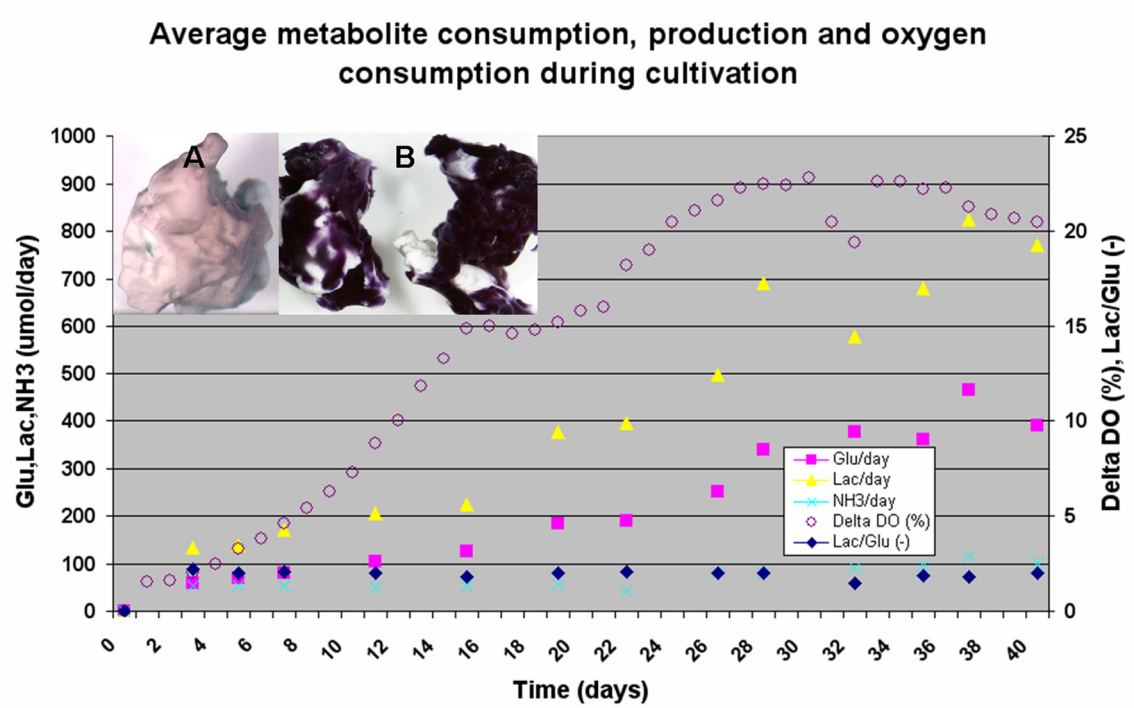

Figure 7. *Net dissolved oxygen consumption (ΔDO = DO,in – DO,out) and metabolite consumption and production during dynamic proliferation of hBMSCs from donor #5 on OsSatura[tm] BCP particles. Picture A shows the scaffold without cells and picture B shows MTT stained hBMSCs after 40 days of proliferation.*

The molar ratio of lactate produced and the amount of glucose consumed (Qlac/glu) is very close to 2 during the entire cultivation period. The same phenomenon was observed in other runs. This finding suggests that anaerobic glycolysis is the prevalent mechanism for glucose consumption as an energy source (37, 38). This was a surprising finding since 100% air saturated medium (containing 20% oxygen) enters the bioreactor. In all cases, we measured dissolved oxygen concentrations at the outlet above 70% of air saturation which does not represent a hypoxic environment for the cells. The fact that this mechanism is occurring in the presence of oxygen is a phenomenon known as the Warburg effect (39). At this time, the significance of this effect is not known to us but future research is going to be conducted in order to unravel the cell metabolism of human BMSCs under different oxygen conditions.

3.2 Characterization of human hybrid constructs produced in a perfusion bioreactor system.

In vitro characterization: SEM, Collagen type I, ALP expression and quantitative PCR

During visual inspection of the hBMSCs on the BCP particles, it was observed that the hybrid structures became interconnected. For all runs, a dense layer of extracellular matrix was present on and between the particles.

SEM

Characterization of the cell layer and the extra cellular matrix was done using SEM microscopy. Figure 8 shows the development of the cell layer on the BCP surface for bioreactor run # 1.

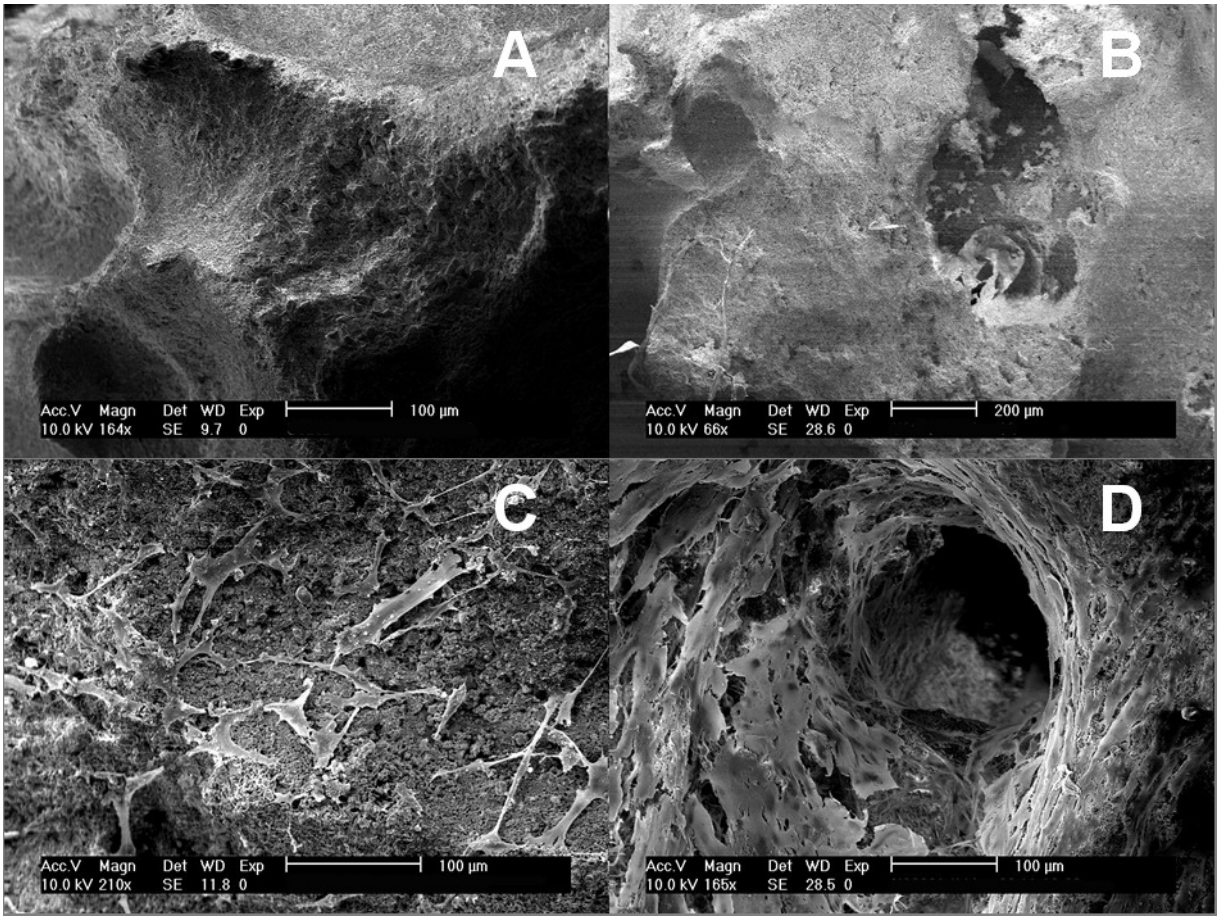

Figure 8. *Scanning electron micrograph of hBMSCs cultured on OsSatura^{tm} BCP particles in bioreactor run # 1 (seeding density 1*10^{6} cells). Scaffold after 0 days (A), after 3 days (B), after 10 days (C) and after 17 days.*

Particles seeded at 1 million cells were visualized during proliferation after 3, 10 and 17 days. Particles seeded at 1 million cells showed few cells after 3 days (fig 8b) but considerably more after 10 and 17 days (fig 8c and d). After 17 days the hybrid constructs of bioreactor run # 1 and 3 (donor # 1, seeding density respectively 1 and 12 million cells) showed no difference with respect to the appearance of the cell layer on the particles. This was in correspondence with the results of the MB staining shown in figure 4. On the SEM images, sphere like structures (+/- 0.5 - 1 µm) were observed throughout the whole construct. In figure 9, these nodules are indicated with a white arrow and circle for different magnifications. EDX analysis showed (red circle) that these nodules consisted of calcium phosphate which is indicative of calcium formation in vitro.

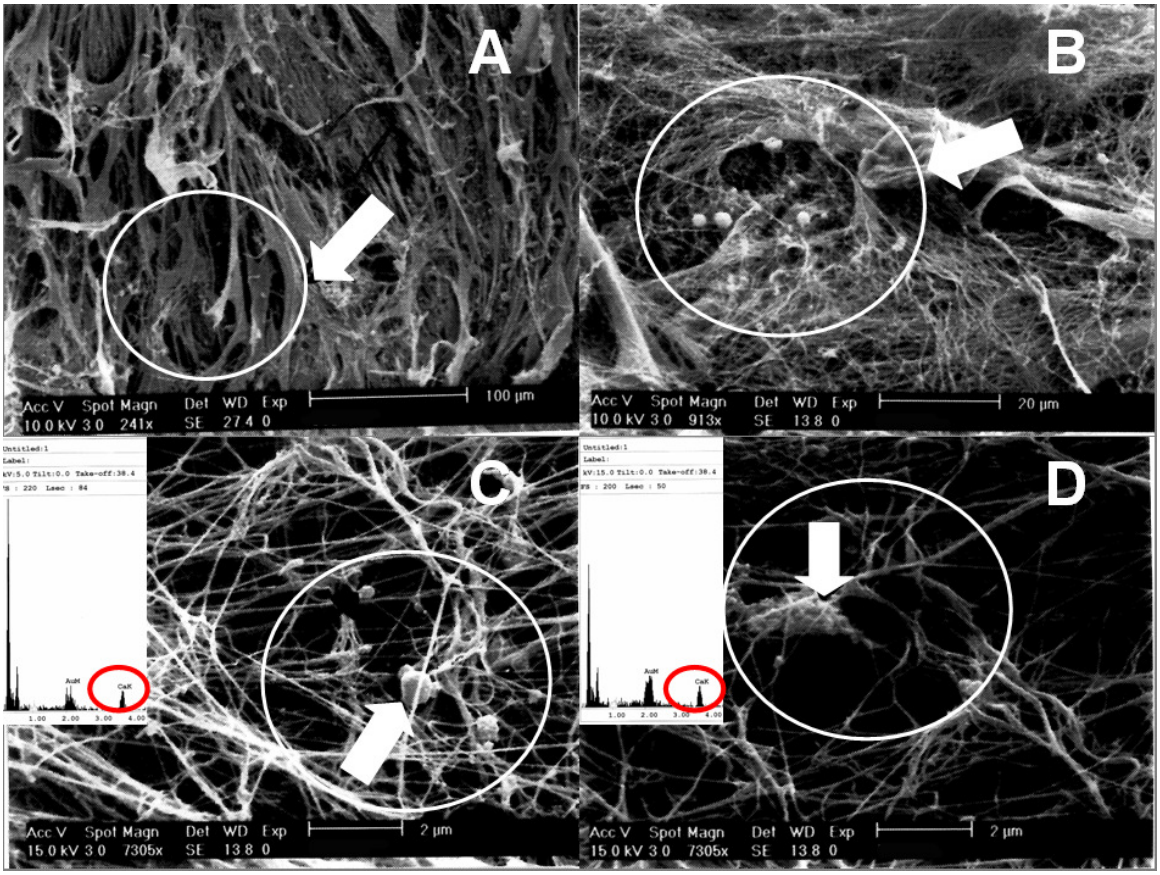

Figure 9. *Scanning electron micrograph of hBMSCs on OsSatura[tm] BCP particles in bioreactor run # 4 after 20 days of dynamic proliferation at different magnifications (A:241x, B:913x, C,D 7305x). White arrows and circles depict calcium phosphate nodules as determined by EDX analysis (fig 9C and D, red circle).*

Collagen type I and ALP expression

In order to confirm if the hybrid constructs show differentiation towards the osteogenic lineage, the presence of alkaline phosphatase (ALP) and collagen type I was investigated. The results are shown in figure 10. Dynamically cultured constructs showed abundant ALP and collagen type I expression (respectively fig 10a and c) whereas the controls with cells without primary antibody and controls without cells showed no ALP and collagen type I expression (fig 10b and d). The presence of extracellular matrix containing calcium phosphate nodules as well as ALP and collagen type I expression proves differentiation towards the osteogenic lineage under in vitro conditions which is in agreement with the previously mentioned studies (28-30). *Quantitative PCR*

We observed no significant differences in osteogenic gene expression profiles between static and dynamic conditions in bioreactor run # 11-13 (data not shown). There seemed to be a slightly higher but statistically insignificant ALP expression under dynamic conditions when compared to the statically cultured hybrid constructs. Other osteogenic genes such as Cbfa1, collagen type I, osteocalcin, osteonectin and negative regulator of mineralization S100A4 showed no significant difference between static and dynamic conditions. Although we observed a slightly higher induction of BMP2 expression under dynamic conditions, it did not reflect on its target gene expression such as Id1, Id2 or Smad6.

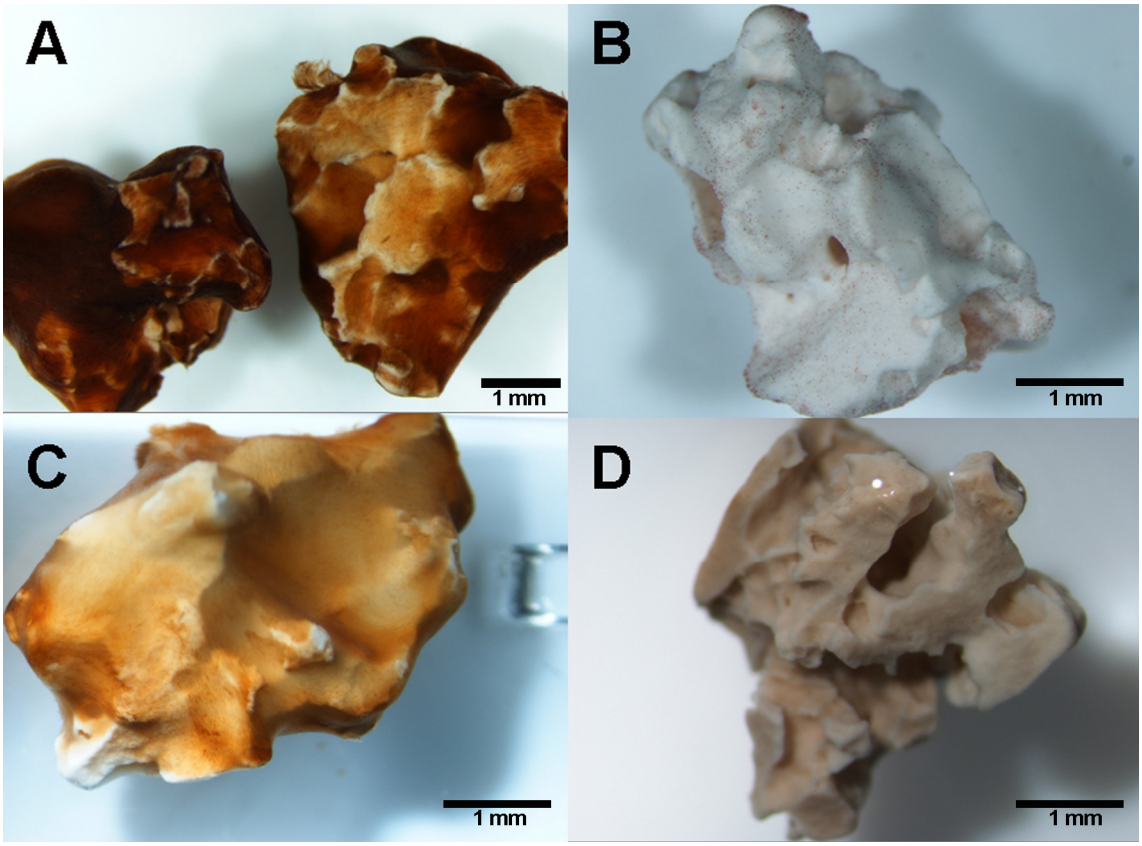

Figure 10. *ALP and collagen type I expression (resp fig 10a and c) on hybrid constructs from bioreactor run # 9 after 40 days of dynamic proliferation. The controls with cells without primary antibody and controls without cells showed no ALP and collagen type I expression (resp fig 10b and d).*

In vivo characterization: bone formation of hybrid constructs in nude mice

After subcutaneous implantation in nude mice for 6 weeks, the hybrid constructs were explanted, histologically processed and bone formation in vivo was assessed. The bone formation for the bioreactor runs is schematically depicted in table 3.

Table 3. *Bone formation for all bioreactor runs.*

Run #	Donor #	Passage #	Amounts of HBMSC seeded (*10^6)	In vivo: number of mice/ number of hybrid constructs implanted	In vivo bone formation: static	In vivo bone formation: dynamic
1	1	2	1	2/12	+	+
2	1	2	6	2/12	+	+
3	1	2	12	2/12	+	+
4	2	2	12	2/12	-	-
5	3	1	1	2/12	+	+
6	3	1	1	2/12	+	+
7	4	1	1	2/12	+	+
8	4	1	4	2/12	+	+
9	5	5	12	6/36	-	-
10	5	2	12	10/30	+	+
11	6	2	12	10/30	+	+
12	7	2	12	10/30	+	+
13	8	2	12	10/30	+	+

In 7 out of 8 donors, in vivo bone formation was observed in nude mice under both static and dynamic conditions. In figure 11, the in vivo bone formation of dynamically cultured hybrid constructs is shown for donors #1 (11A1-3) and #3 (11B1-2).

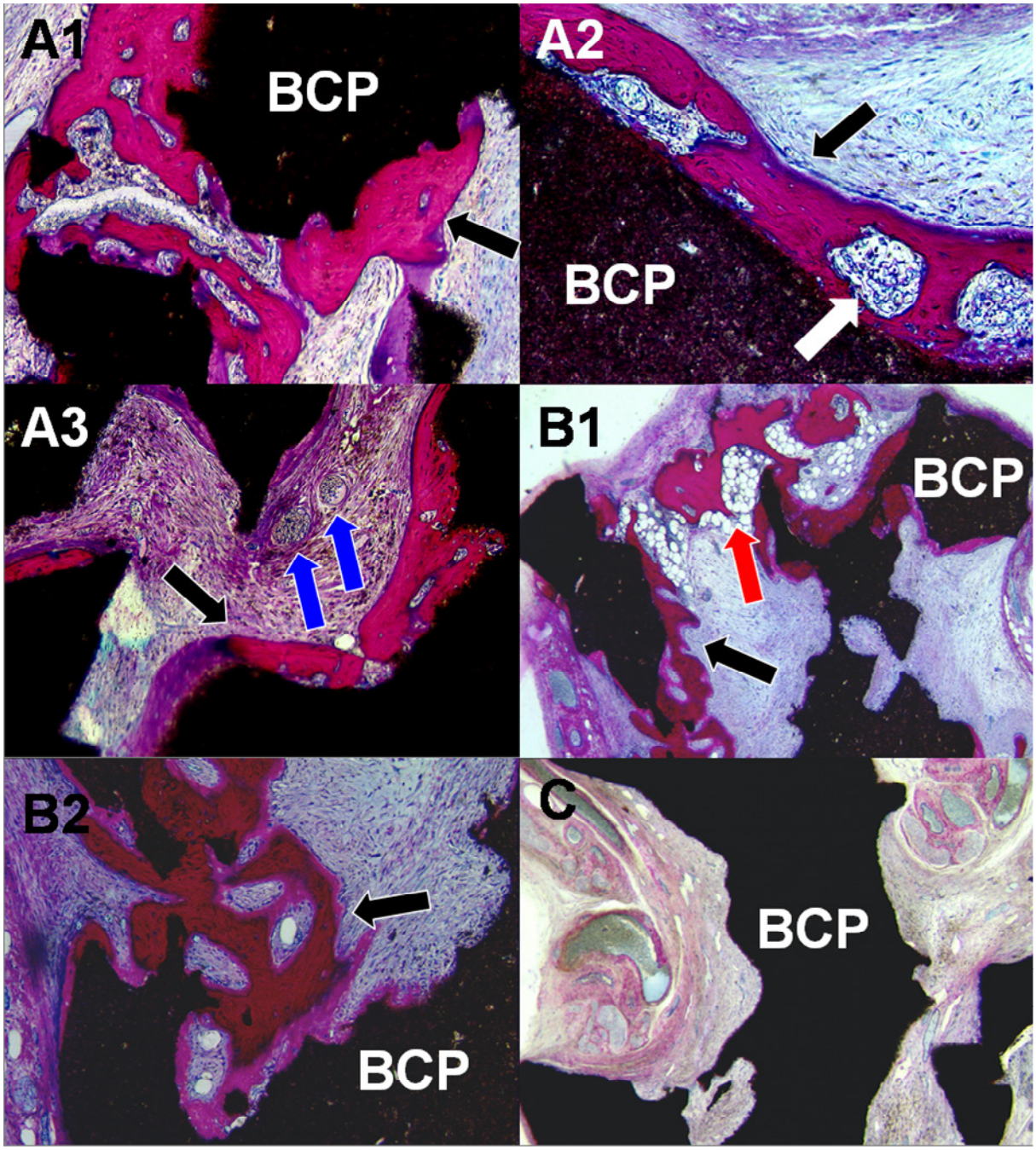

Figure 11. *Bone formation by HBMSCs after subcutaneous implantation of dynamically seeded and cultured hybrid constructs from donor #1 (A1-A3) and #3 (B1-B2). New bone (black arrow) is formed on the surface of the OsSatura[tm] BCP particles (BCP). Bone marrow (white arrow), blood vessel (blue arrow) and fat (red arrow) formation is visible close to newly formed bone tissue.*

The control scaffold without cells is depicted in figure 11C. *De novo* formed bone was deposited against the walls of the scaffold material. In many samples, areas with mineralized bone (fig 11A1-A3 and B1-2, red color) and osteoid (fig 11A3 and B1-2, pinkish color) could be identified. Osteocytes are visible within the bone matrix, and osteoblasts are present in a layer on top of the newly formed bone. Blood vessels

(blue arrow), bone marrow (white arrow) and fat cells (red arrow) were often associated with and in close proximity to newly formed bone.

