Pre-clinical Validation of Bone Tissue Engineering Using Mesenchymal Stromal Cells

Anindita Chatterjea

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DISSERTATION

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on the authority of the rector magnificus,
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by

Anindita Chatterjea

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Summary

The incidence of bone and joint related disorders such as osteoporosis, arthritis, as well as other diseases such as obesity, diabetes, and cancer, which can cause injury to orthopedic tissues and affect the health and capability of the human skeleton is on the rise. In such situations, the body's own regenerative capacities are often exceeded resulting in poor healing of bony defects. Such situations necessitate the use of grafting material to aid the body in its restorative attempts. It has been estimated that globally, one million bone-grafting procedures are performed annually on the pelvis, spine, and other body extremities. 11% of these procedures rely on the use of synthetic bone graft substitutes. According to market analysis this number is expected to rise even further in the coming years due to the aging population, lifestyle issues, risks associated with obtaining autograft bone, the need to achieve superior and optimum bone fusion, speedy patient recovery and the need to eliminate multiple surgeries (in case of bone harvesting from the patient). The challenge is to provide these synthetic substitutes with osteoconductive and osteoinductive properties comparable to autologous bone. While altering the physical and chemical properties of the synthetic graft materials has been partially successful in endowing them with the desirable osteoinductive and conductive properties, till date their performance within the human body is not comparable to that of autologous bone. Adding growth factors such as bone morphogenetic proteins (BMPs) and stem cells have been proposed as alternative strategies to boost the biological properties of these materials. In this thesis, we have mainly focused on optimizing the combination of ceramic materials with stem cells derived from the adult bone marrow (BM derived MSCs) to engineer a bone graft which has potential to be used clinically as a replacement for autografts.

In Chapter 1, a general introduction is given to the field of bone tissue engineering. Chapter 2 reviews the clinical trials using non-genetically

modified, bone marrow derived MSCs, for bone tissue engineering described in literature, to identify factors which are a bottleneck in the successful clinical translation of bone tissue engineering approaches.

The first experimental chapter (Chapter 3) deals with evaluating in vivo, the performance of tissue engineered grafts generated using whole unprocessed bone marrow in place of the mesenchymal stromal cells (MSCs) which are isolated from the bone marrow in an unphysiological environment using labour, time and money intensive protocols. Although our results indicate that grafts generated using the whole bone marrow performed comparable to grafts generated using the expanded MSCs, the amount of bone obtained is still not comparable to the gold standard "autologous bone".

Thus in order to improve the amount of bone formed, we cultured the MSCs in a more physiological 3-dimensional cell aggregation system. Such systems have been reported by other investigators to improve the differentiation potential of the MSCs by promoting better cross-talk between the individual cells (Chapter 4). Our results suggested that the grafts based on the cell aggregation system generated significantly greater amounts of bone as compared to those generated by the conventional system of using single cells. In Chapter 5, we then adapted this protocol in order to generate bone tissue engineered grafts that can be delivered to the defect site via minimally invasive approaches.

In Chapter 6, we investigate another potentially "off-the-shelf" approach to generate tissue engineered constructs. The unpredictable donor-donor variation in the amount of bone formed makes it difficult to guarantee good in vivo bone formation using autologous MSCs. Since data from other research areas suggests that MSCs do not follow the normal rules of allogeneic rejection, in this chapter we tested in vivo the bone forming capacity of allogeneic MSCs. As our results were suggestive of an immune attack on the osteogenically differentiated allogeneic MSCs, within the same chapter, we investigated the possibility of using immunosuppressants to prolong the survival and eventual bone formation by the allogeneic MSCs.

Chapter 7 aimed to determine if the superior osteoinductive potential of the β-TCP is relevant to bone healing in a critical sized orthotopic defect in rats, in comparison with the less osteoinductive HA. Further, it is believed that a mild

contained inflammation positively influences the amount of bone formed, while a stronger inflammatory reaction can make the surrounding milieu hostile. Therefore, in this study, we also compared the inflammatory response elicited by the two ceramics and studied its effects on the dynamics of bone formation.

In conclusion, this thesis tests multiple strategies to develop bone tissue engineered grafts suitable for use in a clinical setting. While we successfully demonstrated the possibility to make significant improvements in the amount of bone obtained using simple, cost effective, clinically applicable techniques, our results suggest that challenges still remain in the quest to develop a replacement for an autologous bone graft. Mimicking the natural in vivo environment, though extremely complicated, is probably the most promising approach. Chapter 8 of this thesis describes the possible future approaches that can be adopted to provide a replacement to one of nature's most dynamic tissues – "the bone".

Samenvatting

Het aantal gevallen van bot- een gewrichtsaandoeningen zoals osteoporose en artritis, alsmede andere aandoeningen zoals obesitas, diabetes en kanker die schade aan het bewegingsapparaat kunnen veroorzaken en de gezondheid en kwaliteit van het menselijke skelet beïnvloeden neemt toe. Het regeneratieve vermogen van het lichaam wordt in zulke gevallen vaak overschreden, wat resulteert in een slechte genezing van bot defecten. Het is in zulke situaties noodzakelijk om gebruik te maken van transplantaten (grafts) om dit herstel te bevorderen. Naar schatting worden er wereldwijd op jaarbasis één miljoen botgrafting procedures uitgevoerd, onder andere in het bekken en de wervelkolom. 11% van deze procedures is afhankelijk van het gebruik van synthetische grafts. Marktonderzoek wijst uit dat dit percentage in de komende jaren nog verder zal stijgen als gevolg van vergrijzing, slechte levensstijl, risico's geassocieerd met het verkrijgen van lichaamseigen bot, de noodzaak voor het verkrijgen van uitstekende en geoptimaliseerde botfusie, snel herstel van de patiënt en het vermijden meerdere operaties (in het geval van bot oogsting van de patiënt). De uitdaging is om deze synthetische vervangende materialen te voorzien van osteoconductieve en osteoinductieve eigenschappen vergelijkbaar met autoloog bot. Hoewel men er al gedeeltelijk in is geslaagd om de fysische en chemische eigenschappen van het materiaal aan te passen door ze te voorzien van de gewenste osteoinductieve en osteoconductieve eigenschappen, is de doeltreffendheid van deze materialen in het menselijk lichaam tot dusverre niet vergelijkbaar met die van autoloog bot. Het toevoegen van groeifactoren zoals bone-morphogenic proteins (BMPs) en stamcellen wordt voorgedragen als alternatieve strategie om de biologische eigenschappen van deze materialen te versterken. In dit proefschrift hebben we ons met name gericht op het optimaliseren van de combinatie van keramieken en stamcellen afkomstig uit het beenmerg voor het ontwikkelen van een botgraft voor klinische toepassing als vervanging van autografts.

In hoofdstuk 1 wordt een algemene introductie gegeven tot het vakgebied van bot weefseltechnologie (of tissue engineering). Hoofdstuk 2 geeft een literatuur overzicht van klinische trials waarin gebruik wordt gemaakt van niet-genetisch gemodificeerde (uit beenmerg verkregen) stamcellen voor bot weefseltechnologie, voor het identificeren van factoren die een knelpunt vormen voor het succesvol klinisch toepassen van bot weefsel technologieën.

Het eerste experimentele hoofdstuk (hoofdstuk 3) behandelt de in vivo evaluatie van de tissue engineered grafts uit onbewerkt compleet beenmerg in plaats van de mesenchymale stromale cellen (MSCs) die in niet fysiologische omstandigheden uit het beenmerg worden geïsoleerd door gebruik te maken van arbeidsintensieve, tijdrovende en kapitaalintensieve protocollen. Hoewel onze resultaten erop wijzen dat de grafts uit compleet beenmerg vergelijkbaar presteren als de grafts geproduceerd uit opgekweekte MSCs, is de verkregen hoeveelheid bot nog niet te vergelijken met de gouden standaard “autoloog bot”.

Zodoende hebben we, om de hoeveelheid gevormd bot te verhogen, de MSCs gekweekt in een meer fysiologisch driedimensionaal cel aggregaat systeem. Deze systemen zijn al eerder door andere onderzoekers beschreven voor het verbeteren van de differentiatie capaciteit van MSCs door de interactie tussen de individuele cellen te bevorderen (hoofdstuk 4). Onze resultaten suggereren dat de grafts gebaseerd op het cel aggregaat systeem een significant grotere hoeveelheid bot vormden vergelen met de hoeveelheid bot gevormd met de conventionele grafts waarbij individuele cellen worden gebruikt. In hoofdstuk 5 hebben we dit protocol vervolgens aangepast om bot tissue engineered grafts te produceren die via minimaal invasieve procedures in het bot defect kunnen worden aangebracht.

In hoofdstuk 6 onderzoeken we een andere mogelijke “off-the-shelf” methode voor het genereren van tissue engineered constructen. De onvoorspelbare variatie in botvormende capaciteit van verschillende donoren bemoeilijkt het garanderen van een goede botvorming in vivo met het gebruik van autologe MSCs. Aangezien ander onderzoek suggereert dat MSCs niet voldoen aan de regels van normale allogene afstotingsreacties, hebben we in dit hoofdstuk de in vivo botvormende capaciteit van allogene MSCs getest. Omdat onze resultaten een immuunreactie op de osteogeen gedifferentieerde allogene MSCs suggereren, hebben we – in hetzelfde hoofdstuk – de mogelijkheid voor

het gebruik van immunosuppressiva voor het verlengen van de overleving en de uiteindelijke botvorming door de allogene MSCs onderzocht.

Hoofdstuk 7 beoogt vast te stellen of de uitstekende osteoinductieve potentie van de beta-TCP relevant is voor botherstel in ratten met een orthopedisch defect met kritische grootte, in vergelijking met de minder osteoinductieve HA. Bovendien wordt aangenomen dat een milde beheerste ontstekingsreactie de hoeveelheid botvorming positief kan beïnvloeden, terwijl een sterkere ontstekingsreactie kan leiden tot een afstotingsreactie van het omliggende weefsel. Daarom hebben we in deze studie ook de ontstekingsreacties die door beide keramieken worden veroorzaakt onderling vergeleken, en het effect op de dynamische processen van botvorming bestudeerd.

Concluderend beschrijft dit proefschrift verscheidene strategieën voor het ontwikkelen van tissue engineered botgrafts die geschikt zijn voor gebruik in een klinische setting. Hoewel we succesvol de mogelijkheid hebben laten zien voor het significant verbeteren van de hoeveelheid botvorming door middel van simpele, kosteneffectieve en klinisch toepasbare technieken, laten onze resultaten ook zien dat er nog steeds uitdagingen zijn in de zoektocht naar het ontwikkelen van een vervanging voor een autologe botgraft. Het nabootsen van de natuurlijke omgeving is waarschijnlijk de meest veelbelovende methode, al is dit uitermate ingewikkeld. Hoofdstuk 8 van dit proefschrift beschrijft mogelijke toekomstige methodes die een vervanging kunnen leveren voor één van de meest dynamische weefsels van de natuur – “het bot”.

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Chapter 1

Introduction

1.1. A brief insight into bone biology

The skeleton's role as internal support system of the body has given bone the reputation of being an inert and static material. However, given the ability of bone to adapt to the functional demands of the body, to continuously remodel itself to maintain tissue homeostasis, to repair itself without a scar and serve as an "on demand" mobilizable store of calcium and phosphate, it is in fact the ultimate "smart material" [1]. Below we describe in short the biology of bone (Fig. 1.1).

Bone tissue in the adult skeleton is arranged in two architectural forms: trabecular also called cancellous or spongy bone (around 20% of the total skeleton) and cortical or compact bone (around 80% of the total skeleton). Cortical bone is almost solid with a porosity of 10% while cancellous bone is a highly porous structure (>75%porosity). The distribution of these two types of bone is strategically arranged to accommodate the input of stresses and strain during weight bearing. For example, the trabecular areas in the metaphysis of the long bones readily distribute the forces and movements to the cortical shell of the diaphysis. Similarly, the vertebral body distributes the axial compressive forces to the sponge-like network of the trabecular bone of which it is composed, thus minimizing fracture risk even under extreme conditions [2].