Most of the statically and dynamically cultivated hybrid constructs showed bone formation in vivo. As reported before, an osteogenic phenotype as shown for the hybrid constructs from bioreactor runs # 4 and 9 in vitro is not predictive for osteogenisis in vivo (40). In 7 out of 8 donors, bone formation was observed in statically as well as dynamically cultured hybrid constructs. Therefore, this effect could not be explained by the difference in cultivation method. In vivo bone formation appears to be donor dependant (run # 4 vs run # 1-3 and 5-9) as well as passage dependant (run # 9 vs 10, of donor 5) which is in agreement with previous studies (20, 41-42). Varying the seeding density and the perfusion rate during dynamic culturing did not have a distinct effect on the in vivo bone formation. In addition, no relationship was found between bone formation and donor age or donor sex. This could be due to the fact that the amount of donors in our study is relatively small (n=8, from which only 5 randomly selected) when compared to previous work in which more donors were used and this correlation was seen (43, 44). On the other hand there are also studies which did not find a correlation between donor age and in vivo bone formation (45-47).

In order to assess bone formation quantitatively, we selected 5 donors (donors 5-8, bioreactor run #10-13) and cultivated hBMSCs under static and dynamic conditions on ceramic particles. The resulting human hybrid constructs were implanted in a statistic relevant number of nude mice. The in vivo bone formation was assessed quantitatively by histomorphometry and is depicted in figure 12.

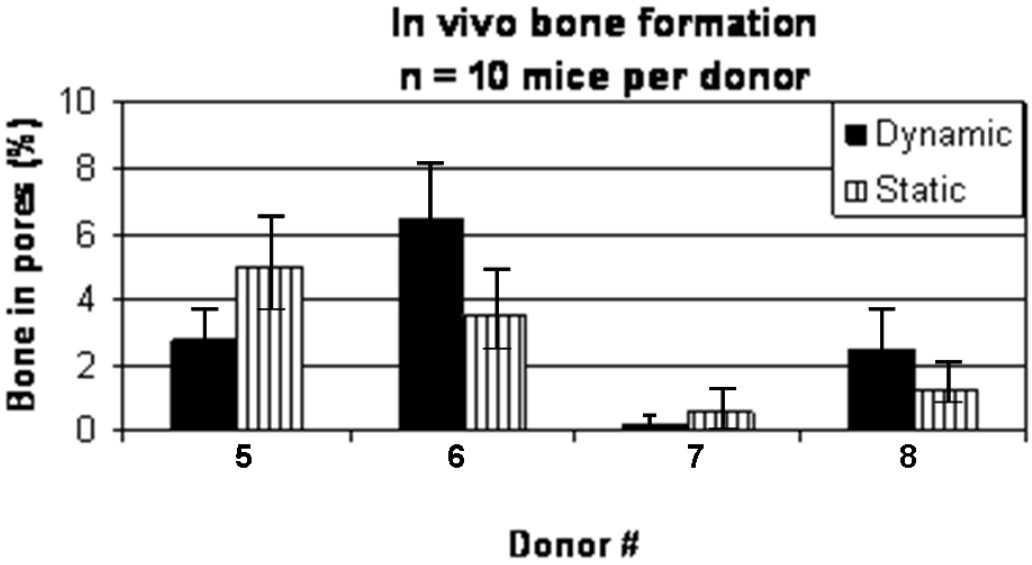

Figure 12. *Bone formation by hBMSCs after subcutaneous implantation of dynamically and statically cultured hybrid constructs from donor # 5-8. Bone formation was quantitatively assessed by histomorphometry. No significant differences ($p<0.05$) were observed between static and dynamic conditions for the donors.*

For these four donors, no statistic significant difference was found between statically and dynamically cultured hybrid constructs ($p<0.05$). These results differ from the results of Braccini et al (48). In their studies, implantation of dynamically cultured human hybrid constructs resulted in higher amounts of in vivo bone formation in nude mice when compared to statically cultured hybrid constructs. It is possible that the

discrepancy between these two studies is caused by the difference in primary isolation of the human BMSCs. In our study, preselection of hBMSCs took place by culturing in 2D tissue culture flasks whereas Braccini et al directly seeded a ficolled fraction on and in the ceramic scaffold. It has been reported before that subculturing cells in vitro in 2D tissue culture flasks induces loss of multipotency and in vivo bone formation (42). In the future, we will therefore attempt to isolate hBMSCs directly from bone marrow aspirates and seed and culture them on and in ceramic particles in our perfusion system.

Eventually, bone formation in a critical size defect of hybrid constructs produced in bioreactors would result in proof of concept in a large animal model. Previous results showed that viable cells on BCP scaffolds resulted in more bone formation when implanted ectopically in goats when compared to the bare BCP scaffold (49). However, vascularity in an ectopic acceptor site is much higher when compared to an orthopic site. Survival of cells in large sized grafts for orthopedic reconstruction will be compromised amongst others due to the absence of vascularisation during the first week after implanting (50). Therefore, the ultimate challenge would be to obtain vascularisation within the osteogenic construct before implanting it in the acceptor site. This concept is currently being investigated by several groups (51-53).

CONCLUSIONS

Dynamic seeding and culturing of human bone marrow stromal cells of different donors on clinically relevant amounts of ceramic scaffold material is feasible by using a semi automated perfusion bioreactor system. We showed that these cells could be seeded and proliferated on ceramic particles in different seeding densities and at different perfusion rates, until the particles were completely covered. After 20 days a homogeneous and viable cell layer could be observed based on MB and MTT staining which corresponded with on line measurements of oxygen consumption during the cultivation period. The hybrid structures became interconnected and a dense layer of extracellular matrix was present as visualized by environmental scanning electron microscopy. SEM images showed within the extra cellular matrix sphere like structures which were identified as calcium phosphate nodules by energy dispersive X-ray analysis (EDX). Furthermore, these cells show differentiation towards the osteogenic lineage as was shown by collagen type I production and ALP expression. We observed no significant differences in osteogenic gene expression profiles between static and dynamic conditions like ALP, BMP2, Id1, Id2, Smad6, collagen type I, osteocalcin, osteonectin and S100A4. Subcutaneous implantation of hybrid constructs in nude mice consisting of OsSatura BCP particles and human BMSCs cultivated under dynamic and static conditions resulted in de novo bone formation in a donor dependent way. In the 7 out of 8 donors that showed bone formation, dynamically cultured hybrid constructs showed the same amount of bone as the statically cultured hybrid constructs. When in vivo bone formation was quantitatively assessed by histomorphometry for 4 donors, no statistical significant difference was found between statically and dynamically cultured hybrid constructs.

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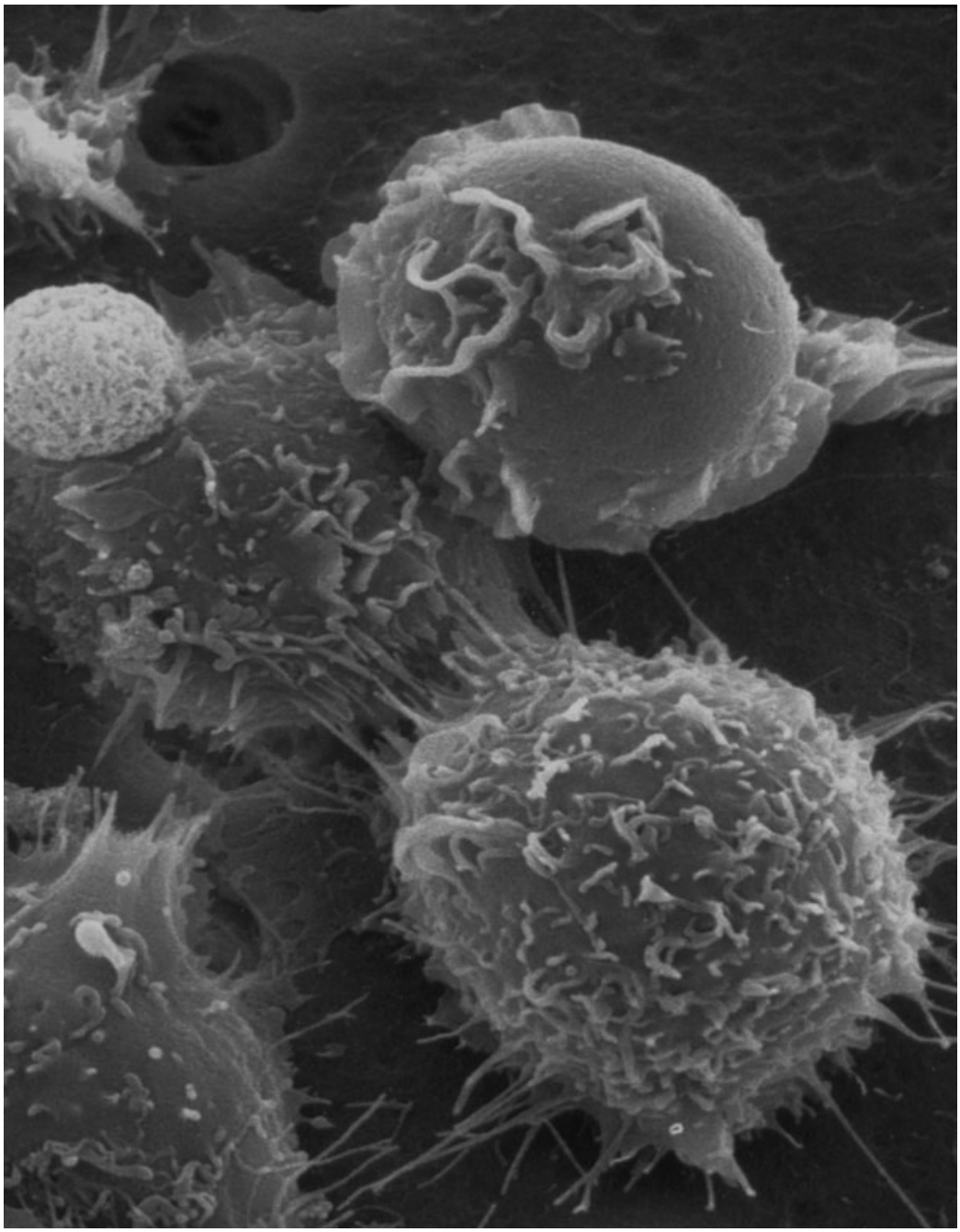
CHAPTER 6



"I like to wait to see how things turn out
If you apply some pressure"

Apply Some Pressure-Maximo Park

Picture: Multipotent bone marrow stem cells form the precursors to various types of blood cells. Adapted from:McLaren, A. Ethical and social considerations of stem cell research. Nature 2001; 414:129-131.

CHAPTER 6

BONE FROM UNPROCESSED BONE MARROW BIOPSIES
A one-step bioreactor approach towards the clinical application of tissue engineered bone

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ABSTRACT

Cell based bone tissue engineering is a promising alternative for replacing the autologous bone graft, but there are still issues to be resolved for it to be widely clinically applicable. Conventional two dimensional (2D) cell expansion in tissue culture flasks is laborious, material intensive, susceptible to contamination and not monitored and controlled. In this paper, we propose an alternative method to produce osteogenic constructs based on a semi automated bioreactor process. We show for the first time that these constructs can be produced directly from unprocessed bone marrow biopsies and conventional 2D cell expansion is completely circumvented. Goat Bone Marrow Stem cells (GBMSCs) could be seeded and proliferated on clinically relevant amounts of 3D ceramic scaffolds. The proliferation of GBMSCs on the scaffolds corresponded with online measurements of oxygen consumption and metabolite consumption and production. The hybrid structures became interconnected and a dense layer of extracellular matrix was present as visualized by environmental scanning electron microscopy (SEM). Within the extra cellular matrix, sphere like structures were observed which were identified as calcium phosphate nodules by energy dispersive X-ray analysis (EDX). Furthermore, these cells showed differentiation towards the osteogenic lineage as was shown by collagen type I production and ALP expression. Subcutaneous implantation of 3D dynamically produced hybrid constructs in nude mice showed at least the same amount of de novo bone formation as the controls. Furthermore, these constructs can be produced in a controlled and more cost efficient way which brings bone tissue engineering one step closer to application in clinical practice.

INTRODUCTION

The autologous bone transplant (autograft) is still the golden standard in many orthopedic interventions. There are however considerable drawbacks with respect to the use of autograft like limited availability, infection, donor site morbidity and post operative pain (1,2). Cell based bone tissue engineering could potentially circumvent these problems and the proof of concept of bone tissue engineering has been shown both in rodents (3-8) as well as in large animal models (9-12). A common approach in bone tissue engineering is the assembly of a hybrid construct consisting of a porous biodegradable matrix or scaffold to which cells can physically adhere. This in vitro tissue precursor is often combined with bioactive molecules to stimulate proliferation and/or osteogenic differentiation during the in vitro culture period. Finally, the hybrid construct is implanted into the defect site to induce and direct the growth of new bone as shown in figure 1 by the red arrows.

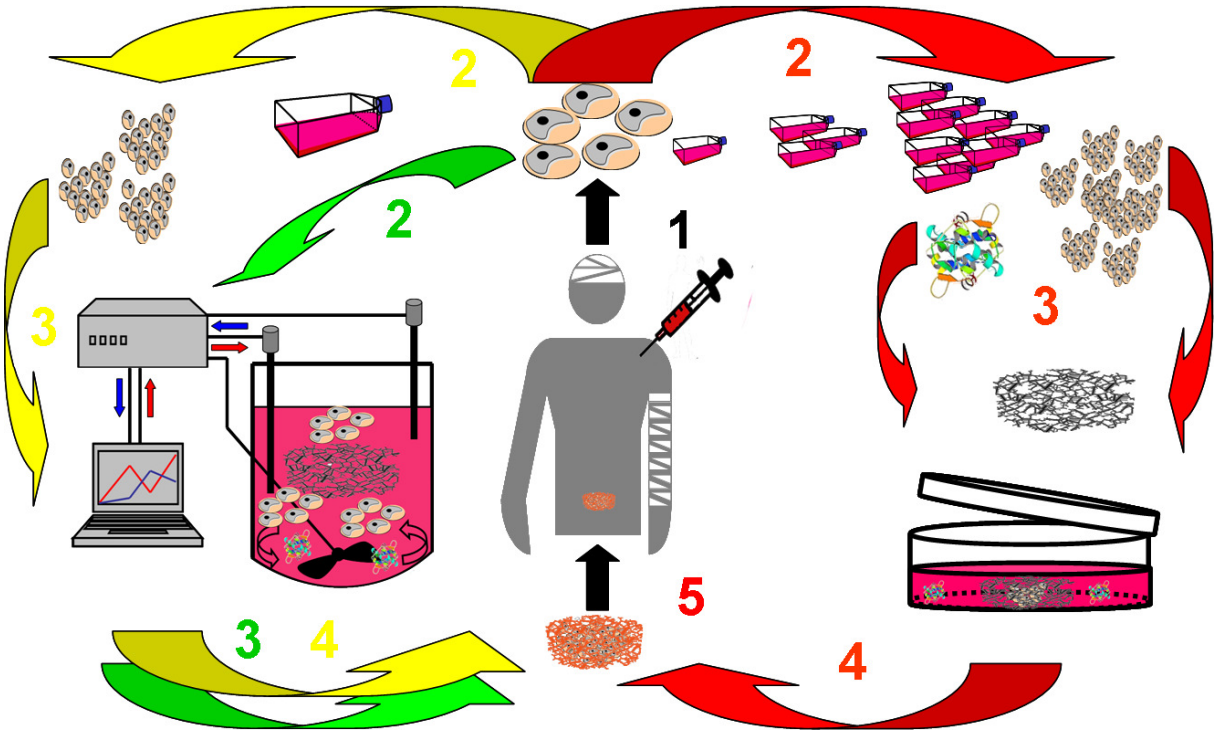

***Figure 1**. Different approaches for cell based bone tissue engineering. All routes begin with harvesting of a bone marrow biopsy of a patient (step 1). **Red)** Conventional cell based bone tissue engineering. 2. BMSCs are selected by adhesion in tissue culture flasks. Subsequently, cells are expanded in vitro in tissue culture flasks. 3. Cells are combined with a suitable scaffold material, often in the presence of bioactive molecules. 4. Cells are cultured on the scaffold material. 5. The hybrid construct is implanted back into the defect of the patient. **Yellow)** Cell based bone tissue engineering using a bioreactor approach. 2. BMSCs are selected by adhesion in tissue culture flasks. 3. Cells are seeded and expanded in vitro, potentially in the presence of bioactive molecules, on the scaffold material in a bioreactor system. 4. The hybrid construct is implanted back into the defect of the patient. **Green)** Optimization of cell based bone tissue engineering using a bioreactor approach. 2. The whole marrow biopsy is directly inoculated in a bioreactor system where BMSCs are seeded on the scaffold material. Cells are expanded in vitro, potentially in the presence of bioactive molecules, on the scaffold material in this system. 3. The hybrid construct is implanted back into the defect of the patient. During the seeding and proliferation period in the bioreactor, the process is monitored and controlled online with respect to culture parameters.*

Although cell based bone tissue engineering is a promising concept, there are still certain issues which have to be addressed for it to be clinically applicable. It has been reported that the average amount of adult stem cells that can be differentiated

into the osteogenic lineage from a patient is only about 1-10 per 100.000 cells present in the bone marrow (13-15). An average bone marrow aspirate of 10 ml yields about 80 million cells and therefore between 800 and 8.000 adult stem cells suitable for bone tissue engineering. On the other hand, the amount of adult stem cells required for clinical use to repair a large bone defect is estimated on 200-800 million adult stem cells [16]. This means that up to a million-fold multiplication is needed to produce sufficient stem cells for clinical treatment. In the conventional approach, this multiplication is executed in 2 Dimensional (2D) tissue culture flasks (17,18). These flasks, however, are limited in their productivity by the number of cells that can be supported by a given area, while repeated handling for culture maintenance makes the process labor-intensive and susceptible to contamination. Moreover, the microenvironment of the cells is not monitored and controlled which may result in sub-optimal culture conditions (19). Another challenge complicating the clinical application is the available amount of a tissue engineered product. Clinically relevant amounts of hybrid construct (defined as a combination of a biomaterial and bone marrow stem cells) for spinal surgery vary depending the approach from 4-6 cm^3 for an Anterior Interbody fusion (AIF) to 15 cm^3 or more when applying a PosteroLateral fusion (PLF) (20). Production of these amounts of hybrid construct is complicated because of potential mass transfer limitations. Especially diffusion of oxygen is relatively slow and oxygen consumption is high when compared to the transport of other nutrients.

In addition to clinical and process technological arguments, there are also scientific reasons to avoid the 2D adherence selection and proliferation of the selected BMSC population. In this conventional approach, only the BMSCs are used for the production of hybrid constructs. Evidence is increasing, however, that the interaction of multiple cell types is necessary for successful (bone) tissue engineering in terms of improved functionality and engraftment capacity. The effect of these other cell types could be either direct or indirect. Coculture of tissue specific cells together with endothelial cells is an example of such a direct approach, since it aims at solving one of the main limitations of tissue engineered grafts: their rapid vascularization (21,22). Another option is to increase the functionality of the grafts indirectly by producing growth factors and hormones e.g VEGF, PTH and BMPs (23-24). Furthermore, the 2D proliferation of BMSCs is not comparable with the in vivo situation. It has been shown that 2D expanded BMSCs have a diminished differentiation capacity in vitro when compared with those found in fresh bone marrow (25, 26). Therefore, it is hypothesized that a 3 Dimensional (3D) culture system may represent a physiologically more favourable environment for BMSCs than a tissue culture flask, as shown for several other cell types (27).

In previous papers we have reported a 3D perfusion bioreactor system in which we produced clinically relevant amounts of tissue engineered bone using both goat (28) and human (29) BMSCs. In this system, the proliferation could be monitored online during the cultivation period and the resulting hybrid constructs were able to form bone in vivo. The initial isolation of the BMSCs from bone marrow biopsies was, however, still done in 2D tissue culture flasks as shown in figure 1 by the yellow arrows. Therefore, in this study, we investigate the feasibility of a one step direct seeding and proliferation method of BMSCs from goat bone marrow biopsies (GBMSCs) which is schematically depicted in figure 1 by the green arrows. Mesenchymal stem cells from the bone marrow are allowed to attach directly on and

into the ceramic biomaterial. For process development reasons, high and low seeding densities are investigated. After the attachment phase, cells are allowed to proliferate in the same system. Cell growth is assessed by means of histological staining techniques, online oxygen measurements and metabolic activity. Constructs were analysed in vitro for their osteogenic capacity by means of collagen type I and ALP activity. Finally, the obtained constructs are implanted subcutaneously in nude mice to evaluate and compare their bone forming capacity in vivo. In this approach, 2D subculture is completely avoided, saving material, space and labour whereas contamination riks are decreased and the culture is monitored online during cultivation.

MATERIAL AND METHODS

One step seeding and cultivation of hybrid constructs

The semi automated bioreactor system

An adapted version of a direct perfusion flow bioreactor was used as described previously (30) and is schematically shown in figure 2. In short, the bioreactor consists of an inner and outer housing, which were configured as coaxially disposed, nested cylinders. The bioreactor system comprised a bioreactor, a sterile fluid pathway (made of γ sterilized PVC tubing, which had low gas permeability) that includes a medium supply vessel, a pump, an oxygenator and a waste vessel. The oxygenator comprised a closed chamber containing a gas-permeable silicon tube. The gas environment in the chamber was kept at a constant level of 20% O_2 and 5% CO_2 and medium was pumped through the gas-permeable tube. This system enables a medium flow through the bioreactor of a constant pH and a constant oxygen concentration. The individual components of the bioreactor system can be detached in a sterile way by using a tube sealer (Teruseal^{tm}, Terumo). After sampling, these components can be attached again in a sterile way using a tube welder (TSCD^{tm}, Terumo). The fluid pathway contained a temperature sensor and two dissolved oxygen sensors (DO), which were placed at the medium inlet and outlet of the bioreactor.

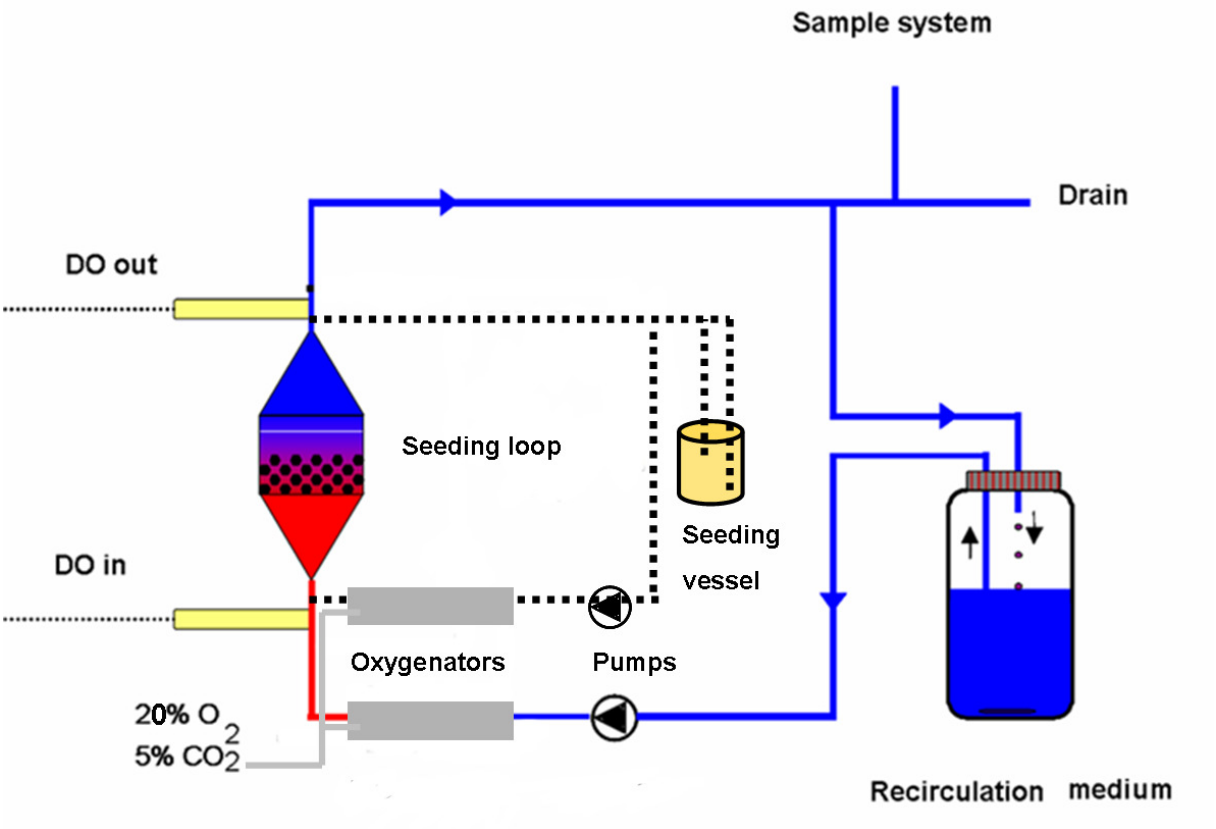

Figure 2. *Process scheme bioreactor system. Medium is perfused from the bottom to the top allowing a mediumflow over and through the scaffold bed (stacked black dots). Two loops can be distinguished: a seeding loop (dashed line) and a proliferation loop (solid line). Both loops contain separate oxygenators. Dissolved oxygen (DO) is measured at the medium in and outlet.*

The whole bioreactor system was placed in a temperature controlled box (incubation unit), which was kept at 37°C. Online monitoring and control was done with Applikon ADI 1060 and 1040-21 consoles. Data were acquired using Bioexpert vs 1.1 sofware.

Scaffolds

Biphasic calcium phosphate scaffolds (BCP, OsSatura^{tm}, IsoTis, The Netherlands) with a macroporosity 36% (pores > 100 µm) were produced using H_2O_2 and naphthalene and the material was sintered at 1200℃ (31). The total porosity of these scaffolds was 59% (average interconnected pore size = 388 µm of all the pores > 100 µm) as measured by Hg porosity measurement. The ceramic consisted of 80 +/- 5% hydroxyapatite (HA) and 20+/-5% tricalcium phosphate (TCP) as confirmed by X-ray diffraction. Fourier Transformed Infrared spectroscopy (FTIR) showed no additional impurities. Granules of ∅ 2-6 mm were γ- irradiated at a minimal dose of 25 Kgy. 10 cc of scaffold material consisted of 230 scaffolds +/- 10%.

Culture medium

Culture medium comprised of α-MEM (Gibco, 22-571-038) supplemented with 15% FBS (Cambrex), 100 U/ml penicillin + 100 mg/ml streptomycin (Gibco, 15140-122), 0.2 mM L-ascorbic acid-2-phosphate (Sigma, A8960), 2 mM L-glutamine Gibco,

25030) and 1 ng/ml basic fibroblast growth factor (Instruchemie PhP105, The Netherlands).

Aspiration of bone marrow biopsies (BMBs)

After anesthesia, the pelvic area of a goat was shaved, disinfected, and a small skin incision was made perpendicular to the longitudinal axis of the cresta Iliaca. After rinsing a syringe and attaching a biopsy needle with 5000 IE/mL heparin, a bone marrow biopsy was taken from the cresta iliaca to which 1500 I.E heparin was added and transported to the laboratory. Clumps of cells were gently broken up by resuspending the bone marrow in a syringe with a 20-G needle attached. Numbers of mononuclear cells (MNCs) were determined with a Bürker Türk counting chamber. Prior to cell counting, red cell lysing buffer had been added to the sample. Cells from the biopsy are refered to as GBMSCs

Seeding and culturing of GBMSCs in the bioreactor system

Before cell seeding, 10 cc of scaffold material in the bioreactor was flushed with cultivation medium in order to pre-wet the scaffolds and allow serum proteins to attach to the scaffold surface. Goat BMBs were diluted tenfold in culture medium and transported into a seeding vessel which was attached to the seeding loop of the bioreactor system described in figure 2. Because of known variety of BMBs with respect to volume and MNC count, we investigated both ranges. BMBs of approximately 4 ml (20 million MNCs, referred to as low seeding density(LSD)) and approximately 25 ml (500 million MNCs, referred to as high seeding density (HSD)) were diluted 10 times with cultivation medium. Subsequently, the diluted BMBs were seeded by circulating the cell suspension through the seeding loop for 48 hours with a perfusion rate of 4 ml/min (108 μm/s), flow direction from bottom to top. After seeding, the seeding loop was closed and fresh medium was flushed through the bioreactor and tubing into the waste vessel (connected to the drain) to remove any non adhered cells. After the flush period, the fluid path towards the drain was closed and medium recirculation was started at 1,3 ml/min (which is equal to an average superficial fluid rate of 35 μm/s), in order to promote proliferation of the attached GBMSCs unless stated otherwise. The culture medium (with a composition as described before) in the recirculation loop was refreshed twice every week. During cultivation at 37°C, 3-5 hybrid constructs were taken from the bioreactor (top, middle and bottom section) at several time points. These samples were used for cell staining in order to monitor cell distribution, growth and viability. At the end of the cultivation period, random hybrid constructs were taken for characterization and in vivo studies.

Static culture, 2D and 3D static controls

For control purposes, GBMSCs were isolated from bone marrow aspirates from the iliac crest by adherence selection and cultured as described in detail (18). After centrifugation and resuspension, the bone marrow population was plated at a density of 500.000 mononuclear cells/cm^2 in tissue culture flasks. Cells were cultured at 37°C in a humid atmosphere with 5% CO_2. When cells were near confluence, the cells were washed with phosphate buffered saline (PBS), enzymatically released by means of a 0.25% trypsin-EDTA solution and replated at a density of 5000 cells/cm^2. After one additional passage, cells were enzymatically released as described before

and statically seeded by applying 100 µl containing 100.000 GBMSCs per scaffold in a bacterial 25 wells plate. Cells were allowed to attach for 4 hours, after which an additional 2 ml of culture medium was added to each well. Cells were statically cultured at 37°C in a humid atmosphere with 5% CO_2 for 5 days. During cultivation, the cultivation medium was changed twice every week. Total culture time of the cells to produce static hybrid constructs were identical to the dynamically cultured constructs. These constructs are referred to as 2D static controls. 3D static controls were produced by seeding the same amount of bone marrow/medium mixture (as the 3D dynamic constructs) on top of the scaffolds. After 48 hours, the bone marrow/medium mixture was removed and 2 ml of culture medium was added to each well. Cells were statically cultured at 37°C in a humid atmosphere with 5% CO_2. During cultivation, the cultivation medium was changed twice every week. Total culture times of the cells on these hybrid constructs were identical to the dynamically cultured constructs. Empty scaffolds (ES) without cells were also used in our in vivo experiments.

Online oxygen measurement

The oxygen concentration was measured online in the medium inlet and medium outlet during dynamic proliferation as can be seen in the process scheme in figure 2. The oxygen electrodes used were sterilizable dissolved oxygen sensors from Applisens (Applikon, the Netherlands). In previous studies we showed that the difference in oxygen concentration between the medium inlet and medium outlet (Δ DO), when assuming a constant specific oxygen consumption (q_o), liquid volume of the bioreactor (V_l) and perfusion flow rate (F_l), is directly proportional to the biomass concentration (28,30).

Measurement of nutrients and metabolites

Nutrients and metabolites in the cultivation medium (glucose and lactate) were measured in time using the Vitros DT 60 medium analyzer.

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Characterization of hybrid constructs

Cell distribution, load and viability

Cell distribution and cell load on the scaffolds in the bioreactor were qualitatively assessed by using Methylene Blue (MB) staining. After sampling, cells on the scaffolds were fixed in 1.5% glutaraldehyde in 0.14 M cacodylic buffer pH 7.4 ± 0.1 adjusted with 1M HCL. After fixation, 1% methylene blue solution was added and incubated for 60 seconds and washed twice with PBS in order to remove non-bound Methylene Blue. Cells on the scaffolds were visualized using light microscopy. For measuring cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining was used. A solution of 1% MTT was applied on the scaffolds containing cells. After 4 hours of incubation, the MTT solution was removed by flushing the scaffolds with PBS. Scaffolds and cells were visualized using light microscopy

Alkaline phosphatase staining

Expression of alkaline phosphatase (ALP) was evaluated by an Azo-dye method. Briefly, hybrid constructs were washed twice with PBS and fixed for 2 hours in 4% paraformaldehyde. After washing the hybrid constructs twice with PBS, the samples were incubated in a Naphtol AS-BI phosphate solution (6-Bromo-2-phosphohydroxy-3-naphthoic acid o-anisidide) containing 0.1% w/w Fast Blue R salt (Sigma,The Netherlands) for 15 minutes at room temperature. Prior to incubation, the samples were incubated; the solution was filtered through a 0.2 μm filter in order to remove non dissolved Fast Blue R salt.

Collagen type I assay

Expression of collagen type I by bone marrow stem cells cultured on scaffolds was determined by immunohistochemistry. Fresh samples of hybrid constructs were hydrated by washing them in 100% PBS at 37°C. PBS was removed and samples were blocked with 100% blocking buffer (BB, X0909, DAKO) for 60 minutes at room temperature. Dilutions of the primary antibody (Mouse monoclonal to Collagen type 1, reacts with human and goat, Abcam Ab23446, CSI 008-01) were made in PBS with 10% BB. Samples were incubated with the primary antibody at 4°C for 16 hours and subsequently washed 3 times with PBS with 10% BB. Samples were incubated with a secondary antibody (Rabit polyclonal to mouse IgG with a Horse Reddish Peroxidase conjugate, Abcam, ab6728) for 60 minutes at room temperature. Samples were washed 3 times with PBS. Subsequently a DAB chromogen solution was prepared by adding 3 drops of DAB solution (3, 3'-diaminobenzidine chromogen solution, DAKO) in 1 ml of DAB buffer (buffersolution pH 7,5 DAKO). Samples were incubated in 100 μl of DAB chromogen solution for 10 minutes at room temperature. Positive (goat bone) and negative (incubations without primary antibody and scaffolds without cells) controls were also included in this experiment.

SEM and EDX analysis

After the in vitro cultivation period, matrix formation was examined by scanning electron microscopy (SEM) and energy dispersive x-ray analysis (EDX). Samples from cell-scaffold constructs for SEM analysis were fixed, dehydrated, gold coated and examined in an environmental scanning electron microscope (ESEM;XL30, ESEM-FEG,Philips,The Netherlands). EDX analysis was used to identify the chemical composition of structures present on the scaffolds.

In vivo bone formation

Random scaffold samples from bioreactors were taken after the in vitro cultivation period in a sterile LAF cabinet and were soaked in α-MEM supplemented with 100 U/ml penicillin + 100 mg/ml streptomycin. Prior to implantation, the samples were washed in PBS. Control scaffold samples which were statically seeded and cultured with cells for the same period of time (2D and 3D controls) were also implanted, as well as empty scaffold (ES). Nude male mice (Hsd-cpb:NMRI-nu,Harlan) were anaesthetized by isoflurane inhalation, and subcutaneous pockets were made. The number of implanted dynamically cultured hybrid constructs and the number of mice used are depicted in table 1.

***Table 1**. Number of implanted dynamically cultured hybrid constructs and the numbers of mice used*

Condition	# cultured hybrid constructs implanted per mouse	Number of mice used	Total number of implanted cultured hybrid constructs	Implantation period (Days)
LSD	4	6	24	28
HSD	4	6	24	28

The incisions were closed using a vicryl 5-0 suture. After 4 weeks the mice were sacrificed using CO_2 and samples were explanted, fixed in 1.5% glutaraldehyde (Merck) in 0.14 M cacodylic acid (Fluka) buffer pH 7.3

Histology and histomorphometry

The fixed samples were dehydrated and embedded in methyl methacrylate (Sigma) for sectioning. Approximately 10µm thick, undecalcified sections were processed on a histological diamond saw (Leica Microtome, Nussloch, Germany). The sections were stained with 0.3% basic fuchsin and 1% methylene blue, in order to visualize bone formation. Histomorphometry was performed by scanning histological slides of stained sections of the whole scaffold. At least four sections (from 4 separate hybrid constructs for the dynamic condition and from 2 separate hybrid constructs for the control conditions) per mouse were made. From these scans, the surface area of the whole scaffold (region of interest, ROI), surface area of the BCP ceramic (MAT) and the surface area of formed bone (BONE) is determined. The ratio of total amount of bone formed as a percentage of the available pore area (BIP) is determined according to equation 1. The obtained results were statistically tested using a two tailed student t-test.

$$BIP = BONE/(ROI-MAT)* 100\% \quad [1]$$

RESULTS

One step seeding and cultivation of hybrid constructs

In this study, unprocessed bone marrow biopsies varying in initial mononuclear cell count were directly seeded in a perfusion bioreactor system. After the seeding phase, the attached cells are allowed to proliferate on and in the scaffold. Figure 3 shows an increase in cell load per scaffold in time during the proliferation phase for low seeding density (LSD, fig 3A-F) and high seeding density (HSD fig 3G-M).

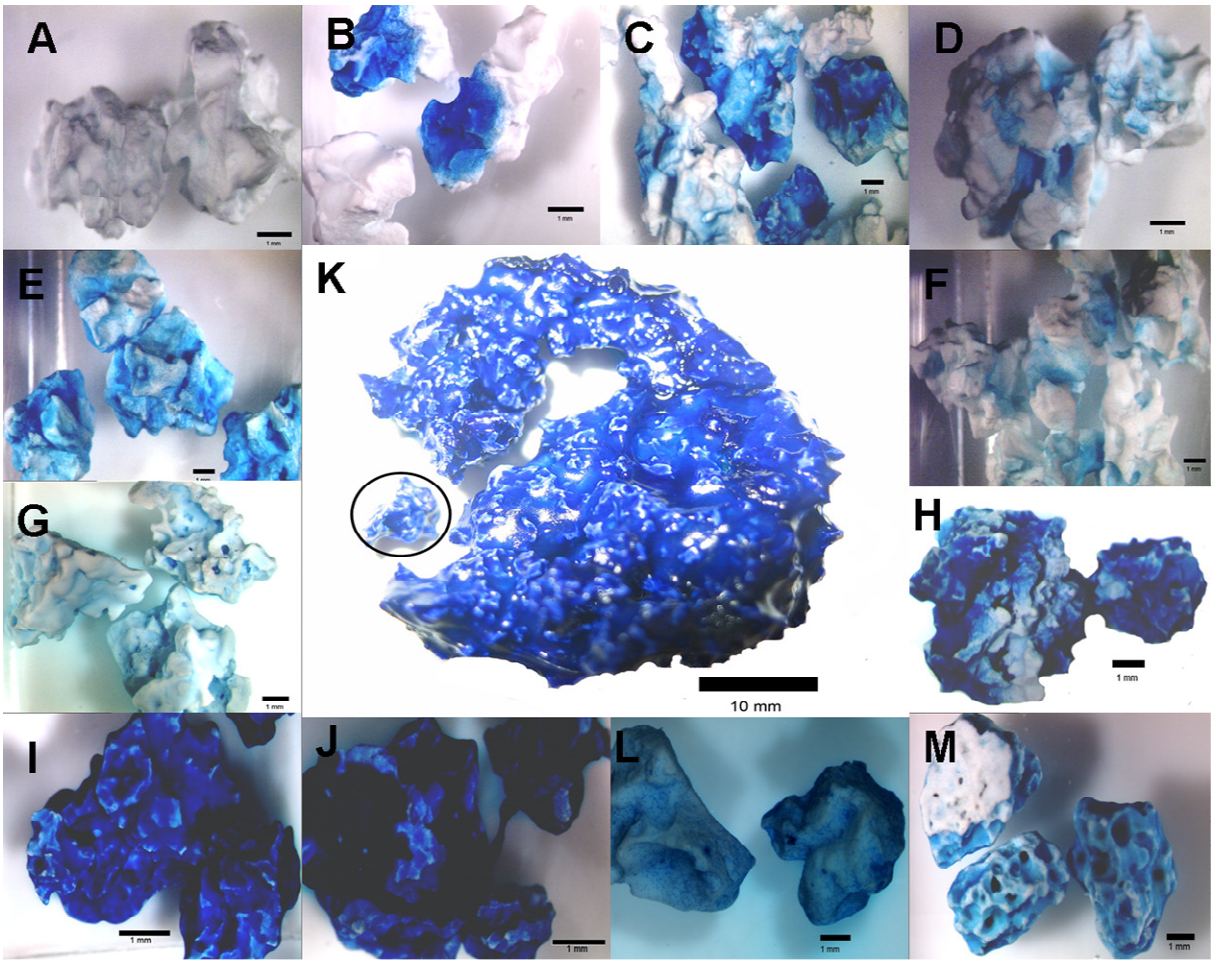

Figure 3. *Methylene Blue (MB) stained GBMSCs on OsSatura^{tm} BCP scaffolds after static and dynamic seeding and proliferation LSD (A-F) and HSD (G-M).* ***LSD****: MB after dynamic seeding (A),after 20 days (B) and after 31 days proliferation(C). MB (2D static control) after seeding (D) and after 5 days proliferation side 1 (E) and side 2 (F).* ***HSD****: MB after dynamic seeding (G),after 12 days (H) and after 31 days proliferation(I,J and overview K). MB (2D static control) after seeding (L) and after 5 days proliferation (M). Circle designates individual particle in overall bioreactor content K.*

After seeding, few cells had attached to the surface of the ceramic scaffold under LSD condition (fig 3A). After 20 days, most scaffolds were partially covered with cells. There were still some scaffolds present with no visible cell attachment (fig 3B). In time, the cell load increased but at the end of the cultivation period the scaffolds were not completely covered with cells (fig 3C). The statically cultured 2D constructs contain cells after seeding (fig 3D), and showed cell coverage on one side of the scaffold (fig 3E) but not on the other side (fig 3F) after 5 days of static cultivation. Under HSD condition, there are also few cells visible after seeding (fig 3G), but cell coverage increased faster compared to LSD (fig 3H). After 25 days the scaffolds were completely and homogeneously covered with cells (fig 3I). The hybrid structures appeared to be connected to each other and a very dense layer of extracellular matrix was present on and between the BCP scaffolds (fig 3J and K). The statically cultured 2D constructs looked similar to those in LSD after seeding (fig 3L) and after 5 days cultivation (fig 3M). The differences with respect to cell coverage in the two experiments could be caused by either different population doubling times (growth rates) or a difference in the initial attached cell amounts. Therefore, online oxygen

consumption was measured in order to calculate the population doubling times for the two experiments (28,30).