Microscopically, bone can be arranged in a lamellar or woven pattern. Woven bone has an irregular, disorganized pattern of collagen fiber orientation and osteocyte distribution. Woven bone is characteristic of embryonic development, although it can also be found in certain locations in the adult skeleton. These include the areas of ligament and tendon insertions and the temporary callus of a healing fracture. Mechanical stimulation can cause rapid production of woven bone which can ultimately remodel into dense lamellar bone. This indicates that woven bone is a rapid response of the body to demands caused by change in functional activity [3]. Lamellar or mature bone on the other hand is found in both cortical and cancellous bone and consists of repeating units called Haversian systems or osteons, which generally run parallel to the long axis of the bone. Each osteon has multiple concentric layers of mineralized matrix called lamellae. They are deposited around a central canal, the Haversian canal, containing blood vessels and nerves. Osteocytes, one of the most abundant cells in the bone, are found between the concentric lamellae and connect to each other and the central canal by cytoplasmic processes called canaliculi. It now appears that through these canaliculi, the osteocyte may actually orchestrate the spatial and temporal recruitment of the cells that form and resorb bone [4]. These cells are further described in the next paragraph.

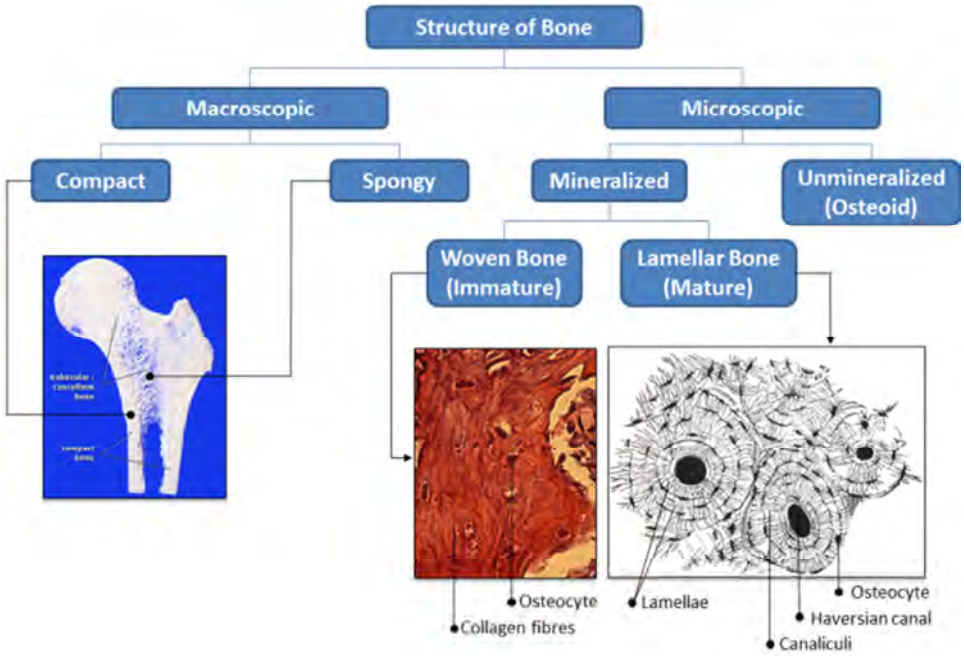

Figure 1.1. Schematic representation of the macroscopic and microscopic structure of bone

Three distinct cell types can be found within bone: the matrix producing osteoblast, the tissue resorbing osteoclast, and the osteogenic precursor cells or bone lining cells. The osteocytes mentioned in the earlier paragraph, accounts for 90% of all cells in the adult skeleton and are actually a highly specialized type of osteoblasts.

The freshly synthesized matrix laid down by the osteoblasts is called osteoid and primarily consists of collagen. 10- 15 days after it has been laid down, the organic matrix begins to mineralize. During this process, the mineral content suddenly increases to 70% of the final amount while the deposition of the remaining 30% takes several months. The calcified bone generated at the end of the mineralization process consists of 25% organic matrix, including cells (2-5%), 5% water and 70% inorganic mineral. Other proteins, some of them unique to bone, such as osteocalcin, are embedded in the extracellular matrix and may have important signaling functions or may play a role during the mineralization process [5].

Bone formation in humans can follow two mechanisms. One route involves direct differentiation of the precursor cells into osteoblasts which then proceeds to form bone. This method of bone formation is called intramembranous bone formation and is found during the development of the skull, maxilla and mandible.

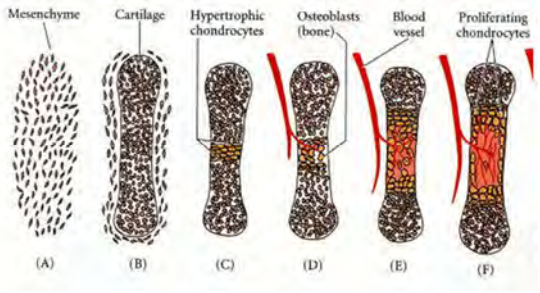

Figure 1.2. Endochondral ossification of bone

The other route of bone formation is via condensation of mesenchymal cells followed by their differentiation into chondrocytes. The chondrocytes hypertrophy, mineralize their matrix and secrete signals leading to invasion by blood vessels. The invading blood vessels bring along hematopoietic cells which interact with the stroma and eventually form the bone marrow. The hypertrophic chondrocytes at some point undergo apoptosis and are replaced by osteoblasts. The osteoblasts ultimately form the bone matrix. The majority of the bones in the body are formed in this manner. This method of bone formation is called endochondral ossification [6] (Fig. 1.2).

1.2. Problem statement

As mentioned above, bone is a dynamic and complex tissue, which plays crucial roles in both mechanical support and mineral homeostasis. Thus, it is not surprising that when bone is injured, it can have major consequences on the quality of the patient's life. Fortunately, bone has a very good regenerative capacity and the majority of bony injuries (fractures) heal without the formation of scar tissue, and bone is regenerated with its pre-existing properties largely restored, and with the newly formed bone being eventually indistinguishable from the adjacent uninjured bone [7].

However, for defects caused by severe trauma, congenital malformations, tumours, infections and non-union fractures, the natural bone regeneration process is not sufficient and thus surgical interventions using bone grafts are required. In addition to the need for bone grafts in cases where the defect is beyond the body's regenerative capacity, bone grafts are also used in spinal fusion and hip revision surgeries.

Spinal fusion surgeries are a treatment option for many orthopedic and neurological conditions. These include correction of spinal deformities such as scoliosis, spinal disc herniation, vertebral fractures and conditions where abnormal motion between the vertebras cause irritation or damage to the adjacent nerves, resulting in pain and

neurological problems. The surgery involves fusion of two or more vertebras using bone grafts.

Hip revision surgeries are needed in patients who experience pain due to loosening of the prosthesis because of wear and tear. The debris from the old prosthesis irritates the surrounding soft tissue causing inflammation. The inflamed tissue in turn results in catabolism of the underlying bone. This results ultimately in the prosthesis loosing contact with the existing bone. The old prosthesis is replaced by a newer one and bone grafts are implanted to make up for the lost bone and re-establish contact of the prosthesis with the surrounding bone.

1.3. Currently available solutions

For all the problems mentioned above, where the natural process of bone regeneration is exceeded, there are a number of treatment options available to the surgeon. These include distraction osteogenesis and bone transport methods [8, 9] and use of bone grafts[10]. A few of the available non-invasive methods include methods of biophysical stimulation such as low intensity pulsed ultrasound (LIPUS) and pulsed electromagnetic field (PEMF) [11-13]. However, these methods are normally used as adjuncts to the invasive methods to enhance bone regeneration.

1.3.1. Distraction osteogenesis and bone transport

Distraction osteogenesis is a biological process of new bone formation between the surfaces of bone segments that are gradually separated by incremental traction. This process is initiated when a traction force is applied to the bone segments generating a tensional stress within the tissues that joins the divided bone segments, which in turn stimulates new bone formation parallel to the vector of traction. A variety of methods are currently used based on this principle, including external fixators and the Ilizarov technique, intramedullary lengthening devices and a combination of intramedullary nails with external distraction devices. However, these methods are technically demanding and have several disadvantages, including associated complications, requirements for lengthy treatment periods which in turn may have consequences on the patient's psychology and wellbeing [8, 14, 15].

1.3.2. Bone grafts

Bone grafting is a commonly performed surgical procedure used to augment bone regeneration. Reconstruction of bone defects using bone grafts is dependent on certain bone-related processes, which can be summarized into osteoconduction, osteoinduction and osseointegration. Osteoconduction is the formation of bone using the pre-existing host osteocompetent cells. Thus, an osteoconductive bone graft is one that provides scaffolding for inward growth and migration of the surrounding cells

involved in bone formation. Osteoinduction is the formation of bone by stimulation and differentiation of the body's undifferentiated precursor cells. Osseointegration is the process by which the bone graft is fixed rigidly and asymptomatically to the pre-existing bone even during functional loading.

Bone grafts, currently available to the surgeon can be of the following types:

1.3.2.1. Natural bone grafts

Natural bone grafts can be obtained from either another part of the patient's own body (autograft), from a human cadaver (allograft) or from another animal species (Xenograft).

- Autograft: The gold standard graft material is autograft as it represents the ideal bone graft substitute. Autologous bone combines all necessary features to induce bone growth and regeneration: osteogenic cells as well as osteoinductive and osteoconductive factors. Live cells and other components within the autografts facilitate integration of the graft with the host tissue. Additionally, autografts are biomechanically stable, serve as scaffolds and allow invading cells and blood vessels to adhere and build up new tissue. However, the supply of suitable bone is limited and its collection is painful, leading to donor site morbidity. Moreover the need for 2 surgeries (one for obtaining bone and the other for the actual implantation in the defect site) makes it an expensive procedure. Besides, there is a risk of infection, hemorrhage, cosmetic disability, nerve damage and a possible loss of function at the donor site [16, 17].
- Allograft and Xenograft: The allograft is typically harvested from a cadaver and then devitalized using freeze drying methods. Absence of viable cells in the allografts makes them a less successful treatment option to autografts. However, advantages to the use of allograft include ready availability and less pain and complications and a more economical option to the patient as an additional surgery does not have to be performed to obtain an autograft. Unfortunately, the grafts are not without controversy, particularly due to their potential to transmit infectious agents. In spite of rigorous donor screenings and tissue treatments, confirmed reports of viral or bacterial infection associated with allografts have been reported. In April 2000, 2 different patients received bone-tendon-bone allografts for anterior cruciate ligament reconstruction from a common donor. Each patient developed septic arthritis from the donor tissue [18]. In November 2001, a patient underwent reconstructive knee surgery, and within 4 days of the surgery, the patient died of infection caused by Clostridium sordellii [19]. After these and similar cases

were reported, the CDC began an investigation that revealed 25 other cases of allograft-related infection or illness [19]. Xenografts have similar advantages and disadvantages as an allograft. However, since the species of origin of the graft material is different, the immunogenicity of these grafts is even higher than with allografts.

The fact that more than 2.2 million bone graft surgeries are performed annually worldwide [20, 21] indicates that bone grafts are a much needed therapeutic option. However, all the aforementioned conventional sources for bone grafts have their limitation. This coupled with the fact that an increase in orthopedic procedures and aging population will further increase the demand for bone grafts, research and development of substitutes which meet the performance of the autografts, without its associated drawbacks, is justified. This sets the stage for bone graft substitutes.

1.3.2.2. Bone graft substitutes

Bone graft substitutes were developed to provide a viable solution to healing bone defects while avoiding the problems associated with natural bone grafts. They consist of scaffolds made of synthetic or natural biomaterials that promote the migration, proliferation and differentiation of bone cells for bone regeneration. A wide range of scaffolding materials can be used, including biological materials like coral or demineralized bone matrix, metals such as titanium or its alloys, glass ceramics, collagen, ceramics such as hydroxyapatite (HA) or b-tricalcium phosphate (b-TCP), calcium-phosphate cements, polymers like poly methyl methacrylate and even composites such as calcium-phosphate coatings on metallic implants[22, 23]. The main difficulty to their wider use remains the absence of osteoinductive properties. Though osteoconductive properties of these materials can be improved by altering their surface character, geometric form as well as the pore size and pore structure, providing the right osteoinductive signals using the biomaterials alone, still remains a challenge.

During the natural course of fracture repair, platelets, inflammatory cells and macrophages arriving at the site of injury secrete cytokines and growth factors, which in turn attract stem cells to the site of the defect [24]. Thus using growth factors in combination with biomaterials is an option to improve the osteoinductivity of the scaffolds. Some growth factors observed at the site of fracture healing include transforming growth factors (TGF-β), insulin like growth factors (IGF-I and II), platelet derived growth factors (PDGF), fibroblast growth factors (FGF) and various types of bone morphogenetic proteins (BMPs). These different growth factors have been studied as an alternative to using the biomaterials alone. However, it is difficult to control precisely the rate of release of growth factors from the scaffolds and thus

their local concentrations. Using supra-physiological doses are an option but this is associated with excessive costs and putative side effects such as unwanted ectopic bone formation [24, 25]. However, in spite of these issues, BMP-2 in combination with a collagen sponge has been approved for use in the clinics and is widely applied [26].