Online oxygen measurement during dynamic proliferation of GBMSCs

Measurement of ingoing and outgoing oxygen concentrations during proliferation in a bioreactor system for both experiments is shown in figure 4.

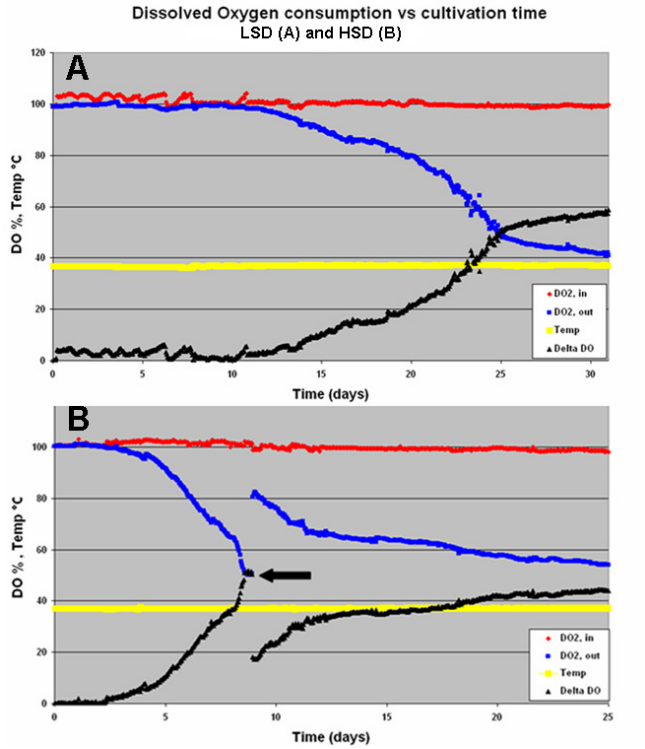

***Figure 4**. Net dissolved oxygen consumption (∆DO = DO,in – DO,out) during dynamic proliferation of GBMSCs from on OsSatura[tm] BCP scaffolds. A (LSD) and B (HSD). 100% indicates the concentration in cultivation medium, which is in equilibrium with the 20% oxygen in air. Black arrow in figure 4B shows an increase in perfusion velocity from 1.3 to 4 ml/min in order to prevent hypoxic conditions in the perfusion system and results in a drop in ∆DO.*

During proliferation, the inlet oxygen concentration was kept at a constant level (red line) by saturation of the medium in the oxygenator and the outlet oxygen concentration (blue line) decreased in time for both experiments. The difference between the ingoing and outgoing oxygen concentration (∆ DO) is also depicted (black line). The ∆ DO increases during cultivation in both experiments in time. In LSD, the ∆ DO is detected after approximately 12 days and reached a maximum of about 60%. In HSD, the ∆ DO is already detected after approximately 2,5 days. In this experiment, the perfusion rate was increased from 1.3 ml/min to 4 ml/min in order to prevent hypoxic culture conditions. At this perfusion rate, a maximum ∆ DO of 40% was reached.

A higher ∆ DO at the same perfusion rate in HSD compared to LSD is caused by higher cell numbers (assuming equal specific oxygen consumption rates) and correlates with the data as presented in figure 3. The exponential parts of the delta

DO curves can be used to calculate the in vitro population doubling times (pdt) in the two experiments. This growth rate is compared to the 2 D growth rate in tissue culture flasks and is depicted in table 2.

***Table 2**. 2D and 3D population doubling times (pdt) for experiment for LSD and HSD*

Condition	2D pdt (days)	3D pdt(days)
LSD	46	49
HSD	28	30

Despite the use two different cultivation methods, the calculated values for the 2D and 3D doubling times match closely. Based on the pdt, the cells seeded from HSD grew faster when compared to LSD. However, this difference in pdt (factor 1.6) can not explain the difference in the Δ DO detection time. We calculated that, when the initial cell amounts on the scaffold were the same, the delta DO detection time in LSD would have been approximately 4 instead of 12 days (calculation not shown). The actual difference (12-2,5=9,5 days) therefore is probably due to a higher number of initially attached cells under HSD conditions.

Cell metabolism during cultivation

Medium samples were analyzed for glucose and lactate concentrations at various time points. Glucose consumption as well as lactate production increased during the cultivation (figure 5).

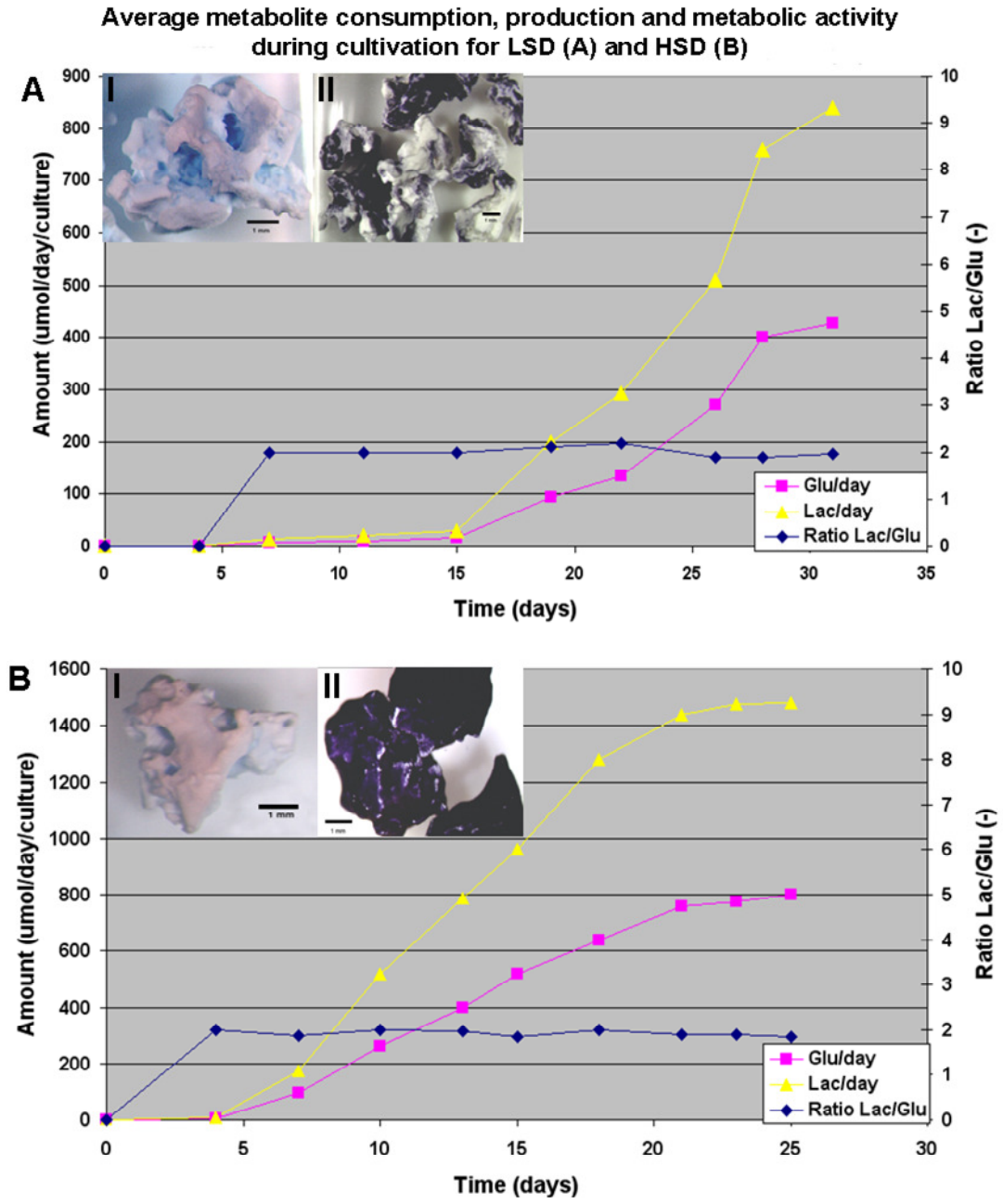

Figure 5. *Metabolite consumption and production during dynamic proliferation of GBMSCs from BMA on OsSatura™ BCP scaffolds: A (LSD) and B (HSD). Picture I shows the scaffold without cells and picture II shows MTT stained GBMSCs at the end of the cultivation period.*

Glucose is used as a carbon and energy source, and lactate is a waste product of cell metabolism under aerobic conditions. This indicated cell growth and was correlated with an increase in Δ DO during cultivation. A thick layer of living cells was present on the scaffolds after respectively 31 and 25 days of cultivation as was shown by MTT staining in figure 5. The molar ratio of lactate produced and the amount of glucose consumed (q lac/glu) is very close to 2 during the entire cultivation period.

Characterization of hybrid constructs

In vitro characterization: SEM, Collagen type I and ALP expression

During visual inspection of the GBMSCs on the BCP scaffolds in HSD, it was observed that the hybrid structures became interconnected (fig 3I and J). A dense layer of extracellular matrix was present on and between the scaffolds, which was characterized by electron microscopy, and ALP and Collagen type I histological staining.

SEM

To evaluate the extra cellular matrix in more detail, samples were gold coated and SEM was performed.

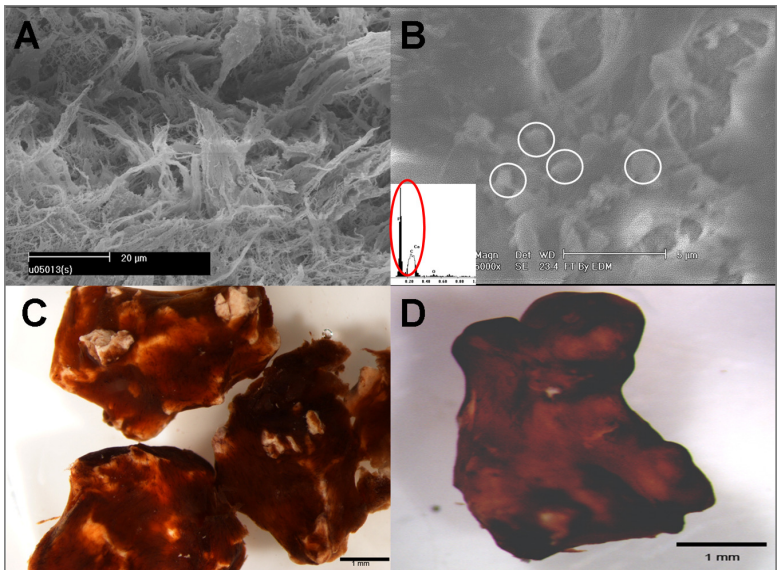

Figure 6. *Characterization of GBMSC hybrid constructs in HSD after 25 days of cultivation. Scanning electron micrograph (SEM) of GBMSCs cultured on OsSatura™ BCP scaffolds showing abundant extracellular matrix (A). SEM picture showing calcium phosphate nodules (white circles) as determined by EDX analysis (red circle)(B). ALP (C) and collagen type I (D) expression on hybrid constructs is visible (brown color). The controls with cells without primary antibody and controls without cells showed no ALP and collagen type I expression (Not shown).*

Figure 6A shows a thick cell layer on the BCP surface for HSD. On the SEM images, sphere like structures (+/- 0.5 - 1 µm) were observed throughout the whole construct. In figure 6B, these nodules are indicated by a white circle. EDX analysis showed (red circle) that these nodules consisted of calcium phosphate which is indicative of calcium formation in vitro.

Collagen type I and ALP expression

In order to confirm if the hybrid constructs show differentiation towards the osteogenic lineage, the presence of alkaline phosphatase (ALP) and collagen type I was investigated. The results are shown in figure 6. Dynamically cultured constructs showed abundant ALP and collagen type I expression (brown colour, respectively fig 6C and D) whereas the controls with cells without primary antibody and controls without cells showed no ALP and collagen type I expression (not shown). The

presence of extracellular matrix containing calcium phosphate nodules as well as ALP and collagen type I expression proves differentiation towards the osteogenic lineage under in vitro conditions.

In vivo characterization: bone formation of hybrid constructs in nude mice

After subcutaneous implantation in nude mice for 4 weeks, the hybrid constructs were explanted, histologically processed and bone formation in vivo was assessed. The bone formation for experiment LSD and HSD is shown in figure 7.

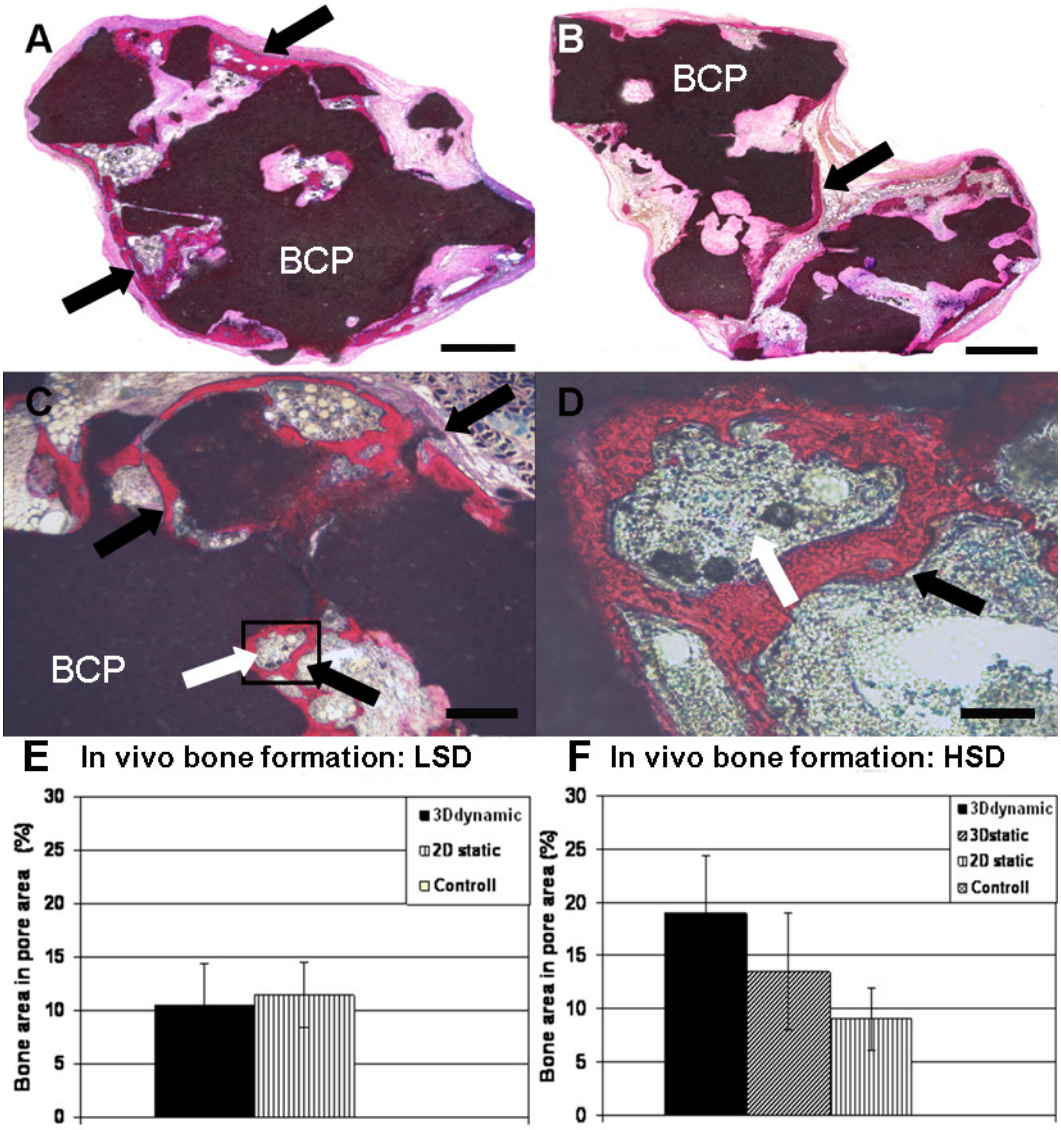

Figure 7. *Bone formation by GBMSCs after subcutaneous implantation of dynamically cultured hybrid constructs in nude mice). New bone (black arrow) is formed on the surface of the OsSatura[tm] BCP scaffolds (A,B,C,D). Bone marrow (white arrow)is often associated with newly formed bone tissue (C,D).Bone formation of statically and dynamically cultured hybrid constructs were quantitatively assessed by histomorphometry in LSD (E) and HSD (F). Size bar A, B = 1mm, C = 200 µm and D = 20µm.*

In both experiments, in vivo bone formation was observed in nude mice. In figure 7A and B, the in vivo bone formation of dynamically cultured hybrid constructs is shown for HSD. *De novo* formed bone was deposited against the walls of the scaffold

material. In all samples, areas with mineralized bone (red color indicated by black arrows) and osteoid (pinkish color) could be identified. Osteocytes were visible within the bone matrix, and osteoblasts are present in a layer on top of the newly formed bone. Bone marrow (white arrow) and fat cells were often associated with and in proximity to newly formed bone as shown for LSD in figure 7C and D. The control scaffold without cells did not show any bone formation in both experiments (not shown). Histomorphometric analysis showed that there was no significant difference in bone formation in LSD between 3D dynamic and 2D static cultivated hybrid constructs ($p=0.7$) (fig 7E). In HSD there was no significant difference between 3D dynamic and 3D static cultivated hybrid constructs ($p=0.076$), but there was a significant difference between 3D dynamic and 2D static cultivated hybrid constructs ($p=0.0008$) (fig 7F).

DISCUSSION

In autologous bone tissue engineering, bone marrow biopsies from different individuals vary enormously with respect to their biological performance (32). This makes standardization and subsequent clinical application of the technique difficult, and it is therefore imperative to investigate the reasons between these differences. Whereas biologists investigate these scientific questions, tissue engineers use various animal models for in vitro and vivo studies such as rats, sheep, dogs and goats. Many of these models have the advantage of higher availability of biological starting materials, as well as lower variability between individuals of the same species. This enables engineers to potentially solve another challenge in clinically relevant bone tissue engineering: increasing the amount of bone formation in an orthotopic site (33).

It has been shown that human hybrid constructs implanted subcutaneously in immuno-deficient mice generally result in 1-3% newly formed bone of the total pore area available for bone growth depending on the donor used (34). It is anticipated however that at least 15-20% of newly formed bone in an orthotopic site is necessary for successful bone tissue engineering in a clinical application. Several strategies are being conducted in order to increase bone formation such as the addition of specific bioactive molecules (23,24,35) and prevascularisation of constructs before implantation (36,37). Currently, little is known about the nature of the required cell types and bioactive molecules. Furthermore, the required dosage and action of these signals may vary between cell types (38,39). For example, we have shown in goats that 2D isolated GBMSCs seeded on scaffolds only yielded about 3 times more bone when compared to scaffolds which were loaded with fresh bone marrow which contained 1000-10000 times less GBMSCs. Therefore, more and more research is focused on maintaining the bioactive molecules (direct or indirect) during stem cell cultivation eg by adding platelet lysates or plasma (40,41) or by alternatively processing of the BMBs (42).

In this study, we chose to not process the BMB at all, and thus remaining all original bioactive molecules and cell types during the initial phase of cultivation. We used goat bone marrow stem cells combined with BCP scaffolds which are known to reproducibly form bone when implanted subcutaneously in nude mice (unpublished data from our group). Perfusion seeding, as demonstrated in this study, can be

integrated in a system which enables seeding and proliferation of goat bone marrow stem cells directly from unprocessed bone marrow aspirates without pre-cultivating of these cells on 2D surfaces like tissue culture flasks. Proliferation of GBMSCs isolated from the BMBs succeeded for lower as well as higher seeding densities, although the cell coverage of the scaffolds was different in the same cultivation period. Cell proliferation could be correlated with increasing oxygen consumption and metabolite consumption and production during cultivation. Additionally, a correlation was found between the amount of MNCs seeded and the cell coverage of the scaffolds reached in approximately the same cultivation period. No difference was seen between 2D and 3D growth rates, which results in equally efficient processes in terms of cultivation time.

With respect to cell metabolism, the molar ratio of lactate produced and the amount of glucose consumed (q lac/glu) is very close to 2 during the entire cultivation period for both experiments. This finding suggests that anaerobic glycolysis is the prevalent mechanism for glucose consumption as an energy source (43,44). This was a surprising finding since 100% air saturated medium (containing 20% oxygen) enters the bioreactor. In all cases, we measured dissolved oxygen concentrations at the outlet above 50% of air saturation which does not represent a hypoxic environment for the cells. Therefore, we expected a q lac/glu ratio close to 1 which is common for aerobic metabolism. The fact that this mechanism is occurring in the presence of oxygen is a phenomenon known as the Warburg effect (45). The same effect was observed when human mesenchymal stem cells were cultured in our perfusion bioreactor system (29). Remarkably, when GBMSCs were cultivated in 2D tissue culture flasks, the molar ratio of lactate produced and the amount of glucose consumed (q lac/glu) was approximately 0.8, which implies more aerobic conditions when compared to our perfusion bioreactor system (46). Future research is going to be conducted in order to unravel the cell metabolism of BMSCs under different oxygen conditions.

The presence of extracellular matrix containing calcium phosphate nodules as well as ALP and collagen type I expression proves differentiation towards the osteogenic lineage under in vitro conditions which is in agreement with the previous studies (47-49). Subcutaneous implantation of hybrid constructs in nude mice cultivated under dynamic and static conditions resulted in de novo bone formation. Under LSD conditions, there was no significant difference between 3D dynamically and 2D statically cultured hybrid constructs. Under HSD conditions, there was a significant more bone formation in the 3D dynamically cultivated hybrid constructs when compared to 2D statically cultured constructs. From these experiments, however, we conclude that the 3D dynamically produced hybrid constructs showed at least the same amount of bone in vivo as the statically cultured hybrid constructs. Furthermore, the constructs can be produced in a controlled and more cost efficient way which brings bone tissue engineering one step closer to application in clinical practice.

CONCLUSIONS

We show for the first time that osteogenic constructs can be produced from unprocessed bone marrow aspirates by using a semi automated perfusion bioreactor system. GBMSCs could be seeded and proliferated on ceramic scaffolds without subculturing these cells on a 2D surface like tissue culture flasks. A correlation was found between the amount of MNCs seeded and the cell coverage of the scaffolds reached in the same cultivation period. The proliferation of GBMSCs on the scaffolds corresponded with online measurements of oxygen consumption and metabolite consumption and production for low and high seeding densities during the cultivation period. Upon cultivation, the hybrid structures became interconnected and a dense layer of extracellular matrix was present as visualized by environmental scanning electron microscopy (SEM). SEM images showed within the extra cellular matrix sphere like structures which were identified as calcium phosphate nodules by energy dispersive X-ray analysis (EDX). Furthermore, these cells showed differentiation towards the osteogenic lineage as was shown by collagen type I production and ALP expression. Most important, subcutaneous implantation of 3D dynamically produced hybrid constructs in nude mice showed at least the same amount of de novo bone formation as the controls. We conclude that direct dynamic seeding and culturing of GBMSCs from bone marrow aspirates on clinically relevant amounts of ceramic scaffold material is feasible by using a semi automated perfusion bioreactor system. Furthermore, the constructs can be produced in a controlled and more cost efficient way which brings bone tissue engineering one step closer to application in clinical practice

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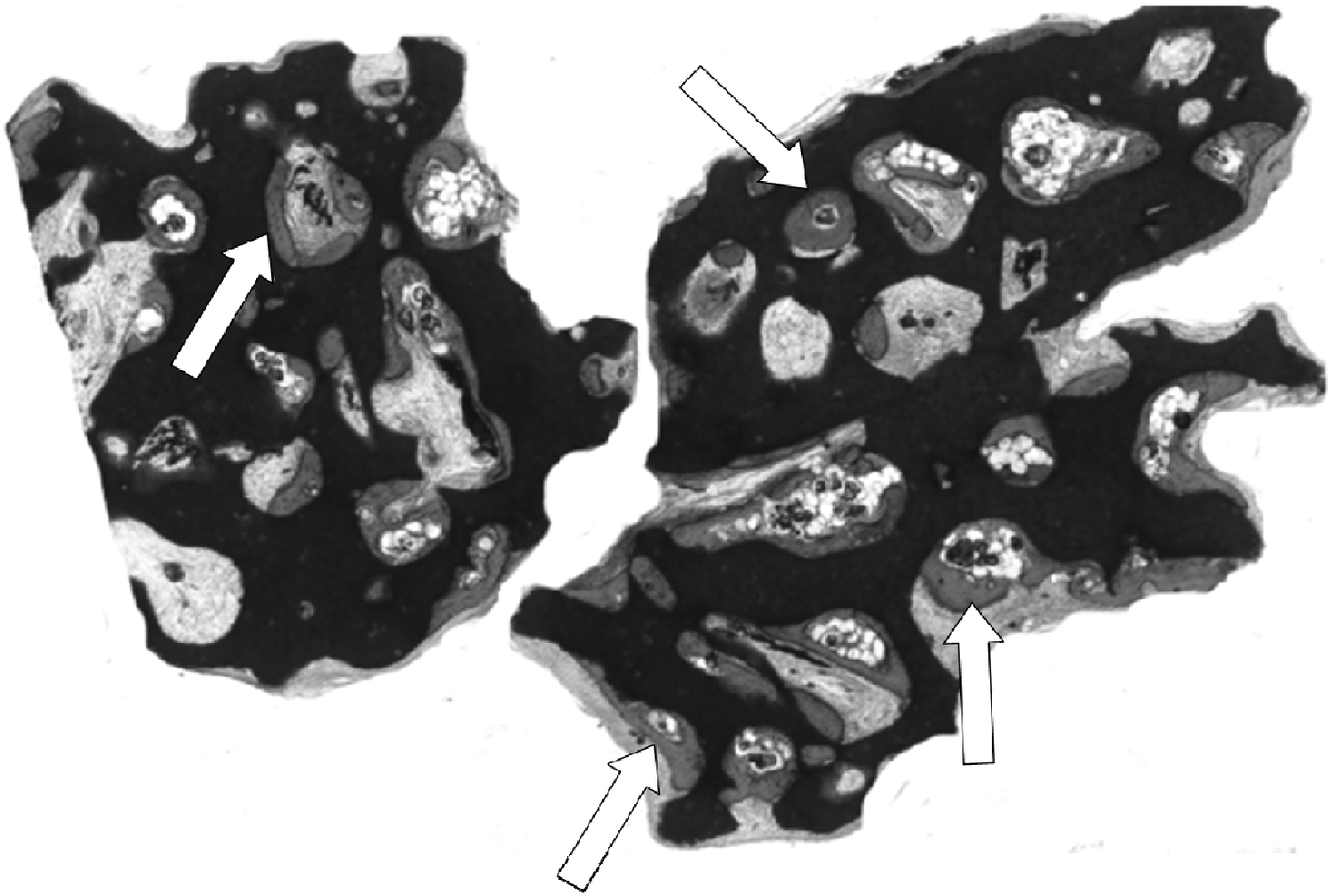
CHAPTER 7



He say "One and one and one is three"
Got to be good-looking 'cause he's so hard to see
Come together right now
over me

Come together-The Beatles

Picture: superiour bone formation(arrows) when combining the synergistic effects of dynamic cultivation combined with PKA signalling by means of cAMP (this thesis).

CHAPTER 7

A MULTIDISCIPLINARY APPROACH TO PRODUCE CLINICALLY RELEVANT AMOUNT OF BONE BY HUMAN MESENCHYMAL STEM CELLS

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ABSTRACT

A key concern in cell-based bone tissue engineering is the limited amount of bone formation in tissue-engineered constructs. We report here a multidisciplinary approach to augment the in vivo bone forming capacity of HMSCs by culturing them in a perfusion-based dynamic bioreactor to optimize nutrient availability during culture. Furthermore, we optimized osteogenic differentiation of the cultured cells, by supplying them with cyclic AMP at the end of the culture period. As expected, cAMP inhibited proliferation both in static and dynamic conditions. cAMP efficiently upregulated the early osteogenic marker ALP in both static and dynamic condition. When the cells were implanted subcutaneously in immuno-deficient mice, newly formed bone covered up to 25% of the total pore area available for bone growth in the dynamic-cAMP group compared to 2-8% bone formation in the other conditions. The improved bone formation by a combination of dynamic culturing and addition cAMP seems a highly efficient method to boost the bone-forming capacity of human mesenchymal stem cells and brings us two steps closer to clinical evaluation of bone tissue engineering.

INTRODUCTION

The easy isolation procedure and the potency of human mesenchymal stem cells (HMSCs) to differentiate into adipogenic, chondrogenic, myogenic, neurogenic and osteogenic lineages has generated a remarkable interest for their effective use in regenerative medical applications (1,2). Cell-based bone tissue engineering includes isolation of HMSCs, in vitro expansion, seeding onto osteo-inductive scaffold materials and implanting tissue engineered construct back into the patient to heal a bone defect. HMSCs are typically isolated from bone marrow and other sources such as adipose tissue and placenta (3-5). When HMSCs are seeded onto porous bioceramics and implanted in animal models, they are able to repair small experimentally induced osseous defects (6,7). Furthermore, the bone forming ability of these cells were also tested in large animal models to mimic a clinical situation (8, 9). Although some clinical trials using HMSCs showed a favorable outcome in fracture healing (10), a common problem seems to be that the amount of newly formed bone is insufficient to fully bridge the implant (10-12). Current studies demonstrate that pre-differentiation of MSCs in vitro into the osteogenic lineage before implanting, augments the in vivo bone forming capacity of the cells (13,14).

The differentiation of multipotent MSCs into a matured osteoblast requires a spectrum of signaling proteins including morphogens, hormones, growth factors, cytokines, matrix proteins, transcriptions factors and their co-regulatory proteins (15-17). Currently, dexamethasone is commonly used to initiate the osteogenic process in HMSCs, thus ignoring the multiple signaling pathways that control osteogenesis. Therefore, understanding the osteogenic process regulated by various signaling cues in time is important to augment the biological activity of HMSCs. In this milieu, we focus on the molecular cues that stimulate in vitro proliferation and differentiation, which in turn improve vivo bone formation. We have reported that stimulation of the Wnt signaling pathway and histone deacetylase inhibitors such as Trichostatin A can be used as a tool to enhance proliferation and differentiation of HMSCs, respectively (18-20). In addition, we recently demonstrated that protein kinase A (PKA) activation in HMSCs using 1 mM cyclic adenosine mono phosphate (cAMP) consistently enhances in vitro osteogenesis and in vivo bone formation by HMSCs (21). Another way to stimulate osteogenesis is through mechanical strain and fluid shear stress (22,23). In vivo, osteoblasts and osteocytes experience interstitial fluid shear stress upon mechanical loading of bone through fluid flow inside the canalicular–lacunar and trabecular spaces within bone tissue (24,25).

To mimic the in vivo mechanical stimulation that cells feel, researchers have developed various kinds of 3D perfusion bioreactors with defined mechanical stimulations. Fluid shear force caused by a perfusion bioreactor system enhances osteogenic differentiation and mineral deposition, suggesting that the mechanical stimulation provided by fluid shear forces in 3D flow perfusion culture induces the osteoblast phenotype. Increased fluid shear forces also resulted in the generation of a better spatially distributed extracellular matrix emphasizing the importance of mechanosensation on osteoblast differentiation in a 3D environment (26). We have recently reported that cell growth can be effectively monitored in time in a perfusion bioreactor system by online oxygen consumption measurement (27,28). The outstanding effect of fluid shear stress on osteogenic differentiation has been also demonstrated in various other cell types. Culturing rat primary calvarial cells in 3D

dynamic flow conditions enhanced cell distribution, early osteogenic marker alkaline phosphatase (ALP), osteocalcin, osteopontin expression and in vitro mineralization compared to static condtions29. Moreover, when rat MSCs where cultured in a perfusion bioreactor they showed enhanced osteogenic differentiation and calcium deposition compared to counterpart static groups (30). The earliest report about HMSCs in a perfusion bioreactor is by Koller et al (31) followed by a number of studies showing ectopic bone formation by HMSCs implanted subcutaneously in nude mice (32). As mentioned earlier, the limited capacity of HMSCs to produce clinically relevant amount of bone and our phase I clinical trial with inadequate bone formation by HMSCs emphasizes the need to improve bone forming ability of HMSCs by supporting osteogenesis at multiple stages of differentiation (11).

Besides efficient control of osteogenic differentiation, the production of bone grafts of clinically relevant size faces another problem. The cells which are deep inside the construct would have insufficient gas and nutrient supply leading to limited survival of the cells in culture and construct failure. In this view, reports demonstrate that culturing these cell-seeded constructs or materials in dynamic conditions with controlled gas and nutrient increases nutrient availability and stimulates cel survival. Besides, bioreactor based technology cuts down the cost and labor consuming in vitro cell-culture protocols (27-29).

In this work, we aimed to combine the beneficial properties of fluid shear stress on osteogenic differentiation with the previously reported effect of cAMP on in vitro differentiation and bone formation in vivo. For clinical application of cell-based bone tissue engineering, the physician would require the readily available tissue engineered constructs at the site of the surgery. As an initial step to bring the technology from bench to bedside, we combined the molecular mechanisms to regulate osteogenesis of HMSCs with controlled culture conditions. We report here the synergistic effect on HMSCs osteogenesis and in vivo bone formation of culturing HMSCs in perfusion flow with cAMP. This opens a window towards successful clinical application of bone tissue engineering using HMSCs by combining mechanical and molecular cues.

MATERIAL AND METHODS

Isolation and culture of HMSCs

Bone marrow aspirates (5-20 ml) were obtained from donors with written informed consent. HMSCs were isolated and proliferated as described previously (33). Briefly aspirates were re-suspended using 20 G needles, plated at a density of $5x10^5$ cells/cm2 and cultured in HMSC proliferation medium containing α-minimal essential medium (α-MEM, Life Technologies), 10% fetal bovine serum (FBS, Cambrex), 0.2 mM ascorbic acid (Asap, Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin (Life Technologies), 10 mg/ml streptomycin (Life Technologies), and 1 ng/ml basic fibroblast growth factor (bFGF, Instruchemie, The Netherlands). Cells were grown at 37°C in a humid atmosphere with 5% CO_2. Medium was refreshed twice a week and cells were used for further sub-culturing or cryopreservation upon reaching near confluence. The frozen P0 cells were expanded and seeded in proliferation medium at 200,000 cells/particle (3 particles per condition) onto 2-3 mm biphasic calcium phosphate (BCP) particles prepared and

sintered at 1150°C as described previously (34). A day after seeding, the particles with cells were either cultured in static conditions or transferred to bioreactor. First 5 days the cells were cultured on BCP particles in proliferation medium and further 4 days the cells were treated with or without 1 mM cAMP (sigma).

Bioreactor and bioreactor system

A direct perfusion flow bioreactor was used as described previously (28). Briefly, the bioreactor comprises an inner and outer housing, which are configured as coaxially disposed, nested cylinders. The bioreactor system consisted of a bioreactor, a sterile fluid pathway (made of g sterilized PVC tubing with low gas permeability) that includes a medium supply vessel, a pump, an oxygenator and a waste vessel. The fluid pathway contains a temperature sensor and two dissolved oxygen sensors, which are placed at the medium inlet and outlet of the bioreactor. The whole bioreactor system is placed in a temperature controlled unit at 37°C. The incubation unit lacks a gas-controlled atmosphere and to supply the cells with oxygen and carbon dioxide an oxygenator was developed. The oxygenator comprises a closed chamber containing a gas-permeable silicon tube. The gas environment in the chamber is kept at a constant level of 20% O_2 and 5% CO_2 and medium is pumped through the gas-permeable tube. This system enables a medium flow over and through the cell-seeded biomaterials with constant pH and a constant oxygen concentration. The bioreactor system is depicted in Figure 1.

Seeding and culturing of HMSCs in static and dynamic (bioreactor) systems

The frozen P0 cells were expanded and statically seeded in proliferation medium at 200,000 cells per three 2-3 mm biphasic calcium phosphate (BCP) particles prepared and sintered at 1150° C as described previously (34). After 4 hours, 2 ml of proliferation medium was added and the hybrid constructs were incubated statically overnight in a CO_2 incubator. The scaffolds were then divided into two groups: a dynamic and a static group. In the dynamic group, the hybrid constructs are cultured in the bioreactor system described above, whereas in the static group the hybrid constructs are cultured in a non-tissue culture treated 25-well plates at 37°C in a humid atmosphere with 5% CO_2. Subsequently, the cell-seeded constructs were transferred into 2 separate bioreactor systems and medium recirculation was started at 4 ml/min (108 mm/s) using 200 ml of proliferation medium for 3 days. This is referred to as the dynamic condition. Then, medium was refreshed with new medium and one bioreactor was supplemented with 1 mM cAMP whereas the other bioreactor was treated as a control. The cells were further cultured for 4 more days. At the same time, in the static group, hybrid constructs were cultured for 3 days under static conditions. At this time point, the hybrid constructs were divided into two groups one of which was supplemented with 1 mM cAMP, whereas the other group was treated as a control and cultured for further 4 days.

Online oxygen measurement

The oxygen concentration was measured real-time in the medium at the inlet and outlet of the bioreactor as explained in Figure1. The oxygen electrodes were sterilized before placing them in the system (Applikon, the Netherlands). We calculated the difference in oxygen concentration between the medium inlet and

medium outlet (Δ DO) and assumed that with constant specific oxygen consumption (qo), liquid volume of the bioreactor (Vl) and perfusion flow rate (Fl) it is directly proportional to the biomass concentration.

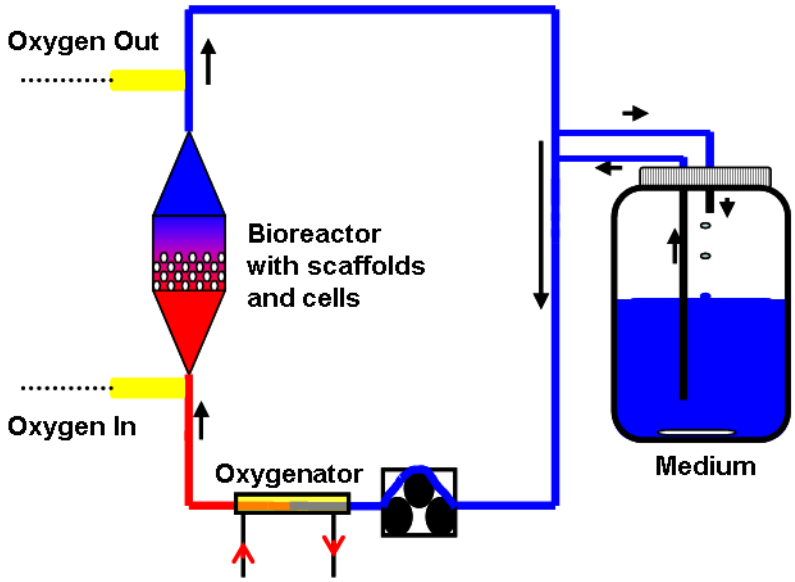

***Figure 1**. Schematic representation of a perfusion bioreactor used in the study.*

Cell distribution, load and viability

Cell distribution and cell load on the scaffolds in the bioreactor were qualitatively assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) staining method. A solution of 1% MTT was applied on the scaffolds containing cells. After 4 hours, the MTT solution was removed the scaffolds were rinsed with PBS. Scaffolds and cells were visualized using light microscopy. Metabolites in the cultivation medium (glucose, lactate and ammonia) were measured using the Vitros DT 60 medium analyzer (Ortho-Clinical Diagnostics, Johnson and Johnson).

RNA isolation and quantitative PCR

The effect of static and dynamic culture systems supplemented with or without cAMP on expression of osteogenic marker genes was analyzed by isolating RNA at the end of the culture period. The RNA was isolated by a Trizol RNA kit (Qiagen) and DNase treated with 10U RNase free DNase I (Gibco) at 37℃ for 30 minutes. DNAse was inactivated at 72℃ for 15 minutes. Two µg of RNA was used for first strand cDNA synthesis using Superscript II (Invitrogen) according to the manufacturer's protocol. One µl of 100x diluted cDNA was used for collagen type 1 (COL1) and 18s rRNA amplification and 1 µl of undiluted cDNA was used for other genes PCR was performed on a Light Cycler real time PCR machine (Roche) using a SYBR green I master mix (Invitrogen). Data was analyzed using Light Cycler software version 3.5.3, using fit point method by setting the noise band to the exponential phase of the reaction to exclude background fluorescence. Expression of osteogenic marker genes are calculated relative to 18s rRNA by the comparative DCT method (35) and

statistical significance was found using student's t test ($P<0.05$). The primers used in the study are listed in Table 1.