1.3.2.3. Tissue engineered bone grafts

Tissue engineering is an interdisciplinary field that applies the principle of engineering and life sciences towards the development of biological substitutes that restore, maintain or improve tissue function. The general principle of tissue engineering involves the association of cells with a natural or synthetic support i.e. a scaffold, to produce a three dimensional living implantable construct, similar to an autologous bone graft [27]. It is expected that the implanted cells will differentiate into osteogenic cells, deposit a matrix and thus form new bone. Thus, the cell based bone tissue engineering approach does not depend on the presence of local osteoprogenitors for new bone synthesis and are therefore particularly attractive for elderly patients or patients with metabolic disorders who have a diminished pool of osteoprogenitors. However, there are reports that the implanted cells contribute to bone formation not just by direct differentiation into bone forming cells but also via the secretion of factors which drives the host cells to contribute to the bone forming process. Moreover, some of the secreted factors include angiogenic cytokines such as VEGF which by enhancing vascularization of the tissue engineered constructs, improves the survival of cells within the constructs, which is of importance in larger sized grafts [28].

Studies comparing grafts with cells and without cells in the goat transverse process model have demonstrated no significant difference in the amounts of bone formed in the area of the construct adjoining the pre-existing bone. However, in the areas of the graft away from the bony sites, there was significantly greater bone in the vital grafts as compared to the grafts without cells. One such clinical scenario where the osteoconduction would be of limited value and use of cells would be beneficial is the posterolateral spinal fusion model where the area of non-union is typically away from the transverse processes [29].

Several classes of cells can be used for the purpose of bone tissue engineering. The first class consists of terminally differentiated primary cells, which in the case of bone tissue engineering would be osteoblasts. Although these cells generally show superior performance regarding tissue specific characteristics, their use for tissue engineering is often limited by laborious isolation protocols and limited proliferation capacities [30, 31].

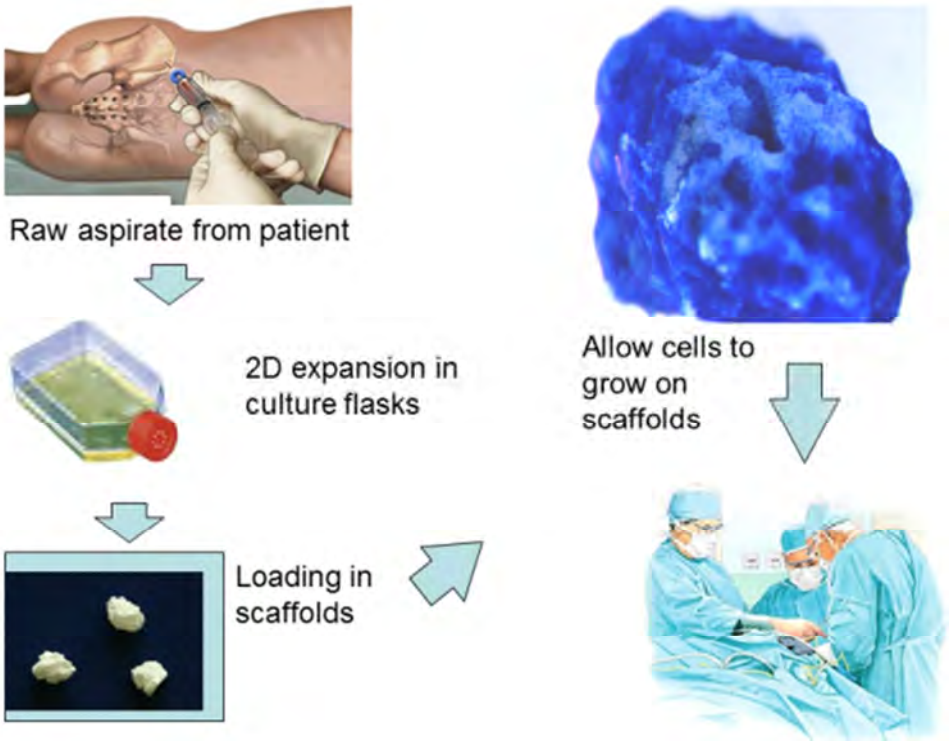

Figure 1.3. Conventional technique of bone tissue engineering

Pluripotent embryonic stem cells exhibit multi-lineage differentiation potential and unlimited self-renewal. However, ethical issues related to their use, coupled with lack of understanding on how best to regulate their differentiation and widely reported tumorogenicity of these cells in various animal models, have fuelled the research for adult cell sources with multipotent potential [32].

More than 30 years ago, Friedenstein et al. first reported evidence of spindle shaped fibroblast-like cells that could be isolated from murine bone marrow via their inherent adherence to plastic in culture [33, 34]. He observed that when the bone marrow was cultured on plastic in the presence of serum, small colonies of cells appeared, each derived from a single cell which he defined as the colony-forming unit fibroblasts (CFU-F). Others extended these early studies and demonstrated that these cells could be differentiated into cells derived from the mesoderm lineage such as adipocytes, chondrocytes, osteocytes and myoblasts. However, since these cells could not give rise to cells from the hematopoietic lineage (which are derived from a distinct cell population, the hematopoietic stem cells), the cells were referred to as non-hematopoietic, multipotent, mesenchymal stem cells or MSCs [35, 36]. Arnold Caplan

was among the first to propose the MSC as a therapeutic concept [37]. A more detailed investigation of MSC raised concerns regarding the term "stem cell", as MSC do not match the criteria defined for stemness without restriction [38]. Therefore nowadays the term mesenchymal stromal cell (MSC) is often used [39].

Because of the ready availability of the MSCs, combined with their multipotent differentiation capacity and possibilities to cryopreserve them for future use, MSCs are now considered a good cell source to generate tissue engineered grafts with in vivo bone forming potential [40, 41].

Typically, to generate a tissue engineered bone graft substitute, a bone marrow biopsy is harvested from the patient and expanded in vitro to obtain a clinically relevant number of cells. These MSCs are then seeded on different types of biomaterials and then implanted in vivo either immediately or after a few days of culture on the biomaterial [42] (Fig. 1.3). The safety and efficacy of such bone tissue engineered grafts have been demonstrated in various animal models as well as a few human clinical trials.

1.4. Location and selection criteria for MSCs

Several studies have demonstrated the MSCs exhibit characteristic features of perivascular cells which encircle small blood vessels within diverse tissues, leading to the conception that the perivascular niche represents a possible site for isolating MSCs [43]. As blood vessels penetrate all tissues in the body, it is not surprising that MSCs have been isolated from several tissues including adipose tissue, liver, muscle, amniotic fluid, synovial tissue, placenta, umbilical cord blood, and dental pulp [44]. Though still referred to as MSCs, cells from each of these sources vary in their proliferative and multi-lineage differentiation potential. However, as the MSCs derived from the bone marrow is the best characterized as compared to the cells from the other sources, bone marrow remains the principal source of MSCs for most preclinical and clinical studies [45]. The MSCs used in this thesis have been all derived from the bone marrow.

The true identity of MSCs has often been confused by different laboratories which employ different isolation and in vitro culture methods. These variables are responsible for the phenotype and function of resulting cell populations. Whether these conditions selectively promote the expansion of different populations of MSCs or cause similar cell populations to acquire different phenotypes is not clear [38]. Since MSCs do not have a specific and unique surface marker that can simplify their enrichment and characterization, The International Society for Cellular Therapy has attempted to address this issue by providing the following minimum criteria for

defining multipotent human mesenchymal stromal cells : a)plastic-adherent under standard culture conditions; b)positive for expression of CD105,CD73, and CD90, and absence of expression of hematopoietic cell surface markers CD34, CD45, CD11a, CD19, and HLA-DR; c) under specific stimulus, cells should differentiate in to osteocytes, adipocytes, and chondrocytes in vitro [46]. Though these guidelines hold true for bone marrow derived MSCs, some adjustments will have to be made as the knowledge about MSCs from other sources increase. For e.g. Short-term cultured MSCs from human adipose tissue, are positive for CD34 unlike MSCs obtained from bone marrow. Recently, the expression of surface molecules like CD146, CD271 or STRO-1 has also been shown to imply self-renewing MSC-like cells with multi lineage differentiation potential [47-49].

1.5. Limitations of conventional MSC based bone tissue engineering approach

In addition to the problems associated with ex vivo enrichment and characterization of human MSC (hMSC), it has been observed that unlike MSCs derived from other animals, hMSCs have a broad variability with relation to their in vitro differentiation capacity as well as their in vivo bone formation. Moreover, using current tissue engineering techniques, hMSCs in most cases do not generate bone in amounts sufficient for most clinical applications. Further, it is as yet not possible to determine a priori the bone forming capacity of a particular donor. Thus, while the proof of MSCs healing critical sized defects were convincingly seen in the orthotopic sites of various animal models such as in segmental defects in dogs or sheep, mandibular defects in sheep, iliac wing defects in goats, only a few case reports of successful reconstructions in humans have been described.

Bone marrow aspiration techniques, in vitro expansion of the cells on tissue culture plastic versus three dimensional scaffolds, the in vitro culture conditions during cell expansion such as hypoxia, composition of the culture medium, cell plating density, addition of osteogenic compounds in the culture medium as well as passage number of the cells used to make the construct can all make a difference to the final in vivo outcome [50-53]. This makes the generation of a graft using hMSCs with guaranteed, reproducible, good bone forming capacity a big challenge. Further, it is difficult to compare findings from various studies as the isolation method and culture conditions differ between various studies [54].

1.6. Aim of thesis

The overall goal of this thesis is to address the various aspects of the generation of a tissue engineered construct using bone marrow derived MSCs, to make it more

applicable to a clinical setting, both with respect to streamlining the generation of the graft and its bone forming capacity. Below, we outline the various aspects of the generation process of a tissue engineered bone graft that have been addressed as a part of this thesis.

1.6.1. MSC isolation and expansion

Bone tissue engineering requires a large number of multipotent MSCs. It is estimated that MSCs represent approximately only 0.01 and 0.001% of the total nucleated cells within isolated bone marrow aspirates [40, 55]. Thus, an extensive in vitro expansion of the MSCs is required prior to utilizing them to generate a tissue engineered construct. Fortunately, MSCs can be easily isolated from a small aspirate of bone marrow and culture-expanded through to significant numbers. This expansion is conventionally performed on 2D tissue culture plastic. However, there are reports demonstrating a loss of replicative ability, colony forming efficiency and differentiation capacity with time in culture [56, 57]. Moreover, after aspirating the bone marrow, the CFU-Fs present in the marrow are plated together with the other cells forming a part of the marrow microenvironment. However, the hematopoietic component of the marrow is made up of cells that do not adhere to the tissue culture plastic and is thus washed away during subsequent medium changes. Thus it is obvious that the expansion of the MSCs during the in vitro culture phase is very different from the expansion that occurs physiologically within the body. Further, the expansion phase on plastic is labor, space and time intensive and thus uneconomical, besides being a barrier to streamlining the generation of tissue engineered grafts for clinical applications. Directly culturing the bone marrow on the ceramic particles, eliminates the expansion phase on plastic and ensures a better preservation of the in vivo milieu that the MSCs are used to in vivo, as cells naturally present in the whole marrow such as the hematopoietic cells get caught in the crevices of the ceramic particles and are not as easily washed away during subsequent medium changes [58]. The aim of chapter 3 is to describe a strategy to generate tissue engineered constructs by using a defined volume of fresh unprocessed bone marrow seeded directly on scaffolds, thus bypassing the expansion phase on plastic.

1.6.2. Use of allogeneic MSCs for bone tissue engineering

Isolation of bone marrow, though far less invasive than harvesting bone grafts, still causes a certain degree of discomfort to the patient. Further, the bone forming potential of MSCs isolated from different donors vary considerably and as discussed earlier, it is as yet not possible to predict the in vivo performance of a particular donor based on in vitro markers or tests. Furthermore, another potential limitation to using autologous bone marrow to generate the constructs is the time required to harvest, select and expand the cells. An alternative approach would be to use MSCs that are

isolated from one or more donors in the past during other surgeries such as total hip replacement surgery. These cells could then be expanded, tested in vivo in animal models and cryopreserved for future "off-the-shelf therapy". The immune phenotype of MSCs (widely described as MHC 1+, MHC 11-, CD40-, CD80-, CD86-) is regarded as non-immunogenic [59, 60] . This could mean that while other allogeneic cells and organs are rapidly rejected in the host, allogeneic cells would escape detection by the immune system and continue to function with efficacy similar to autologous cells. However, as bone tissue engineering demands that the MSCs differentiate into cells of the osteogenic lineage to be of therapeutic use, a concern remains that this differentiation may alter the immunogenicity of the allogeneic MSCs [61]. Conflicting results from previous studies using allogeneic MSCs further complicates the scenario [62-64]. The aim of Chapter 4 is to determine the feasibility of using allogeneic MSCs in bone tissue engineering by comparing the immune response generated by tissue engineered constructs using allogeneic cells with those using autologous cells.