Table 1. *Primer sequences used for qPCR studies.*

Gene	Sequence	Product length (bp)
18s rRNA	F-5'cggctaccacatccaaggaa3' R- 5'gctggaattaccgcggct3'	187
Collagen 1	F -5'agggccaagacgaagacatc3' R- 5'agatcacgtcatcgcacaaca3'	138
BSP	F -5'aggttagctgcaatccagc3' R- 5'ccatcatagccatcgtagcc3'	555
Osteopontin(OP)	F -5'ccaagtaagtccaacgaaag3' R- 5'ggtgatgtcctcgtctgta3'	348
Osteonectin(ON)	F -5'actggctcaagaacgtcctg3' R- 5'gagagaatccggtactgtgg3'	438
Osteocalcin (OC)	F -5'ggcagcgaggtagtgaagag3' R- 5'gatgtggtcagccaactcgt3'	138
CBFA1	F -5'ttacttacaccccgccagtc3' R- 5'cagcgtcaacaccattc3'	536
S100A4	F -5'agcttcttggggaaaaggac3' R- 5'ccccaaccacatcaagagg3'	200
Alkaline Phosphatase (ALP)	F -5'gacccttgacccccacaat3' R- 5'gctcgtactgcatgtcccct3'	70
Id1	F-5'gcaagacagcgagcggtgcg3' R-5'ggcgctgatctcgccgttgag3'	346
Id2	F-5'cctcccggtctcgccttcc3' R-5'ggttctgcccgggtctctgg3'	320
Smad6	F-5'gctaccaactccctcatcact3' R-5'cgtcggggagttgacgaagat3'	336

In vivo bone formation

To evaluate the effect of cAMP and culture condition on in vivo bone formation, the tissue engineered constructs which were seeded and cultured in 4 different conditions as explained earlier were implanted subcutaneously in 10 nude male mice (Hsd-cpb:NMRI-nu, Harlan). The mice were anaesthetized by isoflurane inhalation, four subcutaneous pockets were made and each pocket was implanted with 3 particles of each condition. Each mouse was implanted with four conditions namely static control, static cAMP, and bioreactor control and bioreactor cAMP. The incisions were closed using a vicryl 5-0 suture. After 6 weeks the mice were sacrificed using CO_2 and samples were explanted, fixed in 1.5% glutaraldehyde (Merck) in 0.14 M cacodylic acid (Fluka) buffer pH 7.3, dehydrated and embedded in methyl methacrylate (Sigma) for sectioning. Approximately 10µm thick, undecalcified sections were processed on a histological diamond saw (Leica saw microtome cutting system). The sections were stained with basic fuchsin and methylene blue to visualize bone formation. Histomorphometry was performed by scanning histological slides of stained sections of the whole hybrid constructs. At least three sections (from 3 separate hybrid constructs for all conditions per mouse were made. From these scans, the surface area of the whole scaffold (region of interest, ROI), surface area of the BCP ceramic (MAT) and the surface area of formed bone (BONE) is determined.

The ratio of total amount of bone formed as a percentage of the available pore area (BIP) is determined according to equation 1. The obtained results were statistically tested using a two tailed student t-test.

$$BIP = BONE/(ROI\text{-}MAT)* 100\% \quad [1]$$

RESULTS

cAMP inhibits HMSC proliferation

We have previously demonstrated that cAMP inhibits proliferation, induces osteogenic differentiation in vitro and bone formation in vivo. To further apply the in vivo bone forming capacity of the HMSCs to a clinical setting, we used a multidisciplinary approach by culturing tissue engineered constructs in static and 3D perfusion bioreactor systems in the presence or absence of 1 mM cAMP. We seeded HMSCs onto BCP particles and exposed them to either static or dynamic conditions. First, we allowed HMSCs to proliferate on the scaffolds in proliferation medium for 3 days to allow the cells to completely cover the scaffold and next the cells were further cultured for 4 days in the presence or absence of cAMP. After 7 days, we stained the scaffolds with MTT. As anticipated, cAMP inhibited proliferation as demonstrated by MTT staining of the particles in both static and dynamic conditions while HMSCs cultured in the absence of cAMP showed typical homogeneous cell distribution throughout the scaffolds (Figure 2A). This observation is supported by measurement of oxygen consumption by the cells. The inlet oxygen concentration was kept at a constant level (red line) by saturation of the medium in the oxygenator and the outlet oxygen concentration decreased in time (blue line). The difference in the ingoing and outgoing oxygen concentration shows the oxygen consumption by the cells (ΔDO, Black line). In dynamic control group, the oxygen consumption increased first three days and due to a technical problem cAMP supplemented bioreactor did not show an increase in oxygen consumption as seen in its inability to maintain a constant inlet oxygen concentration and hence the oxygen consumption is graphically concealed. After addition of cAMP to the bioreactor, ΔDO in the cAMP group was significantly lower than control bioreactor, confirming cAMP inhibits cell growth. In contrast, ΔDO increased exponentially in the control group, corresponding to cell growth (Figure 2B). The sporadic peaks indicated by an arrow shows the disturbances caused when the bioreactors are opened for medium refreshments. In line with our earlier observations, MTT assay staining and ΔDO measurements confirm that cAMP inhibits proliferation of HMSCs. Glucose consumption, lactate production and ammonia production showed no significant differences until the addition of cAMP. After 3 days of cultivation, the medium was refreshed and one bioreactor was supplemented with 1 mM cAMP and the other one was treated as control. cAMP addition resulted in growth inhibition which is reflected by decreased glucose consumption by HMSCs (Figure 3). As a consequence of cell confluence on the scaffolds, the oxygen in the bioreactor was insufficient and the cells took the anaerobic pathway to metabolize glucose as reflected by the higher lactate production in the control group compared to the cAMP-supplemented bioreactor (Figure 3). Glutamine is an essential amino acid required for protein synthesis, nucleic acid biosynthesis and cell growth (36). Glutamine metabolism in the cells results in the formation of glutamate and ammonia (37). The total ammonia produced

by the cells is an indication of glutamine consumption by the cells which depends on the cell mass. However, if the medium is not refreshed the produced ammonia may have a secondary effects such as growth inhibition and cytotoxic (38). The higher ammonia production in the control bioreactor indicates the higher cell number compared to cAMP supplemented group (Figure 3). Taken together, it is obvious that cAMP inhibits proliferation of HMSCs.

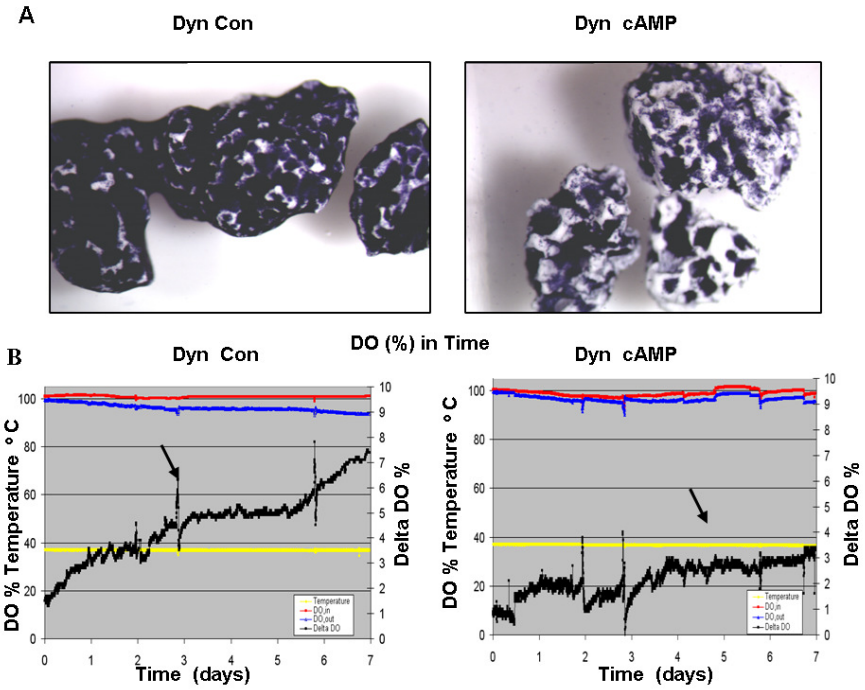

Figure 2. *cAMP inhibits HMSC proliferation. A. MTT staining of the tissue engineered constructs which were cultured in static and bioreactor supplemented with or without 1 mM cAMP. Note the cell number difference in the control (Dyn Con) and cAMP (Dyn cAMP) treated groups. Similar differences were observed in statically cultured groups (not shown here). B. Real time O_2 consumption measurements in the bioreactors. The inlet oxygen concentration was kept at a constant level (red line) by saturation of the medium in the oxygenator and the outlet oxygen concentration decreased in time (blue line). The difference in the ingoing and outgoing oxygen concentration shows the oxygen consumption by the cells (ΔDO, Black line). Note the oxygen consumption increases in the control bioreactor (Dyn Con) while ΔDO stays constant after addition of cAMP to the bioreactor (Dyn cAMP) indicating inhibition of cell growth. The arrows indicate the disturbances caused by medium refreshment regime.*

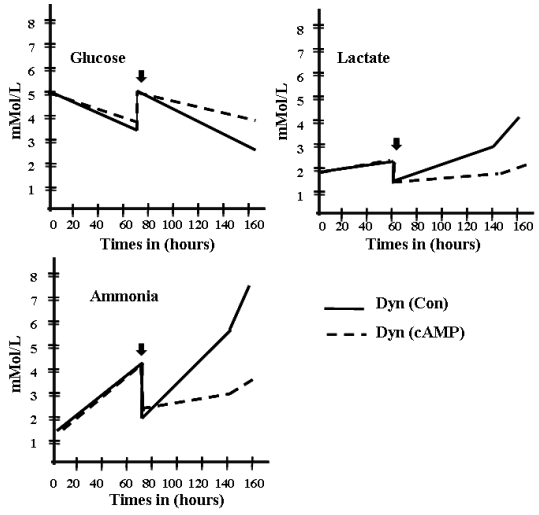

Figure 3. *Nutrient consumption and waste production of HMSCs in control bioreactor (Dyn Con) and cAMP supplemented bioreactor (Dyn cAMP). The black arrow indicates the addition of cAMP to the bioreactor.*

cAMP enhances osteogenesis in vitro

To study the effect of cAMP on in vitro osteogenesis, we isolated RNA from the cells in all condition at the end of the culture period and analyzed gene expression by qPCR. As expected, cAMP induced ALP expression in both static and dynamic conditions compared to their respective groups. However, we did not observe a significant difference in ALP expression between static and dynamic conditions, both in control and cAMP-supplemented groups. Furthermore, we observed a significant increase in BMP2 expression in cAMP supplemented conditions. Consequently, the BMP target genes such as Id1, Id2 and Smad6 were upregulated which is in line with our earlier observations. Osteogenic specific transcription factor Cbfa1, collagen type 1, osteocalcin, calcium binding protein S100A4 and osteopontin expressions were unaffected either by culture conditions or cAMP addition (Figure 4).

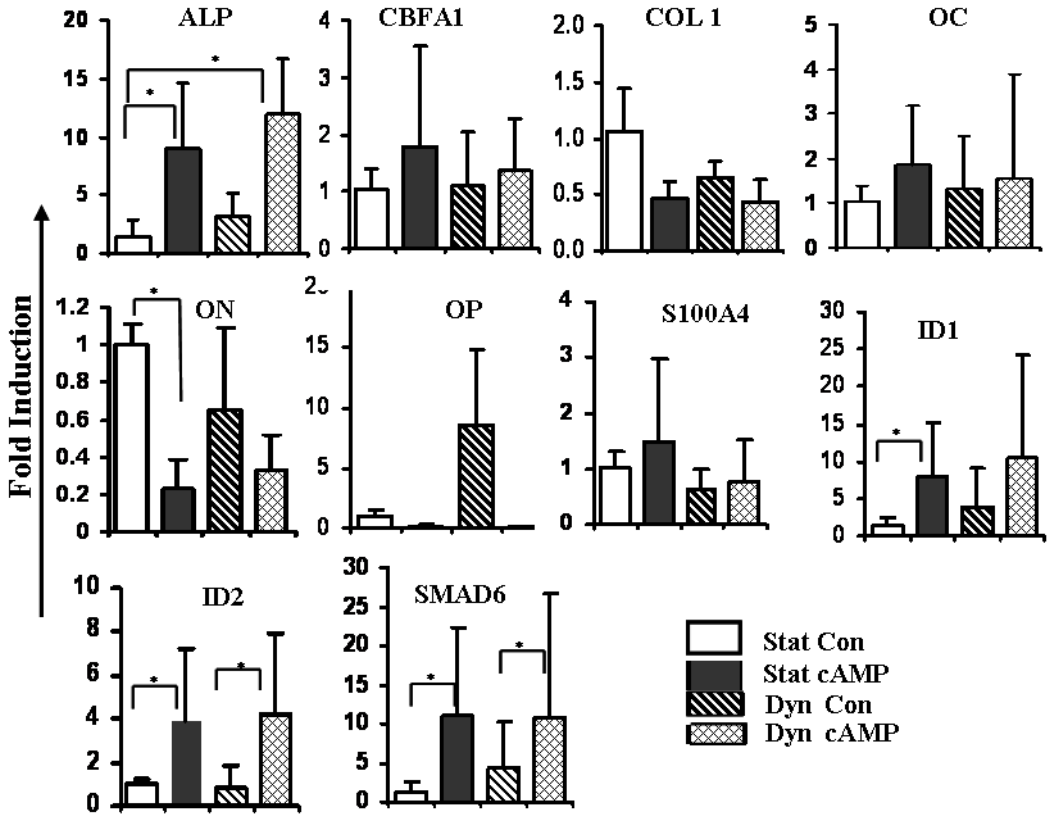

***Figure 4**. cAMP induces osteogenic differentiation of HMSCs. Osteogenic gene expression in tissue engineered constructs cultured in static con (Stat Con), static cAMP (Stat cAMP), dynamic control (Dyn Con) and dynamic cAMP (Dyn cAMP) groups. RNA was isolated from these tissue engineered culture real-time PCR was performed (see materials). The gene expression is indicated as fold induction compared to static control (Stat Con) group and normalized to 18s rRNA. Error bar represent standard deviation.*

cAMP seems to decrease osteonectin expression in static condition, however this decrease was not significant under dynamic condition. In summary, as anticipated, cAMP induced expression of ALP, BMP2 and BMP- target genes Id2, Id2 and Smad6, however no significant differences were found in these gene expression profiles between different static and dynamic culture conditions. In addition, we analyzed extra cellular matrix (ECM) formation on the tissue engineered constructs before implantation in nude mice. Electron microscopic analysis demonstrated that ECM was formed in all the tissue engineered constructs cultivated in different

conditions. No gross differences in ECM formation were observed by electron microscopy (Figure 5).

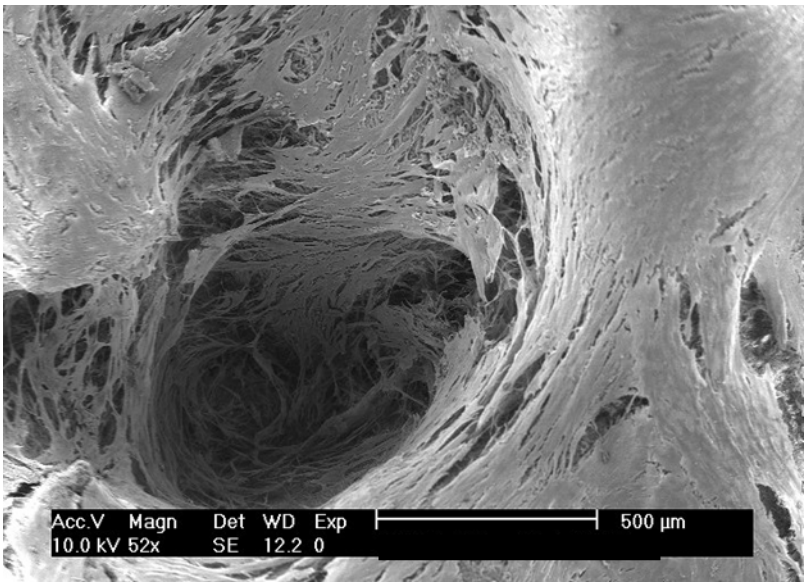

***Figure 5**. A representative electron microscopic image showing extracellular matrix formation on the tissue engineered constructs.*

cAMP in perfusion bioreactor enhances in vivo bone formation

To further investigate the combined effect of 3D perfusion culture system and cAMP-induced osteogenic differentiation, we implanted the tissue engineered constructs of all four conditions in nude mice for 6 weeks. Histological analysis (fig 6B) demonstrated that addition of cAMP in static condition did not enhance bone formation in vivo which is in contrast to our earlier observations. Culturing HMSCs in a perfusion bioreactor system did not either enhance in vivo bone forming capacity significantly compared to static control group. Interestingly, tissue engineered constructs which were cultured in a bioreactor supplemented with 1 mM cAMP significantly enhanced bone formation covering up to 25% of the available pore area for bone growth (Figure 6A). Another imperative observation is that cAMP in dynamic culture conditions resulted in formation of multiple bone marrow-like structures in most of the sample and such structures were virtually absent in other conditions (Figure 6A right panel, white hollow arrow). In the deposited bone tissue, we could typically see osteocytes embedded in the mineralized bone and osteoblast lining periphery of the newly formed bone (Figure 6A right panel, black arrows). These results indicate that culturing HMSCs in the presence of 1 mM cAMP in a perfusion bioreactor significantly enhances bone formation in vivo.

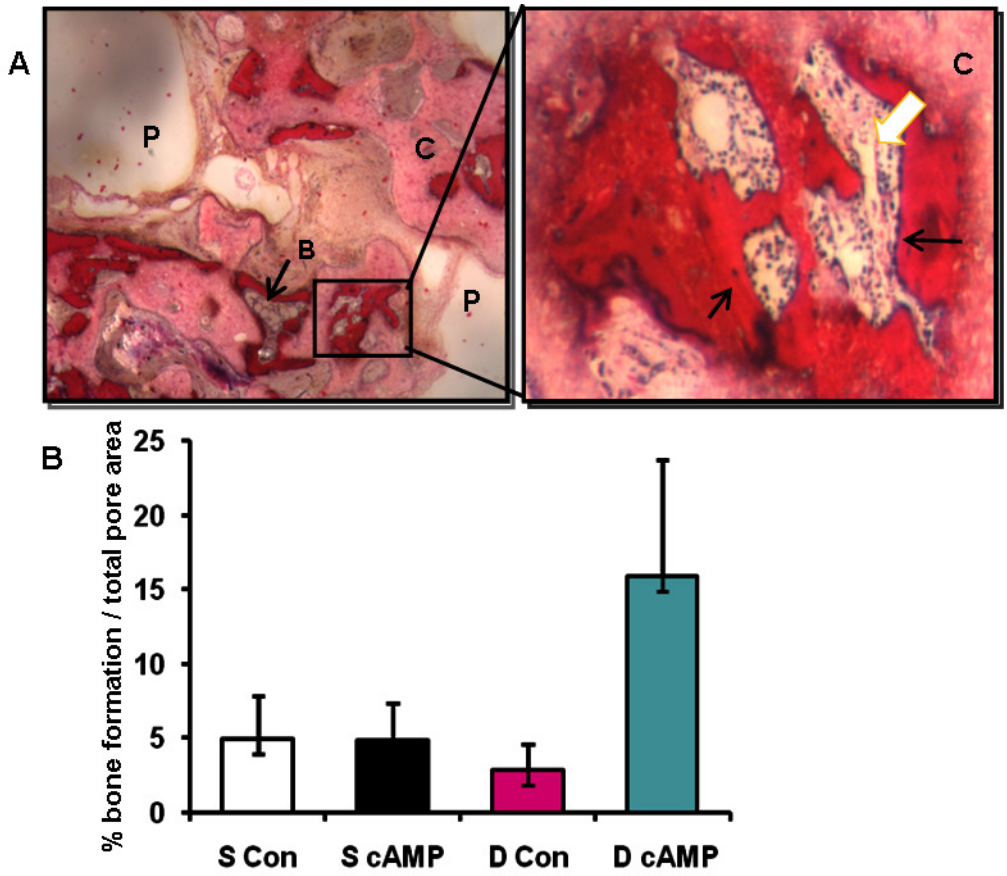

Figure 6. *cAMP enhances in vivo bone formation in a perfusion bioreactor culture system. Tissue engineered constructs cultured in static con (S Con), static cAMP (S cAMP), dynamic control (D Con) and dynamic cAMP (D cAMP) conditions were implanted subcutaneously in nude mice for 6 weeks (same materials). A. A representative histological sample showing bone formation in a tissue engineered construct (Figure A, black arrow with letter B). C indicates ceramic BCP, P indicates available pore area for the bone growth. Magnified image of a bone marrow formation in Dyn cAMP treated condition (right panel). Note the osteocytes embedded in the mineralized matrix, lining osteoblasts on the newly formed bone (black arrows) and the bone marrow-like structures (white arrow).B. Histo-morphometrical analyses for the newly formed bone in various conditions. The newly formed bone was quantified and expressed as percentage bone growth compared to the total available pore area for the newly formed bone. The data were analyzed by using student's t test and P < 0.05 for DcAMP compared to all other conditions.*

DISCUSSION

After successful isolation of MSCs from the bone marrow and discovering their multipotential ability to differentiate into various lineages (1,39), there are number of scientific attempts to prove the proof of concept to regenerate bone tissue in small rodent, sheep, dog and goat models (40-45). Despite, revealing the ability of the MSCs to regenerate bone tissue in animal models, to date there are only few human clinical trials to treat tibial fracture, augmentation of maxilla using HMSCs with moderate outcomes (10,12,46). Recently, Maracci et al. successfully treated patients with large diaphysis defects and the follow up study for 7 years has demonstrated the clinical success of bone tissue engineering using HMSCs. However, the authors had no negative control and did not demonstrate that the bone tissue was formed by implanted cells (47). Our recent clinical trial to treat patients with maxillary defects using autologous HMSCs and hydroxyapatite scaffolds demonstrated that the

implanted cells were incapable of producing bone to a clinically relevant state (11). Overview on the clinical attempts using HMSCs suggest that the key point to consider to augment present bone tissue engineering is to enhance the in vivo bone forming capacity of the implanted cells. As the isolated MSCs are multipotent, unless the cells get proper inductive signal to differentiate into a particular lineage, they would take a default pathway which would limit the bone forming ability of the cells. Therefore, a simple speculation is that by in vitro differentiating the isolated HMSCs into osteogenic lineage would augment the in vivo performance of the cells. Currently, there are a number of osteo-inductive molecules which directs the HMSCs to differentiate into osteogenic lineage such as dexamethasone (48,49), vitamin D (50-52), Trichostatin A53 and indeed many bone morphogenetic proteins(13,54,55). Furthermore, we are currently screening over 20,000 molecules for their ability to induce osteogenic differentiation of HMSCs. Recently, we have demonstrated that PKA activation using cAMP induces osteogenic differentiation of HMSCs and consistently induces in vivo bone forming ability of HMSCs in nude mice model (21). HMSCs isolated from various donors tend to show discrepancy in their in vitro differentiation and in vivo bone forming ability.