1.6.3. In vitro culturing of MSCs

The in vitro culturing phase conventionally associated with the generation of the tissue engineered grafts can represent a foreign and hostile environment for cells. However, this phase can also provide infinite possibilities to direct the behaviour of the cells in a desired manner. Previous researchers have demonstrated the influence of in vitro culture conditions such as cell plating densities, passaging densities, availability of oxygen, presence in the culture medium of compounds known to affect various signaling pathways etc. on the osteogenic differentiation potential of the cells in vitro and the bone formed in vivo [51-53, 65]. Recently, there have been a number of publications which have suggested that culturing MSCs as 3D spheroids can facilitate greater cell-cell and cell matrix contacts [66-69]. This can in turn influence the signaling activity which can alter the differentiation potential of the cells. Chapter 5 attempts to use a novel strategy of employing cell aggregates to generate tissue engineered constructs with a much shorter in vitro generation time coupled with a significantly improved in vivo bone forming capacity.

1.6.4. Delivery of the tissue engineered constructs into the defect site

Conventional TECs usually comprise of a preformed scaffold material loaded with cells. This is then introduced into the defect site using an invasive surgical approach. However, to ensure a proper fit of the TEC into the defect, the surgeon needs to machine the graft or carve the surgical site, which can increase bone loss, trauma and surgical time [70]. Chapter 6 adapts the culture system described in chapter 5 to generate a tissue engineered graft which can be introduced into the defect site using a minimally invasive approach. The autologous platelet gel used as the delivery vehicle,

is liquid at room temperature but jellifies within a few seconds at 37° Celsius (normal body temperature), resulting in a graft which takes the shape of the defect.

1.6.5. Selection of the biomaterial for generation of grafts

Although the main focus of this thesis is on the MSC component of the graft, the properties of the biomaterial on which the cells are seeded also have a crucial role in ultimately determining the success of the final construct. In the last chapter, we therefore compare within a critical sized defect in an orthotopic location, the performance of two commonly used calcium phosphate ceramics, HA and TCP. In previous studies, it has been shown that at an ectopic location, these two materials are at the two ends of the spectra with relation to their osteoinductive properties [71]. The aim of this chapter was to determine if the osteoinductive capacity of the ceramic influenced the outcome in a critical sized defect in an orthotopic location to the same extent as in an ectopic location.

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Chapter 2

Clinical Application of Human Mesenchymal Stromal Cells for Bone Tissue Engineering

The gold standard in the repair of bony defects is autologous bone grafting, even though it has drawbacks in terms of availability and morbidity at the harvesting site. Bone tissue engineering, in which osteogenic cells and scaffolds are combined, is considered as a potential bone graft substitute strategy. Proof of principle for bone tissue engineering using mesenchymal stromal cells (MSCs) has been demonstrated in various animal models. In addition, 7 human clinical studies have so far been conducted. Because the experimental design and evaluation parameters of the studies are rather heterogeneous, it is difficult to draw conclusive evidence on the performance of one approach over the other. However, it seems that bone apposition by the grafted MSCs in these studies is observed but not sufficient to bridge large bone defects. In this review, we discuss the published human clinical studies performed so far for bone tissue regeneration, using culture expanded, non-genetically modified MSCs from various sources and extract from it points of consideration for future clinical studies.

2.1. Introduction

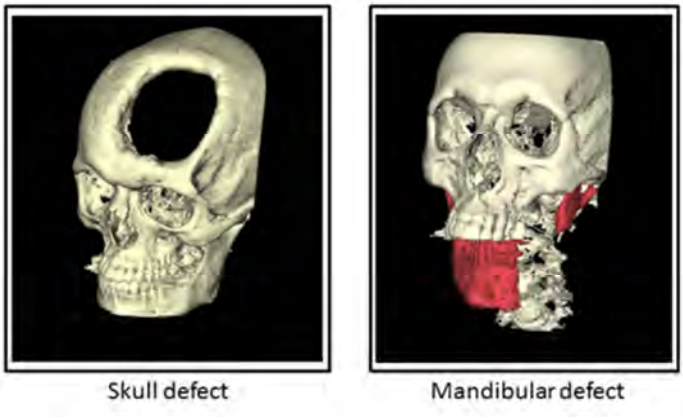

Figure 2.1. 3D reconstruction of a skull and mandibular defect in trauma patients.
Surgeons are often faced with patients having large defects in the bone which do not heal spontaneously. The gaping hole in the skull and the area highlighted in red in the mandible are examples of large sized defects in real patients. Though autografts are the gold standard treatment for such patients, the amount of graft material required is often the limiting factor. Tissue regeneration using synthetic or natural scaffolds seeded with mesenchymal stem cells can be an alternative solution for such patients.

Bone lesions/defects caused by e.g. trauma, bone resection due to ablative surgery or correction of congenital deformities are a common problem in clinical practice. In the majority of the cases the body's self-healing capacity is able to repair the defect. Yet every year, in roughly 1 million cases of skeletal injury, the defect size is too big or conditions not optimal to allow healing (Fig. 2.1, 2.2). In these cases, external help is required in the form of bone graft procedures to achieve union [1].

The most frequently used sources of bone grafting are autologous and allogeneic bone [2]. Autologous cancellous bone grafts are most successful in the present clinical scenario, because in addition to being osteoconductive and osteoinductive they are safe, cheap and easily available to the surgeons. However, obtaining autologous grafts requires the patient to be subjected to additional surgery, thus introducing extra morbidity at the donor site and increasing surgical costs [3] [4] [5]. Besides, the amount of graft material is limited and chances of complications such as infections, instability and paraesthesia at the donor site can affect up to 30% of patients [6] [7] [8]. An alternative is allogeneic bone grafting, which can be obtained from authorized tissue banks which collect and store bone tissue from human cadavers [7]. By this approach, problems associated with harvesting and quantity of graft material is bypassed. To avoid problems associated with immunogenicity, donor grafts can be devitalized via processes such as irradiation and freeze drying. Unfortunately, this processing also eliminates the cellular component, thus reducing the graft's

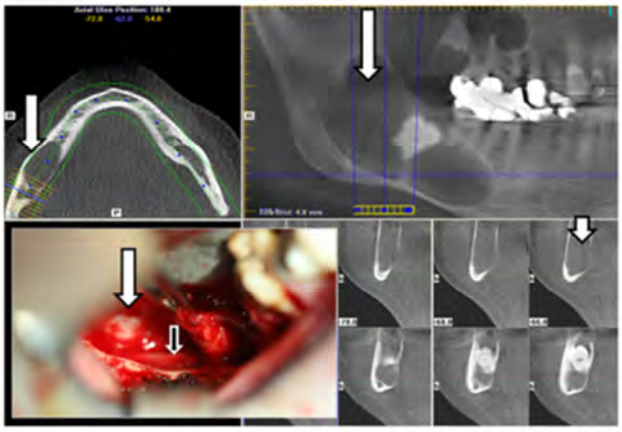

Figure 2.2. Mandibular Defect following Cyst
CT scan of huge cyst in de mandible (see white arrows). The clinical picture represents the situation after removing the cyst revealing the alveolar nerve positioned at the bottom of the cavity (black arrow).

osteoinductivity, thereby resulting in a slower rate of new bone formation as compared to autologous grafts [9].

As an alternative to autologous or allogeneic bone grafts, surgeons may use scaffolds made of synthetic or natural biomaterials that promote the migration, proliferation and differentiation of bone cells. In the last decade, a large number of publications have illustrated the osteoinductive and osteoconductive properties of scaffolds such as synthetic hydroxyapatite (HA) [10-12], coralline HA [13-15], β-Tricalcium phosphate and porous biphasic calcium phosphate[16-19], calcium phosphate cements[20], chemically-treated titanium[21] and glass ceramics[22]. However, the degree of osteogenic and osteoinductive properties provided by the osteoprogenitor cells, as present in the autografts, exceeds that of the scaffolds. To improve osteoinductivity, scaffold materials can be loaded with osteoinductive growth factors such as bone morphogenetic proteins (BMPs). The drawback of the growth factor approach are the supra-physiological concentrations needed to obtain the desired osteoinductive effects, their high costs and more importantly, potential ectopic bone formation [23] [24].

Alternatively, scaffolds can be loaded with osteoprogenitor cells in order to generate a living bone graft in vitro, an approach referred to as bone tissue engineering. Various possible sources for osteoprogenitor cells have been considered. Osteoblasts obtained from autologous bone biopsies and then expanded in vitro were an obvious first choice due to their non-immunogenicity. However, the relatively low number of cells obtained after dissociation of the biopsy specimen, the time consuming nature of the

whole process and the problems associated with obtaining osteoblasts from patients with bone related diseases prompted continuation of the search for better options [1] [25]. Mesenchymal stromal or stem cells (MSCs) which can be obtained from various tissue sources, like bone marrow, adipose tissue, umbilical cord or placenta provide an alternative source of osteoprogenitor cells. MSCs were first identified in the bone marrow by Friedenstein and co-workers in 1966 [26] and were subsequently named mesenchymal stem cell (MSCs) by Caplan [27]. They are very attractive to researchers as they can be extensively expanded in vitro to obtain numbers sufficient to treat large bone defects [28] and they have immunosuppressive effects in vivo, which may make them suitable for allogeneic transplantations [29] [30]. MSCs isolated from different sources share many phenotypical and functional characteristics. However, depending on the tissue source and the isolation methods employed, their differentiation potential varies [31]. The varied tissue sources and isolation methods make it difficult to determine if the resulting cells are sufficiently similar to allow for a direct comparison. Therefore, the International Society for Cellular Therapy proposed a set of minimal criteria to label a cell as a MSC [32]. These include: 1. Cells must be plastic adherent when maintained in standard culture conditions; 2. They must express CD105, CD73 and CD90 and lack expression of CD 45, CD34, CD14 or CD11b, CD79α or CD 19 and HLA-DR surface molecules; 3. They should differentiate into osteoblasts, adipocytes and chondroblasts in vitro.

Haynesworth et al. were the first to combine human MSCs from adult bone marrow with ceramic scaffolds to successfully generate bone in vivo upon ectopic implantation into immunodeficient mice [33]. This provided proof of principle on the feasibility of using hMSCs in bone tissue engineering. Since then a lot of interest has been generated in the field of tissue engineering, resulting in in vitro and in vivo studies with different scaffold/cell combinations. The proof of concept for repair of critically sized bone defects using tissue engineered bone graft substitutes has been provided by a number of animal studies [30, 34-44] and several clinical studies have been conducted to assess the safety and efficacy of this approach in man. Nevertheless, bone tissue engineering did not find its way to routine clinical practice.

Here, we present an overview of all published human clinical studies performed so far to generate bone using constructs seeded with culture expanded, autologous, non-genetically modified MSCs obtained from various human cell sources, suggest possible recommendations for future design of clinical studies and describe future research directions. Cells from the periosteum have not been included in this review because no studies have been performed to determine the MSC nature of the periosteal cells used in the clinical studies. There are previous reports which indicate that the periosteal cells fulfill the minimal criteria to be labeled as an MSC [45-48]. However

there are differences in the isolation and expansion protocols used in these studies and in the studies employing the cells for clinical applications [49-52]. Studies using the mononuclear fraction of the bone marrow or adipose tissue have also been excluded from this review. Although MSCs are present in the mononuclear fraction, other populations of cells also form a large part of this fraction.

2.2. Clinical studies in humans using autologous MSCs from various cell sources for bone tissue engineering

Prior to market release of tissue engineered products, an investigational new drug application (NDA) may have to be submitted to accredited regulatory bodies such as the Food and Drug Administration (FDA) or the European Medicines Agency (EMEA). Following this, clinical trials have to be enrolled as phase 1, phase 2 or phase 3 trials. In phase 1, evidence is obtained about the safety of a particular approach in a selected group of patients. Generally, these are small trials with a number of patients recruited being less than 30. In phase 2, more patients are included to evaluate effectiveness on the possible applications. Phase 3 clinical trials involve multicentre trials on 300-3000 patients and are a definitive assessment of the concerned treatment in comparison with the current gold standard. Following completion of all phases of clinical trials, the regulatory body reviews the results, before making a final decision on the release of the tissue engineered product in the market. A search on clinicaltrials.gov using search terms, "Mesenchymal stromal cells", "autologous MSCs" and "bone tissue engineering", provided 2 relevant studies:

1. Treatment of Osteonecrosis of the Femoral Head with Implantation of Autologous Bone Marrow Cells, a Pilot Study.

This is a phase 1 study, which started in January 1999 and was completed in September 2008. However no publications describing the study results are currently available in literature.

2. Autologous Implantation of Mesenchymal Stem Cells for the Treatment of Distal Tibial Fractures. This is an ongoing, phase 1/phase 2 study. The study started in April 2009 and the expected primary completion date is April 2011. No results from this study have yet been published in literature

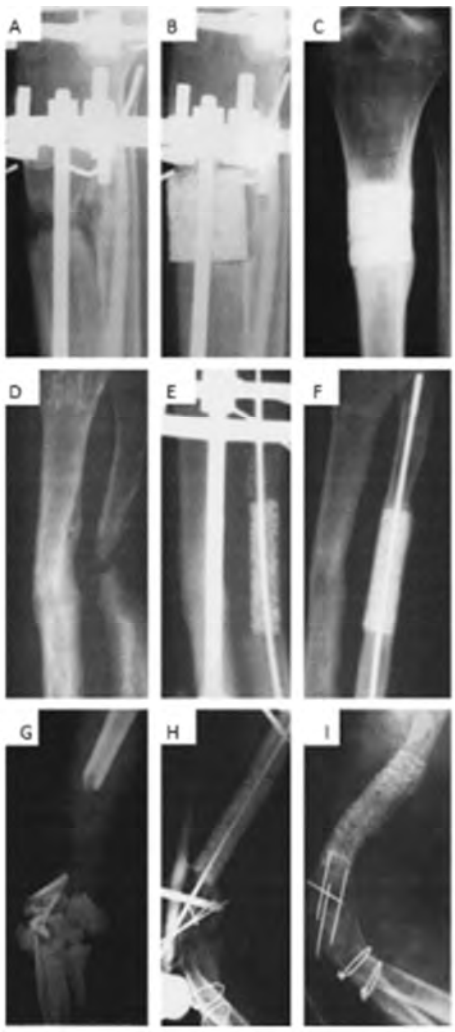

Figure 2.3 Radiographs obtained before and after the repair of large bone defects in three Patients from the study by Quarto et al.

Panels A, B, and C show films obtained from Patient 1 before, immediately after, and 18 months after surgery, respectively. Panels D, E, and F show films from Patient 2 before, immediately after, and eight months after surgery, respectively. Panels G, H, and I show films from Patient 3 before, immediately after and 15 months after surgery, respectively. All the films obtained at the last time point demonstrate bridging of the defect with newly formed bone.

Designs of clinical trials vary from randomized control trials (RCT), replicated single subject experiments, cohort outcome studies, systematic case studies and case reports. In general, the more rigorous the design of a clinical trial, the greater the credibility that can be attached to the conclusions derived from the outcome of a study. Based on the methodological rigor applied, RCTs are generally considered at the top of the hierarchy as randomization in selection of patients for inclusion in the various treatment groups ensures negation of the selection bias while inclusion of controls help rule out the effects of the confounding factors that may have an effect on the treatment outcome. However, due to practical and ethical issues involved in conducting RCTs, most of the trials conducted on human patients and described in

literature for bone tissue engineering, are at the level of cohort outcome studies or case reports. Cohort studies provide information on the percentage of patients which respond positively to a given therapeutic technique while case reports describe the effects of using a particular tissue engineered graft in a single patient. The observed results in the latter can be thus idiosyncratic to the specific patient being evaluated and systematic replications of the experiment would be necessary prior to obtaining conclusive evidence. Absence of controls in cohort studies is a major drawback of such a study design. However, these preliminary attempts also have an important role in the development of scientific research because they generate information that can provide some clues to the safety and potential therapeutic effects of the treatment option and may stimulate researchers to perform the more elaborate, time consuming and costly RCTs in the future. In this review we list all human clinical studies, including case reports that have been published in literature, using autologous, culture expanded, non-genetically modified, human MSCs for purpose of bone tissue engineering. None of these studies have obtained approval from institutions such as FDA or EMEA. The ethical approval for conducting these studies has been provided by their respective local university/hospital ethics committees.

The first clinical case series demonstrating feasibility of using tissue engineered constructs (TEC), as an alternative to autologous bone grafts for treating long bone defects, was reported by Quarto et al [53]. In 2001, they described the preliminary results of 3 patients (27, 16 and 15 months respectively post-surgery) suffering from various segmental defects (Fig. 2.3). The patients were chosen because conventional surgical therapies such as Ilizarov's technique which excludes graft transplant, had failed. The Ilizarov's technique relies on the bone regeneration potential to fill the gap created artificially via osteotomy of the affected segment while maintaining the periosteum intact and then distraction of the two separated halves fixed apart used ring fixators [54]. Autografts were technically difficult to perform because the degree of bone loss would leave the patient with serious donor site morbidity. The first patient was a 41 year old female with a 4 cm large segmental bone defect in the right tibia, the second a 16 year old female suffering from a traumatic loss of a 4 cm segment of the distal diaphysis of the right ulna, while patient 3 was a 22 year old male, who missed a 7 cm segment of the right humerus. For all the patients, macro porous 100% hydroxyapatite (HA) scaffolds were custom made to fit the shape and size of the defect. These were then loaded with ex vivo expanded hMSCs isolated from their own bone marrow. All 3 patients were monitored with radiographs and CT scans, which revealed abundant callus formation by the second month post surgery and good integration of the implants with peri-implant bone formation by the sixth month after surgery. A follow up report 6-7 years after surgery reported that the

implants displayed good osseo-integration with no further complications. Angiographic evaluation performed in patient 3, 6.5 years after surgery also indicated vascularization of the grafted zone suggesting presence of vital bone at the graft site. However, no controls were included in this study and initial follow up was based only on radiological evaluation which the authors admit was not optimal because the high mineral density of the scaffolds used made it difficult to differentiate the new bone from the pre-existing scaffold [55]. Nevertheless, the study showed that the procedure is safe to perform.

In the years after the initial trial, case studies involving single patients treated with tissue engineered constructs were reported in literature. In 2007, Krecic Stres et al. treated 1 patient with a comminuted fracture femur using a combination of TEC and autologous cancellous bone in a ratio of 2:1 [56]. The TEC was generated by seeding bone marrow derived MSCs on porous calcium-triphosphate granules. Clinically, the researchers claim that the patient has been recovering well. However, the combination of autologous bone with the TEC makes it difficult to draw conclusive inferences on the feasibility of using TEC alone for bone tissue engineering as it would be impossible to determine the individual contributions of the TEC and the autologous bone. Moreover, the investigators only relied on clinical evaluation and X-rays to determine new bone formation. No controls or biopsies were planned for the patient. Also, the actual defect size was not mentioned. This is essential as the size of the graft has been found to be crucial in determining the survival of the cells within the core of the graft.

Hibi et al. reconstructed an alveolar cleft defect by injecting culture-expanded and osteogenically-induced bone marrow derived MSC mixed with autologous platelet rich plasma [57]. This study provided a novel approach of using autologous platelet rich plasma as the scaffolding material for the cells. The patient was followed up post operatively with serial CT scans which showed the regenerated bone extending from the cleft walls after 3 months and bridging the cleft after 6 months. It remains unclear whether the defect is filled by bone tissue produced by the implanted cells, or is formed due to osteoconduction from the border of the cleft defect

In 2010, Lee et al. described a successful reconstruction followed by dental implant placement of a 15 cm jaw defect as a result of segmental mandibulectomy due to central hemangioma in a 14 year old boy[58]. Three reconstructive surgeries were performed. In the first surgery, autologous resected mandible obtained during the hemimandibulectomy was used as a tray into which osteogenic- differentiated autologous bone marrow stem cells and fibrin glue was injected. Due to lack of adequate mandibular bone for dental implant placement and recovery of dentition, the

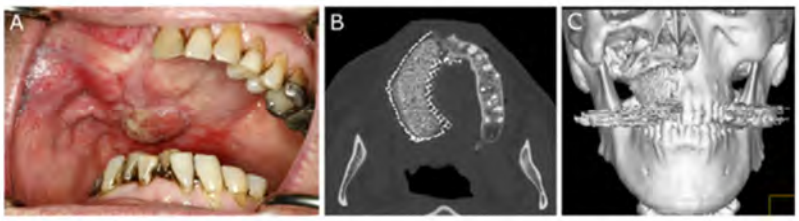

Figure 2.4. Two months postoperative results of the study by Mesimaki et al.
Mesimaki et al reconstructed a major maxillary defect in an adult patient using autologous adipose derived MSCs (ASCs) combined with rhBMP-2 and β-TCP granules in a micro-vascular reconstruction surgery. Two months postoperative results indicate that (A) The rectus abdominis muscle has atrophied nearly totally and epithelialized almost completely. Only a small area in the molar region was non-epithelialized. A well formed buccal sulcus is also noted. Axial (B) and 3D CT scans (C) show the shape and normal bone density of the new maxilla.

second surgery involved vertical distraction osteogenesis with injection of autologous osteoblastic differentiated MSCs. The third and final surgery was 7 months later for implant placement. At the time of implant placement biopsies were taken from the implant site and histological evaluation of the biopsies revealed newly formed viable lamellar bone. Dental CT images taken 4 months after the implant placement confirmed continued presence of mineralized bone at the augmentation zone.

In 2009, Mesimaki et al reconstructed a major maxillary defect in an adult patient using autologous adipose derived MSCs (ASCs) combined with rhBMP-2 and β-TCP granules in a microvascular reconstruction surgery [59]. After isolating the ASCs from abdominal subcutaneous fat in autologous serum using GMP class clean room facilities, the cells were seeded on β-TCP scaffolds. Prior to combining with cells, the scaffolds were incubated for 48 hrs in basal medium supplemented with rhBMP-2. This medium was discarded when the cells were added and fresh medium without rhBMP-2 was added. The cell scaffold combination was kept in culture for 48 hrs prior to their placement in a titanium cage and subsequent implantation in a pouch prepared in the patients left rectus abdominus muscle. The vascular supply of the muscle was not disturbed. 8 months later, the rectus abdominus muscle pouch was opened and the titanium cage filled with the TCP granules and ASCs was macroscopically examined. The new bone formed in the cage was clinically confirmed to be vital and rigid. A biopsy taken from the newly formed bone revealed histology of normal mature bone. Subsequently the vessels were disconnected from the rectus abdominus muscle and the muscle flap together with the tissue engineered bone was placed in the maxillary defect. The abdominal vessels were reanastamosed with the

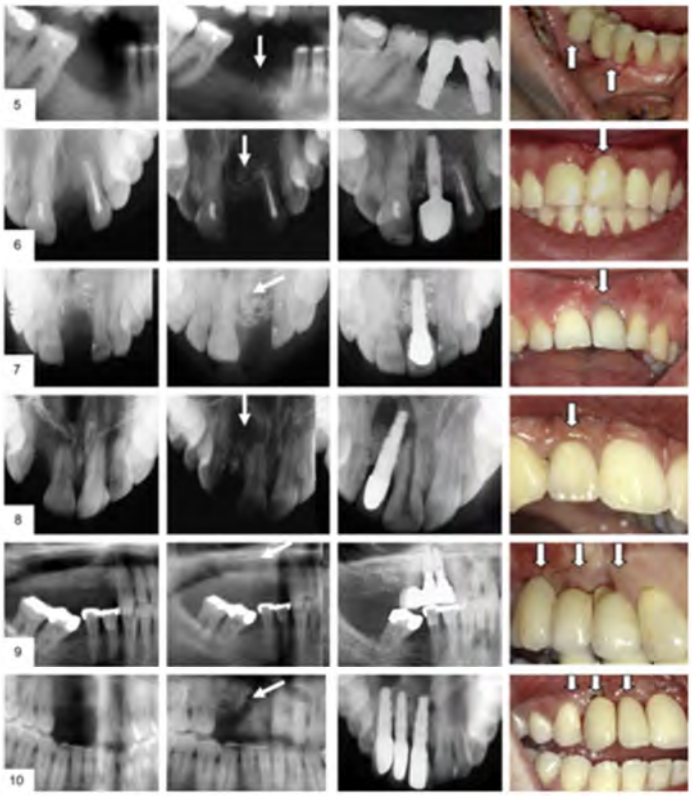

Figure 2.5 Overview for patients 5–10 from the study by Meijer et al to reconstruct a maxillary defect and placement of dental implants.
First column; radiographs showing the alveolar defects. Second column; showing the reconstruction (arrow) by augmentation (5–8) and by sinus elevation procedure (9 and 10). Third column; radiographs showing the dental implants and the prosthetic construction (crown or bridge). Fourth column; clinical situation at the end of the rehabilitations (arrow).

facial vessels. The muscle was left to epithelialize intraorally. The patient was followed up with CT scans. Within two months of the surgery, the muscle flap had almost completely epithelialized and the shape and normal bone density was achieved in the reconstructed maxilla (Fig. 2.4). Four months after placement of the graft, dental implants were placed and their primary stability was reported to be excellent. The implants osseointegrated without any reported adverse effects. This study was the first clinical case where ectopic bone was produced using autologous ASCs in a micro vascular reconstruction study. It demonstrated the feasibility and safety of using ASCs for bone regeneration. However, the relative contribution of the rhBMP-2 and the ASCs in the new bone formed remains to be determined.