Our phase I clinical trial to augment jaw defects using tissue engineered approach yielded insignificant amount of bone to a clinical situation (11), since then we are aiming to improve the in vivo performance of HMSCs. Mechanical strain and fluid shear stress are shown to induce osteogenic differentiation (22,23) which also depicts the in vivo conditions that osteoblasts and osteocytes feel (24,25). Scientists have developed various kinds of 3D perfusion bioreactors with defined mechanical stimulations and their ability to induce osteogenic differentiation in vitro has been demonstrated in comparison with the static conditions (26). In this milieu, we attempted to produce clinically relevant amount of bone in a perfusion bioreactor effectively controlling and monitoring cell growth and differentiation using goat bone marrow stromal cells (27,28). However, with the known over performance of goat and rat MSCs compared to HMSCs limits the extrapolation of the obtained results to a real clinical situation using autologous HMSCs. Therefore, in this study we aimed to augment bone tissue engineering in a multidisciplinary approach by combining cAMP and perfusion bioreactor using HMSCs to closely mimic clinical situation. In line with our earlier observations, cAMP inhibited proliferation both in static and dynamic conditions which typically depicts the inverse relation between proliferation and differentiation (56). Interestingly, even with fewer number of cells in cAMP treated tissue engineered constructs, they produced significantly higher amount of bone indicating the amount of the committed cells into the osteogenic lineage is important rather than just the cell load on the tissue engineered constructs. On the hand, it can be further speculated that the presence of a higher load of differentiated functional osteoblasts on the tissue engineered constructs would even enhance the in vivo bone forming capacity of the cells. We are currently investigating to optimize a practical balance of cell load before addition of cAMP to achieve utmost bone formation. Furthermore, we observed no significant differences in osteogenic gene expression profiles and matrix formation between static and dynamic conditions suggesting that mechanical stimuli in combination with cAMP played the key role in enhanced the in vivo bone forming capacity. There seem to be a slightly higher but statistically insignificant ALP expression in dynamic condition and mostly other osteogenic genes such as Cbfa1, collagen type I, osteocalcin, osteonectin, negative regulator of mineralization S100A4 showed no significant difference between static

and dynamic conditions. Although, we observed a slightly higher induction of BMP2 expression in dynamic conditions, it did not reflect on its target gene expression such as Id1, Id2 or Smad6. Dynamic culturing of HMSCs did not show an increased in vivo bone formation compared to static condition. The mechanical stimulation of the cells by fluid flow has been suggested to impact on intracellular calcium levels (57,58), nitric oxide signaling (59-61) and intracellular messengers and transcription factors (62,63). The improved bone formation by a combination of dynamic culturing and cAMP seems to be very unique effect, since just culturing HMSCs in dynamic condition did not show an added effect on either osteogenic gene expression profile or bone formation. The effect of cAMP on in vivo bone formation in static conditions is although apparent from our earlier studies; our results suggest that there is distinct combination effect which cAMP otherwise able to deliver its effect on in vivo bone formation in static condition.

The donor variation in response to an osteogenic signal is a well-known phenomenon. We have demonstrated that HMSCs from a number of donors respond differently to osteogenic signals (15,64). Our earlier studies using HMSCs isolated from a number of donors showed consistently enhanced bone formation by cAMP treatment (21) however, in this case cAMP failed to enhance in vivo bone forming capacity of HMSCs which is most likely a donor dependent effect. On the other hand, when cAMP was presented in dynamic condition, together it enhanced in vivo bone forming ability of the cells to a greater extent which expands the application of cAMP as an osteogenic inducer for bone tissue engineering purposes.

A balanced concentration and context effect of osteogenic stimuli are important induce osteogenesis in HMSCs. For instance, it is known that HMSCs respond differently to key osteogenic signals such as bone morphogenetic proteins (BMPs) and dexamethasone compared to some of the most frequently used osteogenic model cell lines (65). Further, the required dosage and action of these signals may vary between cell types (65,66). Moreover, the response of HMSCs should always be considered in the light of the heterogeneous nature of this cell population and donor variation (64,67). The crucial role of cAMP in cell fate decision has become apparent from the current studies and the induction depends on the concentration and duration of cAMP and dexamethasone to which HMSCs are exposed (21,68). 10^{-7} M dexamethasone and 0.5 mM cAMP is shown to inhibit osteogenic process and induce adipogenic differentiation (68), while we demonstrate that 10^{-8} M dexamethasone and 1 mM cAMP significantly enhances dexamethasone induced osteogenesis in vitro and bone formation in vivo (21) and in agreement with the authors a balanced context, concentration and species dependent effect of osteogenic stimuli need to be investigated for their effective use in bone tissue engineering. In conclusion, our data reveals a multidisciplinary approach using a perfusion bioreactor system in combination with a precise blend of osteogenic signals to augment in vivo performance of HMSCs to form bone far beyond any current tissue engineering protocols would produce and we are currently structuring clinical trials.

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CHAPTER 8

"Living in dreams of yesterday, we find ourselves still dreaming of impossible future conquests"

Charles Lindbergh.

Picture: Charles Lindberg and Alexis Carell exibiting their organ perfusion pump (Time magazine, June 1938)

CHAPTER 8

General discussion and conclusions

Although bioreactors are being used for decades now in modern food and pharmaceutical industry, they are considered relatively new in regenerative medicine. However, the concept of culture parameter control and increased mass transfer *in vitro* with respect to human tissue culture existed already more than 70 years ago. Charles Lindbergh (the aviator!) and Alexis Carrel designed a prototype bioreactor (they called it perfusion pump) which was able to perfuse tissues and even organs outside the human body (1). The ultimate goal of their secret quest was to achieve immortality: a human body in which one could remove, repair and finally replace all parts. Although this high striving goal was not achieved, it resulted amongst others in the invention of the modern heart lung machine. Furthermore it provided an excellent example of interdisciplinary research in the field of regenerative medicine.

Since then, the integration of technology and biology has advanced in a rapid pace resulting in numerous applications. In regenerative medicine, the use of automated systems as a model or production system has received increasing attention. The importance of these systems was also shown when assessing “Strategic directions in Tissue Engineering” (2). When creating strategic concepts, manufacturing and scale up yielded the second most raw ideas supporting these concepts. Manufacturing and scale up as well as standardized models were ranked seventh with respect to the normalized dominant concept, indicating their immediate priority in the field. However, it also showed that manufacturing and scale up is one of the areas in which the least progress has been achieved until now (2). The overall aim of this thesis was therefore to develop and evaluate a bioreactor approach towards controlled and monitored bone tissue engineering resulting in clinically relevant amounts of bone.

As already mentioned in chapter 2, the main functions of the bioreactor are to provide control over environmental conditions and the nutrient, product and waste concentrations during the bioprocess. Additionally, they can establish standardization, automation and scale up of tissue engineered products for clinical applications. The role of bioreactors with respect to cell based tissue engineering has been discussed in general in that chapter. Here, we will discuss bioreactors for stem cell seeding, cultivation and their application for bone tissue engineering.

Bioreactor systems: from cell seeding to hybrid osteogenic construct

When considering regenerative medicine, many groups are investigating if (undifferentiated) stem cells can be seeded and multiplied in a bioreactor system. The relevant amounts of stem cells would be harvested at the end of the cultivation period and are subsequently used for cell regenerative purposes. In the case of bone tissue regeneration, these multiplied cells would be combined with a suitable scaffold before implantation. One of the most obvious bioreactor systems to cultivate these cells in suspension are stirred vessels. Although some groups report the feasibility such an approach (3,4), most systems are based on the adherent dependant cultivation of stem cells. Major attention is drawn to the cultivation of embryonic stem cells because of their well known proliferative capacity while maintaining their

pluripotency (5-9). However, there are still technical (immune rejection of transplanted embryonic stem cells) and ethical problems (use of human embryos) before these cells can be clinically applied. Since adult stem cells do not have these drawbacks, several groups investigated and reported the feasibility of adult stem cell propagation in bioreactor systems (10-13).

In this thesis, we have focused on a cell based tissue engineering approach. For reasons explained previously, we chose mesenchymal stem cells to pursue our bioreactor approach. Although in the classic tissue engineering protocol, 2D cell seeding and cultivation are still the golden standard, 2D tissue engineering is certainly not! A common approach in tissue engineering is to combine cells with a designated 3D scaffold. When creating a 3D hybrid construct, cell seeding on and into this construct is the first step and does play a role in the progression of tissue formation (14). Although static loading is the most commonly used method, low seeding efficiencies and non-uniform cell distributions are often reported (15). Higher seeding efficiencies and more uniform cell distributions were achieved when compared to static seeding or stirred flask bioreactors (16). For individual scaffolds, these findings were confirmed by our own data: visually more cells and more homogeneously distributed cells were observed on and in the BCP granules (data not shown). When considering the entire packed bed of scaffolds, chapter 3 showed that the cells were not distributed homogeneously over the scaffold surface. However, a homogeneous and viable cell layer was detected in and over the individual scaffolds after the cultivation period as was shown in chapter 4.

Perfusion does not only play a key role in the seeding phase, it is also a powerful tool to supply nutrients to and remove waste products from cells in clinically sized tissue engineered constructs. The implications of inadequate mass transfer can often be observed following the culture of 3D constructs under conventional static conditions (i.e. with unmixed culture media). Due to diffusional limitations, statically cultured constructs are frequently inhomogeneous in structure and composition, containing a necrotic central region and dense layers of viable cells encapsulating the construct periphery (Figure 1).

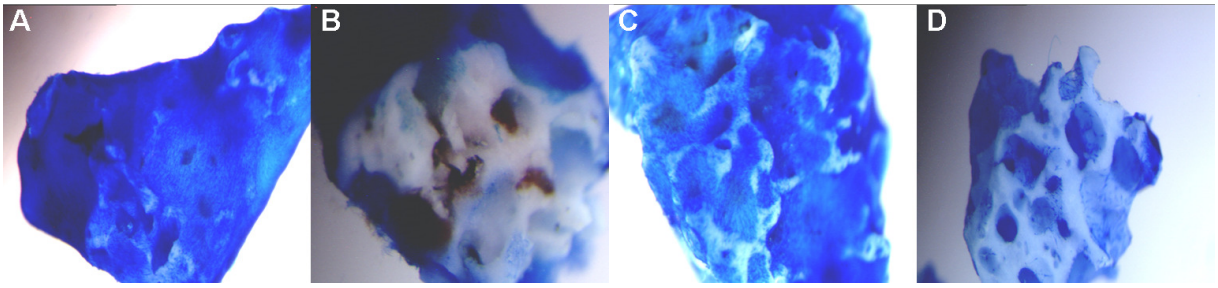

Figure 1*.Untreated bone marrow aspirates under static (A, B) and dynamic (C, D)conditions on BCP scaffolds after 21 days of cultivation. A and C represent the outside whereas B and D show sections of the hybrid constructs. Statically cultured constructs show abundant cell growth on the outside of the scaffold (A) whereas no cells are present in the inside of the scaffold (B). Dynamically cultured constructs show cell growth on the outside (C) and inside (D) of the scaffolds demonstrating the effect of nutrient and oxygen supply to cells by medium perfusion (from our unpublished data).*

Because of the advantages mentioned before, more and more groups have developed perfusion bioreactor systems for cell seeding and cultivation for bone tissue engineering (17-20). Apart from the variety in bioreactor systems, different cell

sources and animal models are used for in vitro and in vivo studies such as rats, sheep, dogs and goats. Many of these animal models have the advantage of higher availability of biological starting materials, as well as lower variability between individuals of the same species. In our own studies (chapter 3, 4 and 6), we used goat MSCs in a nude mice model to investigate the in vivo osteogenicity of the produced hybrid constructs. In summary, the main conclusions that can be drawn from chapter 3 and 4 are:

- A bioreactor system was designed which enables the seeding and proliferation of MSCs in one system for the production of clinically relevant amounts (10 cm^3) of tissue engineered bone.
- During the seeding and proliferation phase, the cell attachment and cell growth could be monitored online by means of oxygen consumption
- The produced hybrid constructs are metabolically active, showed abundant matrix production, calcium phosphate nodules and their osteogenic potential in vivo in a nude mice model system.

Although these studies show proof of concept for a bioreactor based bone tissue engineering approach, it is known that human hybrid constructs often behave differently with respect to vivo osteogenicity when compared to animal model systems (21). Additionally, patient variability with respect to proliferation and differentiation capacity of human MSCs is complicating the investigation of human cell based bone tissue engineering (22-23).

Many researchers have developed various kinds of 3D perfusion bioreactors with defined mechanical stimulation to mimic the in vivo mechanical stimulation that cells undergo. Fluid shear force caused by a perfusion bioreactor system was found to enhance osteogenic differentiation and mineral deposition, suggesting that the mechanical stimulation provided by fluid shear forces in 3D flow perfusion culture induces the osteoblast phenotype. Increased fluid shear forces also resulted in the generation of a better spatially distributed extracellular matrix emphasizing the importance of mechanosensation on osteoblast differentiation in a 3D environment (24). The outstanding effect of fluid shear stress on osteogenic differentiation has also been demonstrated in various other cell types (25-26).

Therefore, we designed a study producing hybrid constructs from 8 different human donors in 13 individual runs in our developed bioreactor system. We investigated the influence of dynamic flow conditions on the in vitro and in vivo bone formation and compared them to statically cultured constructs. The main conclusions that could be drawn from this study (chapter 5) are:

- Viable human tissue engineered bone could be produced in clinically relevant amounts (10 cm^3) from MSCs in different seeding densities for different donors and at different perfusion rates showing the robustness of the system
- The produced hybrid constructs show their osteogenic potential in vitro and in vivo (in a nude mice model system), Dynamically and statically cultured constructs showed similar in vivo bone formation.

In our perfusion system, we could not demonstrate the positive effect of fluid perfusion on in vivo bone formation for human MSCs, which was for example shown

by Braccini et al (27). Several factors complicate the comparison of our results with other bioreactor perfusion systems. Local flow mediated shear stresses are a function of media flow rate, dynamic viscosity, bioreactor configuration and porous scaffold microarchitecture (28). Even though culture medium and superficial velocities (+/- 100 μm per second) are the same for Braccini's and our study, there are still two variables that can cause major differences with respect to the applied shear stresses. 3D computational fluid dynamics coupled to microcomputed tomography can help estimating shear stresses in porous scaffolds (28). Research is being conducted in order to couple experiments and models to study the effects of shear stress on 3D human MSC construct development (29). Another cause for the observed discrepancy between the two studies could be the initial cell source for the production of hybrid constructs. In our study, we used 2D tissue cultured expanded human MSCs whereas Braccini chose to select the mono nucleated fraction from bone marrow and subsequently seeded and expanded these on ceramic scaffolds. They found that the final hybrid constructs did not only contain cells from the mesenchymal lineage but also hematopoietic cells. It is possible that these cocultured hematopoietic cells played a critical role in the osteoinductivity of the constructs (30,31). This direction should be investigated in more detail in future studies.

As a first step we investigated if seeding and cultivation of MSCs directly from the bone marrow aspirate was feasible in our perfusion bioreactor system. Additional advantages would be the avoidance of the "unnatural" 2D cultivation and the possible positive effect of other cell types and bioactive molecules present in the bone marrow. In summary, the main conclusions that could be drawn from this study (chapter 6) are:

- Viable tissue engineered bone was produced in clinically relevant amounts (10 cm^3) from bone marrow biopsies with low and high mono nucleated cell content in a semi automated bioreactor system.
- The produced hybrid constructs show their osteogenic potential in vitro and in vivo (in a nude mice model system), and the 3D dynamically produced hybrid constructs showed at least the same amount of bone in vivo when compared to the statically cultured hybrid constructs.
- Future research should be focused on human bone marrow biopsies to asses whether this approach is feasible in the clinic.

Eventually, bone formation in a critical size defect of hybrid constructs produced in bioreactors would result in proof of concept in a large animal model (32). Previous results showed that viable cells on BCP scaffolds resulted in more bone formation when implanted ectopically in goats when compared to the bare BCP scaffold (33). However, vascularity in an ectopic acceptor site is much higher when compared to an orthopic site. Survival of cells in large sized grafts for orthopedic reconstruction will be compromised amongst others due to the absence of vascularisation during the first week after implanting (34). Therefore, the ultimate challenge would be to obtain vascularisation within the osteogenic construct before implanting it in the acceptor site. This concept is currently being investigated by several groups (35-38).

Bioreactor systems: Monitoring, control and model systems

Bioreactors provide us with a tool to conduct monitored and controlled studies. Therefore, they are also used to conduct investigations on cell function and tissue development in an area of ongoing research like tissue engineering. As we already discussed, they are for instance used to study the influence of shear forces or the application of multiple cell types in a 3D environment. Since perfusion bioreactors create an option to monitor and manipulate the metabolite composition of the medium, we designed a study to combine the potential positive effects of shear forces (due to continuous perfusion) and PKA activation using cAMP (39). Therefore, our hypothesis was that by in vitro differentiating the isolated HMSCs into osteogenic lineage, this would augment the in vivo performance of the cells. In summary, the main conclusions that could be drawn from this study (chapter 7) are:

- In line with our earlier observations, cAMP inhibited proliferation both in static and dynamic conditions which typically depicts the inverse relation between proliferation and differentiation.
- Even with fewer cells, cAMP treated tissue engineered constructs produced a significantly higher amount of bone indicating the amount of the committed cells into the osteogenic lineage is important rather than just the cell load on the tissue engineered constructs.
- Dynamic culturing of HMSCs did not show an increased in vivo bone formation compared to static condition (as observed before), but the combination of dynamic cultivation and cAMP did significantly enhance bone formation compared to all other groups.
- The effect of cAMP on in vivo bone formation in static conditions is apparent from our previous studies; the results suggested that there is distinct combination effect which cAMP otherwise able to deliver its effect on in vivo bone formation in dynamic condition.
- Future research should proof if this combination effect is universal or donor dependant with respect to enhanced bone formation in vivo

We are currently investigating to optimize a practical balance of cell load before addition of cAMP to achieve utmost bone formation (40). Interestingly, there are also reports stating that the final number of clonogenic human BMSCs in the constructs was positively correlated to the initial number of BMSCs seeded, and was significantly higher in osteogenic than in non-osteogenic constructs. These results indicate that clonogenic cells play a crucial role in determining the osteogenicity of engineered bone substitutes (41).