In 2007, a study was performed by Soleymani et al. for posterior maxillary sinus augmentation involving 6 patients [60]. In this study, the cell source was the bone marrow from the iliac crest and the carrier material was hydroxyl apatite/β-tricalcium phosphate (HA/TCP) particle. After 3 months, biopsies were taken and results showed a mean bone formation of 41%. Although biopsies were taken, no information of the bone distribution in the scaffold or the source of the newly formed bone (donor or recipient) was provided.

Another clinical study was reported by our group in order to test the potency of bone tissue engineering using bone marrow derived MSCs seeded onto hydroxyapatite particles in 6 patients, requiring reconstruction of bony jaw defects prior to dental implant placement (Fig. 2.5) [61]. Culture expanded bone marrow derived MSCs were seeded on hydroxyapatite particles varying in size from 1-4 mm3. Similar to the work of Schimming et al, the cells were grown on the scaffolds for another 7 days in order to allow further osteogenic differentiation and extracellular matrix deposition and then placed under the muco-periosteal flap in the defect site. In this study, both the in vitro osteogenic capacity and the in vivo bone forming potential of the constructs was assessed using representative samples of cells and constructs respectively. The in vitro potential was tested by performing alkaline phosphatase staining, while the in vivo bone forming capacity of the constructs was confirmed by implanting representative constructs, prepared in an identical fashion to the constructs actually used for the defects, in subcutaneous locations in nude mice. Although no quantification of the bone formed by these hMSCs in the mouse subcutaneous model has been performed, we noted that all the constructs with cells implanted in the nude mice showed bone formation. Four months after application of the construct in the jaw of the human subjects, and before placement of the implant, a biopsy was taken from the operation site. Bone formation was evaluated histologically in the human patients, and in 3 of them no new bone formation was observed. Of the remaining 3, in 2 patients bone tissue in the scaffolds was observed in close contact with the pre-existing bone of the bony defect. This can likely be attributed to migration of osteoblasts from the surrounding bone tissue. In only 1 patient, bone formation was observed more than 7 mm from the pre-existing bone tissue. We consider this to be strongly suggestive for de novo osteogenesis induced by the implanted cells.

An overview of the above mentioned clinical trials are presented in Table 1.

2.3. Experimental design of clinical studies

The clinical studies conducted so far have demonstrated that it is safe to use hMSCs in bone grafting procedures. None of the reports mention adverse effects such as inflammation or excessive tissue growth, despite the fact that there are in vitro studies

Table 2.1. Overview of the clinical studies performed on humans using tissue engineered constructs

Principal Investigator	Year	Cell source	Scaffold	Patients	Area of reconstruction	Salient features	Evaluation	Reported outcome
R.Quarto	2001	Bone marrow	100% hydroxyapatite	3	Long bone defects (1 tibia, 1 ulnar, 1 humerus)	1. First clinical trial in humans using hMSCs 2. Patients with long bone defects selected 3. Patients had good clinical recovery 4. No side effects even after 6-7 years follow –up	Radiology CT scan Angiography	No quantification of new bone formed. Good integration of the host bone with the implanted scaffolds
H. Hibi	2006	Bone marrow	Platelet gel	1	Alveolar cleft defect	1. First study using platelet gel as the scaffolding material	Serial Ct scans	Comparable bone formation to that described in literature with autolgous bone grafts
Y.Soleymani	2007	Bone marrow	HA/TCP	6	Maxillary sinus augmentation	1. Good bone formation in all scaffolds 2. Mean amount of new bone regenerated was 41.3% 3. When compared to the Vacanti study, stark difference in the amount of bone formed, probably due to location of defect or cell source	Radiology Biopsy	Reported successful with mean bone regenerate as 41.34% and good osseointegrati on
H.Krecic-Stres	2007	Bone marrow	Porous calcium triphosphate granules	1	Femoral defect	1. Autologous bone graft was mixed with TECs made with MSCs and scaffolds in ratio of 1:2 to fill the defect	Radiology	Good clinical recovery. No bone quantification performed
Gert Meijer	2008	Bone marrow	Hydroxyapatite scaffolds	6	Intraoral osseous defects	1. Only study which performed a biopsy to not just to quantify the amount of bone formed but also the location of bone on the scaffold. This helped identify if the bone was formed due to osteoconduction alone or as a result of osteo conduction with de novo bone synthesis. 2. Demonstrated the donor-donor variation with hMSCs	Radiology Biopsy	5 patients had no new bone
K. Mesimaki	2009	Adipose tissue	β-TCP	1	Maxillary reconstruction	1. First clinical study to use autologous MSCs derived from adipose tissue and expanded employing good manufacturing protocols (GMP) to heal a bone defect 2. Use of rhBMP-2 to promote bone formation in vivo. 3. Use of a micro vascular flap reconstruction surgery for bone tissue engineering	Radiology Biopsy	8 month follow up indicated presence of mature bone. No quantification of the amount of bone formed is provided. Good clinical course
Jun Lee	2010	Bone marrow	Freeze dried Autologous cancellous bone	1	Mandible reconstruction	1. Repair of a large segmental defect (15cm) 2. Injection of MSCs with fibrin glue into the defect site. 3. Use of autologous cancellous freeze dried bone as a tray to hold the MSCs in place.	Biopsy Radiology	New bone formation after 4 months. No quantification provided. Goo clinical recovery

which suggest that MSCs which have been extensively cultured (4-5 months) can develop genomic instability, which can be an indicator of malignant transformation [62-64]. For most clinical applications, a 6-8 week expansion phase provides sufficient cell numbers. This may account for the fact that no malignant potential of the TECs

has been observed in the clinical cases performed so far. However, to ensure safety for the patient, we propose that in future clinical studies, chromosomal analysis of implanted cells is assessed. Secondly, most of the clinical studies published have a short follow-up period. We recommend using longer follow up periods to obtain data on the long term safety of TEC.

The data presented in the clinical studies make it likely that the grafted hMSCs were able to contribute to bone regeneration, which provides proof of concept for the potential use of tissue engineered grafts in bone regeneration. However, the lack of "gold standard" controls and objective evaluation measures such as bone quantification using histology makes it difficult to draw strong conclusions. The studies where biopsies have been used to evaluate the percentage of bone formed seem to suggest that the contribution of the grafted cells is very limited and certainly not sufficient to bridge critical sized defects. Thus, in order to be able to normalize the efficacy of a given bone TE strategy with respect to that of other trials, we recommend the use of a reference for the bone forming potential of a tissue engineered graft. Given that immune-deficient mice have been used by many researchers in the field, we would like to propose that the tissue engineered grafts to be implanted in patients will be evaluated in mice in parallel and bone formation will be quantitatively assessed.

Future studies should attempt to include comparisons of the TECs with autologous bone grafts for the same application. This type of study design can thus provide conclusive evidence on the efficacy of the new treatment method as compared to the established standard treatment option. When possible, the two types of implants should be implanted in the same patient. A possible situation when this can be performed without raising ethical issues is when a patient with bilateral defects needs quantities of autologous bone graft which may be difficult to obtain without putting the patient at high risk of complications and morbidity. In such cases, the autograft can be used to treat one defect while the other defect is treated simultaneously with the TEC.

Objective evaluation methods should be used to determine the amount of new bone formed. The sample size of the patients should be large enough to allow statistical analysis of the data obtained. We also recommend choice of a surgical site or a tissue engineered scaffold which allows quantification of bone tissue formation without added inconvenience to the patient. For instance, we implanted tissue engineered grafts in the jaw, where we were able to obtain a biopsy in the routine course of the procedure. Other possibilities include tissue engineered grafts where MRI, micro-CT or other non-invasive imaging strategies can be applied to quantify bone formation.

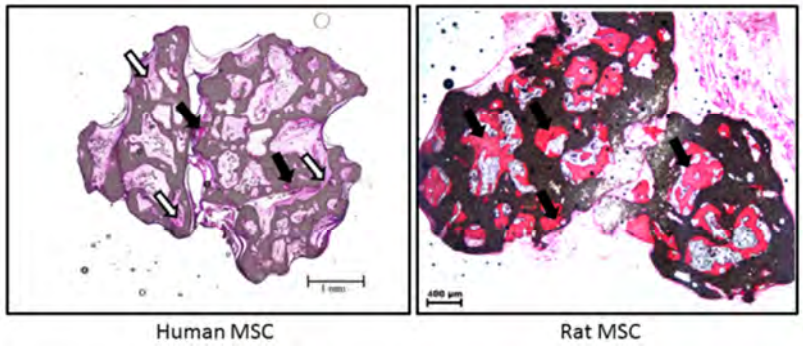

Figure 2.6. Representative section of scaffold seeded with human bone marrow compared to that seeded with rat bone marrow.
Calcium phosphate ceramic scaffolds were seeded with equal number of cells derived from either human or rat bone marrow and implanted subcutaneously in nude mice for 6 weeks. Almost all the pores of the scaffold seeded with rat cells are filled with newly formed bone while the pores of the scaffold seeded with human cells have only one pore with a small amount of bone while the rest of the pores are filled with fibrous tissue. The sections are stained with basic fuschin and methylene blue. The newly formed bone is stained red with basic fuschin (black arrows) while the remaining fibrous tissue is stained pink (white arrows). The black areas represent the scaffold.

In all the clinical studies described, culture expanded MSCs have been combined with a scaffolding material to generate TECs. Expansion of hMSCs can have unfavorable effects on their differentiation potential [64] [65]. For instance, Banfi et al. demonstrated that as early as after the first passage, the bone forming potential was reduced by about 36 times as compared to fresh marrow [66]. Future studies should employ methods to generate TECs which completely bypass the expansion phase of MSCs on plastic. Studies by Warnke et al. [67], Wongchuensoontorn et al. [68] Gan et al. [69] and Aslan et al. [70] have already demonstrated the feasibility of seeding either mononuclear or enriched populations of MSCs obtained on scaffold material for enhancing the osteogenic potential of the cells.

2.4. Concluding remarks

Bone tissue engineering may alleviate problems associated with the current standard treatment used to heal bone defects. However, the success with TECs generated using human MSCs is currently limited. In the majority of the cases, the human MSCs fail to produce clinically relevant amounts of bone while MSCs from other species convincingly generate sufficient bone volume (Fig. 2.6). It is therefore necessary to identify donors with good osteogenic potential and invest research efforts in improving the bone forming capacity of the obtained hMSCs to the level of those obtained from the other species using the widely available ectopic mouse models before embarking on future clinical studies.

Identification of a donor having cells with good osteogenic potential still poses a major hurdle for bone tissue engineering. So far, no literature evidence of a positive correlation between hMSC osteogenesis in vitro and bone formation in vivo has been reported [71]. Our group isolated hMSCs from 62 donors and assessed the in vitro lineage differentiation capacity with gene expression signature and in vivo bone forming capacity. We are currently investigating a gene which we believe could be used as a reliable diagnostic marker for in vivo bone forming capacity (unpublished data).

This is especially attractive as the knowledge that MSCs lack certain surface markers responsible for the host T cell response opens up possibilities for using such allogeneic cells with proven bone forming potential [72] [73]. In addition to being a ready source of guaranteed bone forming cells the patient would also have the benefit of not having to undergo immunosuppressive therapy. Moreover, combining allogeneic cells with scaffolds would then make it possible to have a standardized off the shelf bone tissue engineering product which then can be routinely applied to the clinic.