In our studies, we frequently monitored the metabolite and nutrient concentrations off line. A next step would be to continuously in line monitor and control the medium composition. The medium composition should therefore be tailored for optimal cell expansion and/or proliferation. Whatever cell type is responsible for the bone formation in vivo, a high number of those cells is desired in both tissue engineering as well as in a cell therapy approach. In order to obtain an efficient cell proliferation process, basic knowledge of MSC metabolism is imperative. We have shown that MSC metabolism is species dependant (42) when cultured in 2D tissue culture flasks. Surprisingly, These differences are not only species but also cultivation system

dependant. When culturing goat MSCs on 2D tissue culture flasks or a microcarrier based system, these cells exhibited different metabolisms when compared to perfusion cultured cells on BCP scaffolds. Ratio's of glucose consumption and lactate production (q lac/glu) in tissue culture flasks and microcarriers were close to 1 which is common for aerobic metabolism (11,42). In our perfusion cultures, we always observed molar ratios q lac/glu of 2 during the entire cultivation period as presented in chapter 6. This finding suggests that anaerobic glycolysis is the prevalent mechanism for glucose consumption as an energy source, although no hypoxic environment was created for the cells in the bioreactor. Human MSCs mainly used the glycolytic pathway to catabolize glucose as well, which is less energy efficient. This effect was observed in all cultivation systems (13,43). The fact that this mechanism is occurring in the presence of oxygen is a phenomenon known as the Warburg effect (44).

With respect to growth rate of goat MSCs, we observed significantly higher proliferation rates both in 2D tissue culture as well as 3D microcarrier and perfusion cultivations when compared to human MSCs. Goat MSCs use a larger proportion of the oxidative phosphorylation pathway for energy generation, except for the goat MSCs expanded in our perfusion system. Nevertheless, we did not observe a difference in growth rate between 2D tissue culture expanded and 3D perfusion cultivated MSCs in our study (chapter 6). Additional research with respect to metabolic measurement and modeling is recommended in order to unravel the catabolic and anabolic mechanisms of mesenchymal stem cells.

As mentioned before, we used our perfusion bioreactor system to control the oxygen tension in the reactor at a stable level. Oxygen consumption in the bioreactor could be related to cell mass (chapters 3-6). Bioreactors have also proven to be useful when investigating the influence of e.g. oxygen on (mesenchymal) cell proliferation and differentiation (13, 45-47). Although evidence increases that proliferation rates are higher and differentiation is inhibited under hypoxic conditions (48,49), there are also groups who could not confirm these findings (13,50). Moreover, advanced bioreactor design shows the feasibility of monitoring cell differentiation online by means of micro CT and Raman spectrometry (51,52). In future, advanced (micro) bioreactor systems for e.g. high throughput screening will enable tissue engineers to investigate complex relationships between different cell types, signal molecules and physical stimulation, administered either spatial and/or temporal.

Bioreactor systems: the transfer towards clinical application

In our research we developed a perfusion system in which clinically relevant amounts of bone could be cultured in a controlled environment. With respect to design, the developed system consisted of PVC and silicon tubing whereas the bioreactor itself was designed from polycarbonate. The individual components of the bioreactor system could be detached in a sterile way by using a tube sealer (Teruseal^{tm}, Terumo). After sampling, these components can be attached again in a sterile way using a tube welder (TSCD^{tm}, Terumo). The fluid pathway contained a temperature sensor and two dissolved oxygen sensors (DO), which were placed at the medium inlet and outlet of the bioreactor. Therefore, except for the DO sensors, the entire system was made of disposable material so the risk of contamination and expensive validated cleaning procedures is avoided. Our system was able to control multiple

cultures in parallel which is imperative to be cost effective. Needless to say that the presented bioreactor system, in order to be used in a decentralized production facility (e.g in a hospital facility), would require adaptations and should de developed according to the relevant GMP guidelines.

Although several systems are currently under development and/or used for clinical testing (e.g. Octane Biotech Inc, Canada, Aastrom Biosciences Inc., USA and Xpand Biotechnology BV, The Netherlands), the widespread application of bioreactor systems for cell based processes resulting in clinical and commercial viable products has yet to be realized (53). This seems to be a paradigm because the level of control, reproducibility and automation that an optimized bioreactor system enables is essential to manufacture products that must meet specific regulations and criteria regarding efficacy, safety and quality, in addition to being cost-effective.

So, why is it then that the applications of these systems do not seem to find their way into the clinic yet? Several factors are contributing to this phenomenon, including scientific, clinical, technological, regulatory and commercial ones as reviewed by Martin et al (53). For instance, limited fundamental understanding of cellular and molecular cues with respect to tissue regeneration is causing major variability in the final clinical outcome. In many cases, it is not known if the performance of a cell based product is related to the number of cells implanted, the amount of extracellular matrix produced or the cytokine release profile. Another challenge is to obtain control and knowledge over the cell source used for cell based products with respect to e.g aspiration (54) and human cell batches or donors (55-57), since they are causing intrinsic variability. Bioprocess and sensor technology could contribute by developing sensor technology which can measure import culture parameters like cell proliferation, differentiation and metabolic activity non invasively, locally and online. With respect to regulatory aspects, the lack of sound and clear guidelines is hindering the design of bioreactors that comply with specific and clear specifications. From a commercial point of view, models for commercialization of tissue engineered products are not well established resulting in uncertainties related to markets, reimbursement and overall clinical acceptation. It can therefore be concluded that, although we have made significant progress in our development of a bioreactor system producing clinically relevant amounts of bone, several hurdles have still to be taken prior to clinical application.

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SUMMARY

At present, the autologous bone graft is the gold standard for reinforcing or replacing bone in many orthopedic interventions. Unfortunately, complications of the harvest procedure during harvest operation often occur. Other disadvantages are the elaborate surgical procedure and the limited availability of autologous bone. Tissue engineering of bone by combining bone marrow stromal cells (BMSCs) with a suitable ceramic carrier provides a potential alternative for autologous bone grafts. Although this technique is promising, there are still issues which have to be solved for the technique to be clinically applicable. Currently, in bone tissue engineering, BMSCs are commonly isolated from a marrow aspiration biopsy, multiplied in tissue culture flasks and seeded on and into a three-dimensional scaffold. Subsequently, these cells are induced to differentiate to form an osteogenic hybrid construct.

For large scale-production, the current two dimensional (2D) multiplication process in tissue culture flasks has some serious drawbacks. The tissue culture flasks are limited in their productivity by the number of cells that can be supported by a given area, while repeated handling for culture maintenance makes the process labor-intensive and susceptible to human error (e.g. infections). As a result, the manufacturing costs of these conventional processes are limiting the clinical use of tissue- engineered products. Additionally, the microenvironment of the cells is not monitored and controlled in these tissue culture flasks which results in sub-optimal cell culture conditions. Furthermore, the available amount of these hybrid osteogenic constructs when conventionally produced is complicating its application. Clinically useful volumes of hybrid construct for spinal surgery and orthopedic applications vary from 4 to 60 cm^3. Production of these amounts of hybrid construct is complicated because of potential mass transfer limitations with respect to the supply of oxygen and medium components. Finally, the proliferation on a 2D surface of these cells is not comparable to the in vivo situation. It has been shown that 2D expanded BMSCs have a diminished differentiation capacity in comparison with those found in fresh bone marrow.

In this thesis we developed a bioreactor system for bone tissue engineering which can drastically reduce the amount of space and handling steps involved and has the potential to achieve considerable cost reductions. In addition, this system is closed, largely disposable, semi automated and culture conditions like oxygen concentration, pH and temperature can be monitored and controlled online. In chapter 3 and 4 we used goat BMSCs as a model system and showed that

- A perfusion bioreactor system was designed which enables the seeding and proliferation of these BMSCs in one system for the production of clinically relevant amounts (10 cm^3) of tissue engineered bone.
- During the seeding and proliferation phase, the cell attachment and cell growth could be monitored online by means of oxygen consumption
- The produced hybrid constructs were metabolically active, showed abundant matrix production, calcium phosphate nodules and their osteogenic potential *in vivo* in a nude mice model system.

Although these studies show proof of concept for a bioreactor based bone tissue engineering approach, it is known that human hybrid constructs often behave differently with respect to vivo osteogenicity when compared to animal model systems. Additionally, patient variability with respect to proliferation and differentiation capacity of human BMSCs is complicating the investigation of human cell based bone tissue engineering. Therefore, in chapter 5, we designed a study producing hybrid constructs from 8 different human donors in 13 individual runs in our developed bioreactor system. We investigated the influence of dynamic flow conditions on the *in vitro* and *in vivo* bone formation and compared them to statically cultured constructs. From this study we concluded that:

- Viable human tissue engineered bone could be produced in clinically relevant amounts (10 cm^3) from BMSCs in different seeding densities for different donors and at different perfusion rates showing the robustness of the system
- The produced hybrid constructs showed their osteogenic potential in vitro and *in vivo* (in a nude mice model system). Dynamically and statically cultured constructs showed similar in vivo bone formation.

As a first step towards clinically applicable bone tissue engineering, we investigated if seeding and cultivation of BMSCs directly from the bone marrow aspirate was feasible in our perfusion bioreactor system to reduce the amount of handlings. Additional advantages would be the avoidance of the “unnatural” 2D precultivation and the possible positive effect of other cell types and bioactive molecules present in the bone marrow. In summary, chapter 6 showed that:

- Viable tissue engineered bone was produced in clinically relevant amounts (10 cm^3) from bone marrow biopsies with low and high mono nucleated cell content in a semi automated bioreactor system.
- The produced hybrid constructs showed their osteogenic potential *in vitro* and *in vivo* (in a nude mice model system), and the 3D dynamically produced hybrid constructs showed at least the same amount of bone *in vivo* when compared to the statically cultured hybrid constructs.
- Future research should be focused on human bone marrow biopsies to asses whether this approach is feasible in the clinic.

In addition to their clinical applicability, bioreactors provide us with a tool to conduct monitored and controlled studies. Therefore, they are also used to conduct investigations on cell function and tissue development in an area of ongoing research like tissue engineering. For instance, the influence of shear forces or the application of multiple cell types in a 3D environment can be studied in bioreactor systems. Since perfusion bioreactors create an option to monitor and manipulate the metabolite composition of the medium, we designed a study to combine the potential positive effects of shear forces (due to continuous fluid perfusion) and PKA activation using cAMP. This study is described in chapter 7 and our hypothesis was that by in vitro differentiating the isolated human MSCs into osteogenic lineage, this would augment the *in vivo* performance of the cells. The main conclusions that could be drawn from this study are:

- In line with earlier observations, cAMP inhibited proliferation both in static and dynamic conditions which typically depicts the inverse relation between proliferation and differentiation.
- Even with fewer cells, cAMP treated tissue engineered constructs produced a significantly higher amount of bone *in vivo* indicating the amount of the committed cells into the osteogenic lineage is important rather than just the cell load on the tissue engineered constructs.
- Dynamic culturing of human BMSCs did not show an increased in vivo bone formation compared to static condition (as observed before), but the combination of dynamic cultivation and cAMP did significantly enhance bone formation compared to all other groups.
- Future research should proof if this combination effect is universal or donor dependant with respect to enhanced bone formation in vivo

Overall, we conclude that bioreactor based bone tissue engineering is feasible generating in vitro and in vivo clinically relevant amounts of hybrid osteogenic constructs in a more efficient and controlled way. Therefore, the semi automated disposable perfusion system presented in this thesis could potentially facilitate the introduction of bone tissue engineered products in clinical practice, although several hurdles have still to be taken prior to clinical application including scientific, clinical, technological, regulatory and commercial challenges.

SAMENVATTING

In veel orthopedische interventies is de autologe bottransplantatie tegenwoordig nog steeds de gouden standaard om bot te vervangen of te versterken. Helaas ontstaan er regelmatig complicaties tijdens deze uitgebreide chirurgische ingreep. Bovendien is de beschikbare hoeveelheid donorbot bij autologe bottransplantatie beperkt. Bot weefselkweek, waarbij beenmerg stroma cellen (BMSCs) van een patiënt gecombineerd worden met een geschikt keramisch drager materiaal, is een mogelijk alternatief dat het intrinsieke vermogen heeft om deze nadelen te overkomen. Hoewel deze techniek veelbelovend is, moeten er echter nog problemen worden opgelost om deze techniek klinisch toe te passen. In conventionele botweefsel kweektechniek worden deze BMSCs geïsoleerd en gekweekt in weefselkweekflessen en vervolgens in en op een driedimensionaal drager materiaal gezaaid. Hierna worden ze gestimuleerd om te differentiëren naar een botvormend hybride construct.

Voor grote schaal productie heeft deze 2D vermeerdering in kweekflessen aanzienlijke nadelen. De kweekflessen waarin deze stamcellen worden gekweekt zijn gelimiteerd in de hoeveelheid cellen die erin geproduceerd kunnen worden, terwijl herhaaldelijk menselijk handelen het proces arbeidsintensief en gevoelig voor fouten maakt (bv infecties). Dit heeft als gevolg dat de productiekosten van deze conventionele processen de introductie van weefselkweek voor klinisch gebruik bemoeilijken. Bovendien worden de kweekomstandigheden tijdens dit proces niet gemeten en gecontroleerd, hetgeen resulteert in suboptimale kweekomstandigheden. Verder is de beschikbare hoeveelheid van deze geproduceerde osteogene hybride constructen op conventionele manier een factor die de toepassing ervan bemoeilijkt. Klinisch relevante hoeveelheden van deze hybride constructen voor chirurgie met betrekking tot de ruggengraat en orthopedische toepassingen variëren van 4 tot 60 cm^3. Productie van deze hoeveelheden hybride constructen wordt bemoeilijkt door mogelijke massa transport beperkingen met betrekking tot de voorziening van zuurstof en voedingsstoffen. Tenslotte is de vermeerdering op een 2D oppervlak van deze cellen niet vergelijkbaar met de in *vivo* situatie. Er is bewezen dat 2D vermeerderde BMSCs een verminderd vermogen hebben om te differentiëren vergeleken met dezelfde cellen uit vers beenmerg.

Wij presenteren in dit onderzoek een bioreactor systeem voor bot weefselkweek dat het aantal stappen, de benodigde hoeveelheid ruimte en hierdoor de kosten drastisch kan beperken. Verder is het bioreactor systeem gesloten, semi geautomatiseerd, grotendeels “disposable” (voor eenmalig gebruik) en kunnen kweek condities zoals zuurstofverbruik, pH en temperatuur online worden waargenomen en gecontroleerd. In hoofdstuk 3 en 4 gebruikten we geiten BMSCs als model systeem en toonden aan dat:

- Een perfusie bioreactor systeem werd ontworpen dat het zaaien en vermenigvuldigen van deze BMSCs in een systeem mogelijk maakt en dat klinisch relevante hoeveelheden (10 cm^3) botweefsel kan produceren.
- Gedurende de zaai en vermenigvuldiging fase kunnen de celhechting en vermeerdering online waargenomen worden door de zuurstofconsumptie te meten.
- De geproduceerde hybride constructen waren metabolisch actief, lieten matrix productie en calcium fosfaat nodules zien en zijn in staat om bot te vormen in een *in vivo* muizenmodel.

Hoewel deze studies het bewijs van het concept laten zien is het welbekend dat humane hybride constructen zich anders gedragen met betrekking tot bot vorming *in vivo* vergeleken met diermodellen. Verder bemoeilijkt de patiënt variatie met betrekking tot vermeerdering en differentiatie vermogen van humane BMSCs het onderzoek naar humane cel gebaseerde bot weefselkweek. Daarom hebben we in hoofdstuk 5 een studie ontworpen waarbij hybride constructen van 8 verschillende humane donoren in 13 afzonderlijke runs geproduceerd werden in ons bioreactor systeem. We onderzochten de invloed van dynamische vloeistofstromen op de *in vitro* en *in vivo* botvorming en vergeleken deze met statisch gekweekte constructen. Uit deze studie konden we concluderen dat:

- Levend humaan botweefsel geproduceerd kon worden in klinisch relevante hoeveelheden (10 cm^3) van BMSCs in verschillende zaaidichtheden van verschillende donoren bij verschillende perfusie stroom snelheden hetgeen de robuustheid van het systeem laat zien.
- De geproduceerde hybride constructen lieten hun botvormend vermogen *in vitro* en *in vivo* zien (in een naakte muis model systeem) en dynamisch en statisch gekweekte constructen vormen gelijke hoeveelheden bot *in vivo*.

Om een eerste stap te maken in de richting van een klinische toepassing, onderzochten we of het mogelijk was de BMSCs direct van het beenmerg biopt te zaaien en te vermeerderen in ons perfusie bioreactor systeem om het aantal handelingen te reduceren. Het bijkomende voordeel is dat de “onnatuurlijke” 2D vermeerdering vermeden wordt en dat er mogelijk positieve effecten zijn van andere celtypes en bioactieve moleculen die aanwezig zijn in het biopt. Samengevat laat hoofdstuk 6 zien dat:

- De geproduceerde hybride constructen hadden botvormend vermogen *in vitro* en *in vivo* zowel met laag als met hoog aantal startcellen in het semi geautomatiseerde bioreactor systeem.
- De geproduceerde hybride constructen lieten hun botvormend vermogen *in vitro* en *in vivo* zien (in een naakte muis model systeem) en dynamisch en statisch gekweekte constructen vormen gelijke hoeveelheden bot *in vivo*.
- Toekomstig onderzoek zal zich moeten richten op het verder uitwerken van deze aanpak om vast te stellen of hij toepasbaar is in de kliniek.

Behalve klinisch toepasbaar, voorzien bioreactoren ons van een middel om gecontroleerde studies uit te voeren. Daarom worden ze ook gebruikt om celfuncties en weefselontwikkeling te bestuderen in een continue onderzoeksomgeving zoals weefselkweek. Zo kunnen bijvoorbeeld de invloed van afschuifkrachten of de

toepassing van meerdere cel types in een 3D omgeving bestudeerd worden in bioreactor systemen. Omdat perfusie bioreactor systemen de mogelijkheid bieden om de metabolieten samenstelling van het medium waar te nemen en te manipuleren, hebben we een studie ontworpen om de mogelijk positieve effecten van afschuifkrachten (veroorzaakt door continue vloeistof perfusie) en PKA aktivatie door cAMP te bestuderen. Deze studie is beschreven in hoofdstuk 7 en onze hypothese was dat, door de humane BMSCs *in vitro* te differentiëren richting botcellen, de *in vivo* botvorming vergroot zou worden. De belangrijkste conclusies van deze studie zijn:

- In overeenstemming met eerdere observaties remde cAMP de vermeerdering onder statische en dynamische condities hetgeen de kenmerkende omgekeerde evenredigheid tussen proliferatie en differentiatie laat zien.
- Zelfs met minder cellen laten cAMP behandelde weefselkweek constructen significant meer botvorming *in vivo* zien. Dit geeft aan dat de hoeveelheid cellen die toegewijd zijn richting de osteogene lijn belangrijker is dan simpelweg de totale hoeveelheid cellen op de weefselkweek constructen.
- Dynamisch kweken van humane BMSCs liet geen verhoogde botvorming *in vivo* zien vergeleken met statische condities (zoals we reeds eerder waargenomen hadden), maar de combinatie van dynamische kweekcondities en cAMP verhoogde significant de botvorming *in vivo* ten opzichte van alle andere groepen.
- Toekomstig onderzoek zou moeten aantonen of dit positieve effect van de combinatie algemeen optreedt of dat dit donor afhankelijk is met betrekking tot *in vivo* botvorming.

Op basis van bovenstaande resultaten, concluderen we dat bioreactor gebaseerde bot weefselkweek mogelijk is en dat in vitro en in vivo hybride osteogene constructen in klinisch relevante hoeveelheden op een efficiëntere en meer gecontroleerde wijze geproduceerd kunnen worden. Daarom kan het semi geautomatiseerde systeem zoals gepresenteerd in deze dissertatie de introductie van bot weefselkweek in de kliniek mogelijk dichterbij brengen. Voordat deze introductie in de kliniek kan plaatsvinden, moeten er echter nog verscheidene stappen gezet worden op onder andere wetenschappelijk, klinisch, technologisch, regulatoir en commercieel gebied.

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Frank

CURRICULUM VITAE

Frank Janssen (8th April 1973) completed his MSc in Food Science and Technology at the Wageningen University in 1997 with a specialisation in microbiology and biochemistry. Subsequently he participated in a postdoctoral education program (BODL) at the Technical University Delft in the field of bioprocestechnology and graduated in 1999. Thereafter, he worked as a process development engineer at Campina Melkunie (a dairy company) developing new methods for the production of probiotic dairy products (e.g. Biogarde). Fascinated by the complex mechanisms of eukaryotic systems, his interest for mammalian cell culture kept growing and he joined the tissue engineering company IsoTis in early 2001. As a process engineer, he enforced the bioreactor team which was dedicated to developing a bioreactor based production process for tissue engineered bone. Unfortunately, the company redirected its strategy and decided to stop all cell based programs within the company. In June 2004, he joined the Tissue Engineering department of the University of Twente as a PhD student to follow up on his previous research on bioreactors in bone tissue engineering. In January 2006, he also joined the biotech company Progentix as a process scientist where he participated in the development of a bioreactor process to cultivate mesenchymal stem cells for cell therapy. Since November 2008, he is a project leader in the Bacterial Bioproces and Technology (BTS-B) group at Intervet Shering Plough in Boxmeer where he is developing processes for animal vaccine production.