Other areas of pre-clinical research focus should include identification of more potent sub fractions of hMSCs, in vitro and in vivo studies with MSCs isolated from "waste" tissues such as umbilical cord, human placenta, amniotic fluid and aborted fetuses, alternative seeding strategies to avoid the unphysiological expansion of MSCs on plastic and genetic manipulations of MSCs [74] [75] [76] to enhance the expression of osteogenic genes and priming of MSCs using growth factors such as BMPs [77-79] or compounds such as cAMP [43] or vitamin D [80] to enhance the bone forming capacity while maintaining acceptable costs and safety profile. When the stage is set again for clinical studies, attempts should be made to optimize the experimental design. With the imminent need for bone graft substitutes and the good results obtained with animal-derived MSCs, bone tissue engineering using human MSCs is likely to re-enter the clinic once their biological performance is enhanced.

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2

Chapter 3

Streamlining the Generation of an Osteogenic Graft by 3D Culture of Unprocessed Bone Marrow on Ceramic Scaffolds

Mesenchymal stromal cells are present in very low numbers in the bone marrow, necessitating their selective expansion on tissue culture plastic prior to their use in tissue engineering applications. MSC expansion is laborious, time consuming, unphysiological and not economical, thus calling for automated bioreactor-based strategies. We and others have shown that osteogenic grafts can be cultured in bioreactors by seeding either 2D - expanded cells or by direct seeding of the mononuclear fraction of bone marrow. To further streamline this protocol, we assessed in this manuscript the possibility to seed the cells onto porous calcium phosphate ceramics directly from unprocessed bone marrow. Using predetermined volumes of bone marrow from multiple human donors with different nucleated cell counts, we were able to grow a confluent cell sheath on the scaffold surface in 3 weeks. Cells of both stromal, endothelial and haematopoietic origin were detected, in contrast to grafts grown from 2D expanded cells where only stromal cells could be seen. Upon implantation in nude mice, similar quantities of bone tissue were generated as compared to that obtained by using the conventional number of culture expanded cells from the same donor. We conclude that human osteogenic grafts can be efficiently prepared by direct seeding of cells from unprocessed bone marrow.

3.1. Introduction

Adult mesenchymal stem or stromal cells (MSCs) obtained from bone marrow have great potential in tissue engineering applications as they can be easily isolated and differentiated into osteogenic, chondrogenic and adipogenic lineages. [1] [2] [3]. One of the many tissue engineering applications is the field of bone defect reconstruction. For this application, MSCs are differentiated into osteoblasts in vitro as evidenced by the deposition of a mineralized matrix and expression of osteogenic markers such as alkaline phosphatase (ALP). They are then implanted in vivo to bridge bone defects [4] [5] [6] [7]. However, MSCs represent a rare population of cells with a reported incidence of 0.01 to 0.001% within the bone marrow [8] [1] and large numbers are required to heal bone defects. For instance, spinal fusion surgery requires a minimum of 4 cc of graft material when performed using the anterior interbody fusion method [9]. In our lab, we typically use porous calcium phosphate ceramics, 2-3 mm in size with 200,000 cells per particle to generate an osteogenic graft. To generate 4 cc of graft material, at least 36 million cells would be required. The need for such large cell numbers coupled with the low frequency of MSCs in bone marrow, necessitates their expansion prior to their use in bone tissue engineering and also other applications. Isolation and expansion of MSCs relies on their ability to adhere to plastic [10]. When bone marrow is plated onto tissue culture plastic, a population of cells is obtained which is referred to as MSCs, based on their multipotency and CD expression profile. MSCs are then further expanded to obtain a sufficient number of cells to load on scaffolds for tissue engineering applications [3] [6] [11].

However, it is well recognized that culturing cells in a monolayer on plastic, bereft of the company of the heterogeneous cell populations normally present within the bone marrow, is not physiological. The monolayer culture does not provide an ideal milieu for cell-cell and cell-extracellular matrix interactions, thereby limiting the mechanical and biochemical cues required for the optimal functioning of the cells [12] [13] [14].

The number of clonogenic MSCs present in bone marrow varies greatly from donor to donor. For instance, we tested 27 donors with varying nucleated counts. On plating the aspirates on tissue culture plastic at a density of 500,000 mononuclear cells/cm2 and trypsinizing them at 80% confluency, we obtained on average, 4 million MSCs (data not shown). As previously mentioned, 9 million cells are required for obtaining 1cc of graft material and for clinical application at least 30-40 million cells are required. Thus, in the conventional method of tissue engineering using tissue culture plastic, multiple cycles of medium change, cell trypsinizing, counting and replating are required to obtain a sufficient number of cells. Semi-automation of the process to engineer bone grafts using bioreactor technology is under investigation [14-22]. Here,

MSCs are isolated from the bone marrow and expanded in 2D to obtain the required numbers. Next, seeding and proliferation on scaffolds are performed within the bioreactor. Bioreactors provide a closed standardized culture system which requires minimal operator handling and good physicochemical environmental control, which is crucial for cell survival and proliferation. However, the 2D expansion phase still presents a barrier to complete automation. Based on these considerations, researchers have tried to culture bone marrow- derived MSCs directly on 3D scaffolds, bypassing the need for the 2D expansion phase [23] [24] [25] [26]. The nucleated cells within the bone marrow were separated using a density gradient separation method. Then, mononuclear cells were seeded in the bioreactor on scaffolds to grow the cell ceramic constructs. These studies demonstrated that osteoinductive grafts could be generated within an exclusive 3D system with results comparable to the conventional 2D method of generating grafts [23] [24]. This possibility of expanding MSCs within 3D scaffolds opens new frontiers in the streamlining of the process for therapeutic use.

Whereas the mononuclear cell count is typically used to express the cellularity of a bone marrow aspirate, it does not accurately represent the number of colony forming units (CFU-Fs) contained within the particular marrow [27]. The best predictor of the osteogenic capacity of cell-laden scaffold constructs is the estimation of the final number of clonogenic MSCs implanted [23]. Cell surface markers such as STRO-1 and nerve growth factor (NGF) receptor may be used to prospectively isolate clonogenic MSCs from the crude marrow [28, 29]. However, for cell isolation the marrow is subjected to cell separation strategies, which is again labour intensive. Based on this, we decided on another approach to standardize and streamline the generation of an osteogenic graft.

The aim of the work described in this manuscript is to further streamline the generation of osteogenic grafts by using volume of bone marrow rather than the concentration of nucleated cells in the bone marrow as a guiding parameter. This facilitates the clinical translation without affecting the bone forming potential of the engineered constructs. We demonstrate that within the same total culture time frame, a defined volume of fresh unprocessed bone marrow seeded directly on scaffolds in a static set up, could reproducibly produce grafts with similar osteogenic potential as those obtained by seeding and culturing 2D expanded cells.

3.2. Materials and methods

3.2.1. Bone marrow aspirates

Bone marrow aspirates (10-25 ml total volume) were obtained from 4 healthy donors during hip replacement surgery (donor information in Table 3.1) with written informed consent. Part of the bone marrow was used to isolate and proliferate hMSCs in 2D on tissue culture plastic [30] while the rest of the bone marrow was directly seeded onto the scaffolds.

Table 3.1. Information on donors used in the in vivo study

Donor	Age	Sex	Source	Nucleated cell/ml x10^6
1	65	Female	L. acetabulum	8.3
2	72	Female	L. acetabulum	15
3	66	Female	R. acetabulum	26.6
4	60	Male	L. acetabulum	28

3.2.2. 2D isolation and expansion of cells

hMSCs were isolated and proliferated from the start of the culture period in hMSC osteogenic medium comprising (α-Modified Eagles Medium, Gibco) supplemented with 10% foetal bovine serum (FBS, Lonza), 0.2 mM ascorbic acid (Sigma), 2 mM L-glutamine (Gibco), 100 units/ml Penicillin (Gibco), 100 µg/ml Streptomycin (Gibco) and 10¬¬-8 M dexamethasone (Sigma) to commit the cells towards the osteogenic lineage [31]. To isolate the hMSCs on tissue culture plastic, aspirates were resuspended using a 20 gauge needle, plated on tissue culture flasks at a density of 500,000 cells per square centimetre and cultured in hMSC osteogenic medium. Cells were grown at 37°C in a humid atmosphere with 5% CO2. Medium was changed after 5 days at which moment all the non-attached cells were removed. Thereafter, medium was refreshed twice a week for a total period of 2 weeks. The cells were then trypsinised, counted and seeded at a density of 200,000 cells/porous biphasic calcium phosphate scaffolds (kindly provided by Dr. Huipin Yuan, University of Twente, The Netherlands) produced according to the H2O2 method including naphthalene as described previously [32]. The material was sintered at 1300 °C. The average size of the granules was 2-3 mm with the specific surface area being 0.2 m2/g. The composition of the particles is 20TCP/80HA. The microporosity (volume percentage of micropores smaller than 10 µm within the ceramic) is 8.7% while the calcium release is 4.2 ± 0.4 ppm.

3.2.3. Direct seeding of bone marrow on scaffolds

For direct seeding, 200 µl of unprocessed bone marrow was gently dispersed over the surface of 3, 2-3 mm (BCP) scaffolds. After 4 hours, 2 ml of osteogenic medium was slowly added to each set of 3 scaffolds. Medium was changed after 5 days, and the cells were cultured for 3 weeks on the BCP scaffolds with regular medium change. As a control, expanded cells from the same donor were trypsinised after a 2 week culture period on tissue culture plastic. 200 µl aliquots were then made such that each aliquot contained 600,000 cells. The cells were then dispersed slowly over the surface of the 3 scaffolds. After 4 hours, osteogenic medium was slowly added to the scaffolds. The expanded cells were cultured on these scaffolds for a period of 1 week with one medium change after 3 days.

3.2.4. Cell proliferation, distribution, viability and cell morphology on scaffolds

Cell numbers on the scaffolds were qualitatively assessed at the end of week 1, 2 and 3 of the culture period by methylene blue (MB) staining. Cells were fixed with 1.5% glutaraldehyde in 0.14 M cacodylic buffer, pH 7.3. After fixation, 1% MB solution (Sigma) was added and incubated for 60 seconds. The scaffolds were washed twice with PBS in order to remove non-bound MB. Attached cells were visualized using light microscopy. A quantitative assessment of the number of cells on scaffolds was obtained by determining the DNA content on the scaffolds from both conditions using the Cyquant cell proliferation assay kit (Molecular Probes, Invitrogen detection techniques) according to the manufacturer's instructions. For cell viability 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma) staining was used. A solution of 1% MTT was applied to the scaffolds containing cells. After 4 h of incubation, the MTT solution was removed by flushing the scaffolds with PBS. Scaffolds and cells were visualized using light microscopy. The morphology of the cells was studied using scanning electron microscopy (SEM). Scaffolds seeded directly with bone marrow and with 2D expanded cells were fixed in 4% paraformaldehyde, dehydrated with a series of graded ethanol washes and critical point dried with liquid CO2. Fixed scaffolds were sputtered with gold and examined.

3.2.5. Cell characterization

The cells were characterized by immunostaining with antibodies against CD105 (Endoglin; Dako), CD31 (PECAM-1; Dako) and CD45 (leukocyte common antigen; Dako). The scaffolds were fixed in 10% formalin, embedded in agarose, decalcified using 2% formic acid for 24 hours and then embedded in paraffin. The paraffin samples were sectioned at 5 µm, mounted to the slide, deparaffinised and rehydrated. For staining with CD31, antigen retrieval was performed by boiling the slides in

sodium citrate buffer pH 6.0 for 20 minutes. No antigen retrieval was required for CD45 whereas pre-treatment of the sections with proteolytic enzymes was performed prior to staining with CD105. The sections were blocked using 1% BSA (Sigma) in PBS. Primary antibody incubation was performed for 1 hour in humidified chambers using 1:10 dilution of either monoclonal mouse anti-human CD105, CD31 or CD45. Next, the endogenous peroxidise activity was blocked and the slides were incubated with goat anti mouse HRP conjugated secondary AB (Immunologic). The cells were counterstained with haematoxylin and eosin to stain the cytoplasm and nuclei of cells. The slides were visualised with a light microscope (Leica). Immunostaining was performed on a total of 54 sections obtained from scaffolds seeded directly and with 2D expanded cells from donor 1, 2 and 3. Thus, there were 3 sections per antibody per condition for each of the 3 donors.

3.2.6. In vivo studies

To compare the amount of bone formed by either direct seeding of bone marrow or seeding of 2D - expanded bone marrow cells, in vivo studies were designed in ectopic locations in immune-deficient mice, a model widely used for assessing bone forming capacity of hMSCs [7, 33-36]. The total culture time for the cells in both the direct seeding and the 2D expanded seeding was kept at 3 weeks. In the former, the cells were on the scaffolds from the start while in the latter they were first cultured for 2 weeks on plastic and then transferred to the scaffolds. Prior to in vivo implantation, some scaffolds were used to stain with MB to confirm the presence of cells on the surface of the scaffolds. Six immune-deficient male mice (Hsd-cpb:NMRI-nu, Harlan) were used for the first donor and 6 immune-deficient male mice (Crl:NMRI-Foxn1-nu-,Charles river) were used for each of the last three donors. The mice were anesthetized by inhalation of isoflurane and oxygen. Two subcutaneous pockets were made and each pocket was implanted with 3 scaffolds, of each condition. The incisions were closed using a vicryl 5-0 suture. After 6 weeks the mice were sacrificed using CO2 and samples were explanted. The experiments were approved by the local animal experimental committee.

3.2.7. Bone histomorphometry

The explanted samples were fixed in 4% paraformaldehyde and embedded in methacrylate (L.T.I, Bilthoven, The Netherlands) for sectioning. Approximately 300 μm-thick, undecalcified sections were processed on a histological diamond saw (Leica saw microtome cutting system). At least 6 sections were made from each sample and the sections were stained with basic fuchsin and MB to visualize new bone formation. The newly formed mineralized bone stains red with basic fuchsin while all other cellular tissues stain light blue with MB, and the ceramic material remains black and

unstained by both the dyes. Histological sections were qualitatively analysed using light microscopy (Leica), and each histological section was scored either positive or negative for bone formation. Quantitative histomorphometry was performed as described previously [7]. Briefly, sections were scanned using Minolta Dimage Scan and high-resolution digital photographs (300 dpi) were made from three randomly selected sections from each tissue-engineered graft. For histomorphometrical analysis, bone and material were pseudo-coloured green and red, respectively, using Photoshop CS2 (Adobe Systems). Image analysis was performed using a PC-based system with KS400 software (version 3, Zeiss). A custom-made programme was used to measure bone/ceramic surface ratios.

3.3. Results

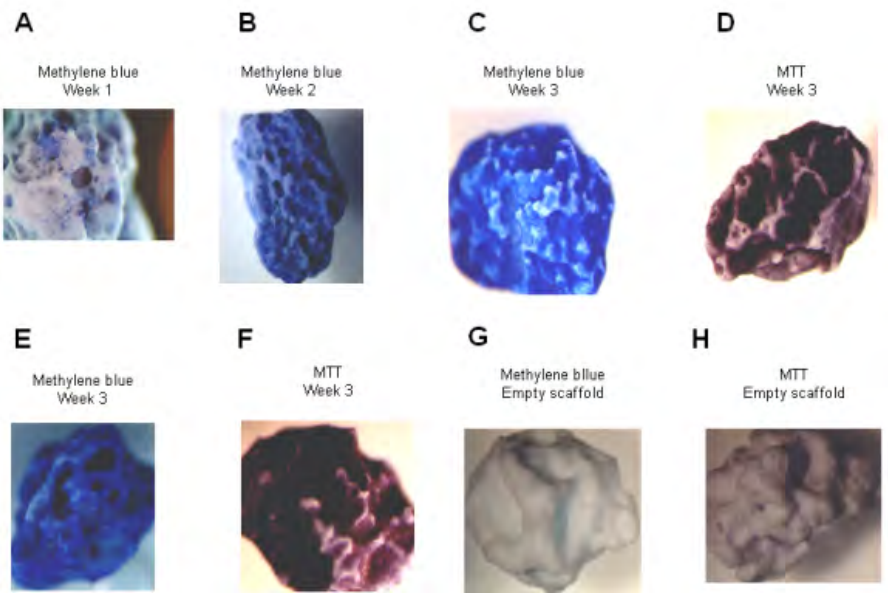

Figure 3.1. Cell proliferation and viability on porous ceramic scaffolds.
Methylene blue staining of scaffolds seeded with unprocessed bone marrow after 1 (A), 2 (B) or 3 (C) weeks of culture. The blue dots represent cells on the scaffold. MTT staining at week 3 of a scaffold seeded with unprocessed bone marrow (D). As a control, cells from the same donor were expanded and seeded on the scaffold and after 1 week stained for methylene blue (E) and MTT (F). To verify that scaffold by itself does not stain with methylene blue or MTT, a representative scaffold without cells was stained for methylene blue (G) and MTT (H).

We were interested in assessing the feasibility of using unprocessed bone marrow for direct seeding on scaffolds for bone tissue engineering applications. To do so, we performed studies to determine the volume of unprocessed fresh bone marrow, which

when cultured on scaffolds for 3 weeks, reliably and reproducibly generated tissue engineered constructs with cell numbers and in vivo bone forming potential similar to that obtained by seeding 2D expanded cells. For all the studies performed, our aim was to have a scaffold covered with a confluent layer of cells.

3.3.1. hMSC expansion on 3D scaffolds

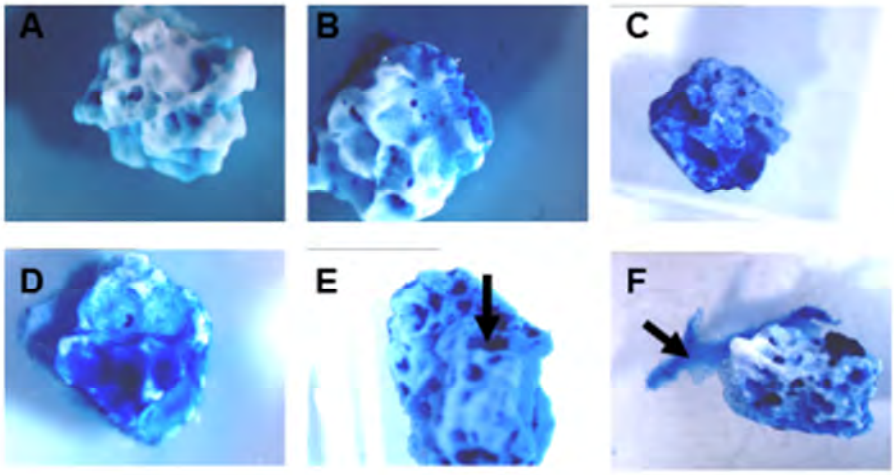

Figure 3.2. Optimisation of volume of bone marrow to scaffold ratio
Methylene blue staining of 1 scaffold from groups of 3 scaffolds seeded with 50 µl (A), 100 µl (B), 200 µl (C), 400 µl (D), 800 µl (E), 1 ml (F) of unprocessed bone marrow, after 3 weeks of culture. The blue dots represent cells on the scaffold while the arrow in Fig E denotes a cell clump. 200 µl of bone marrow per 3 scaffolds gave optimum cell coverage on the scaffold surface while lower volumes did not form a cell sheath. Seeding higher volumes on the scaffolds resulted in the cell sheath detaching from the scaffold surface. Arrow in figure F denotes the detaching cell sheath.

We first aimed at determining the possibility of growing hMSCs directly on scaffolds using unprocessed fresh bone marrow. To do this, we dispersed 1 ml of fresh, unmanipulated bone marrow directly onto 3 BCP scaffolds. After a week, we examined the presence of cells using MB and tested their viability using a MTT assay. Already after one week of culture, we observed foci of cells growing on the ceramics (Fig. 3.1A) and after three weeks of culture, the surface of the scaffold was completely covered by viable cells (Fig. 3.1C, Fig. 3.1D) similar to the condition where expanded cells were allowed to grow on the scaffolds for one week (Fig. 3.1E, F). Our results show that it is possible to grow cells directly on scaffolds using fresh unmanipulated bone marrow. We then tried to determine the amount of bone marrow required to yield a cell sheath on the scaffolds after 3 weeks in culture. We chose a 3 week period

because that is the time required to produce an osteogenic graft with 2 weeks of culture on plastic and one week on the scaffolds. We conducted studies with hMSCs derived from 8 different donors and the volumes of bone marrow used for seeding ranged from 50 µl to 1 ml with varying concentrations of nucleated cell counts (see Table 3.2). Different volumes of bone marrow from different donors are dispersed over the scaffolds and after an initial 4hours incubation period the osteogenic medium was added. The scaffolds were left in culture for a period of 3 weeks. An average of 200 µl of bone marrow per 3 scaffolds produced a confluent cell sheath. Lower volumes did not form a cell sheath in 3 weeks while using higher volumes either did not provide significant benefits or led to detachment of the cell sheath from the scaffold surface (Fig. 3.2).

Table 3.2. Information on donors used in the in vivo study

Donor	Age	Sex	Nucleated cell/ml x10^6
1	65	Female	8.3
2	72	Female	26.6
3	66	Female	15
4	60	Male	28
5	71	Female	25
6	45	Male	10.4
7	69	Male	15.4
8	83	Female	17.8

Based on these results, we used 200 µl of unprocessed bone marrow per 3 scaffolds with a 3 week culture period for all our experiments. To compare the direct seeding method with the conventional approach using 2D expanded cells, 600,000 2D expanded cells were seeded per 3 scaffolds. In our experience, this number results in a confluent layer of cells on the scaffolds after 1 week [11, 37].

3.3.2. Cell quantification and viability

We quantified the number of cells present on the scaffolds seeded directly with bone marrow to those seeded with 2D expanded cells using the Cyquant assay. In two of the three donors tested, the number of cells was similar on both scaffolds (Fig. 3.3). In only one of the donors, two times more cells were found on the scaffolds seeded with 2D -expanded cells as compared to those seeded directly with bone marrow. In all the 4 donors, the viability of the cells present on the scaffolds was tested using the MTT assay. This test confirmed that most of the cells in all 4 donors and in both conditions were metabolically active at the time of in vivo implantation (Fig. 3.1D, 3.1F).

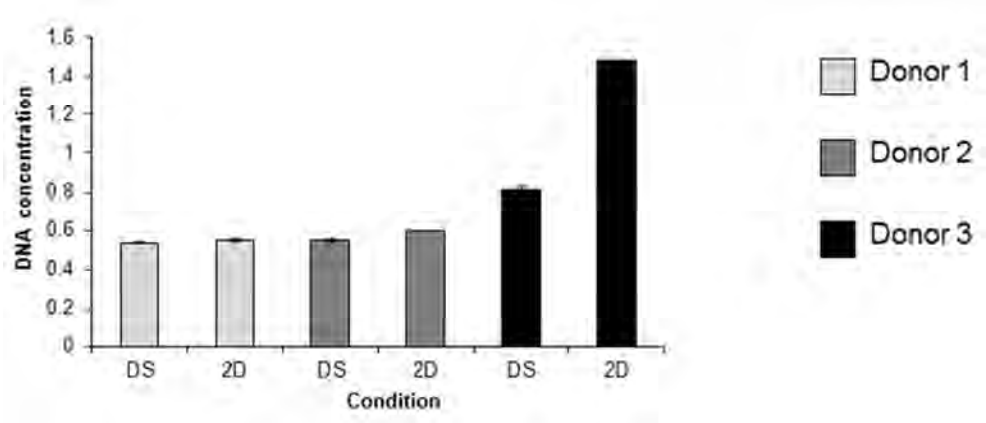

Figure 3.3. Quantification of cell growth

Estimation of DNA content to quantify the number of cells present on scaffolds cultured with either unprocessed bone marrow (DS) or 2D expanded cells (2D). In two of the three donors tested, the number of cells was similar in both groups. In donor 3, 2 times more cells were found on scaffolds seeded with 2D expanded cells as compared to those seeded directly with unprocessed bone marrow.

3.3.3. Cell characterization

Scanning electron microscopy of the ceramics at the end of the 3 week culture period demonstrated that all cells on the scaffolds seeded with 2D expanded cells had a fibroblastic morphology (Fig. 3.4E). In contrast, on the scaffolds directly seeded with bone marrow, we noted the presence of spheroidal cells interspersed by predominantly fibroblastic cells (data not shown), suggesting that during direct seeding of bone marrow, populations of cells other than MSCs grew on the scaffold. To identify the population of cells on the ceramics from both groups, we performed immunostaining using antibodies against cells of mesenchymal (CD105), endothelial (CD31) and hematopoietic (CD45) lineage. For immunostaining, 3 scaffolds were included in the direct seeded and 2D expanded groups each and sections were obtained from each scaffold. 3 sections from every scaffold were stained for each of the 3 antibodies, i.e. CD31, CD45 and CD105. In the sections from scaffolds seeded with 2D expanded cells, we observed no positive staining for CD31 or CD45 (Fig. 3.4C). When stained with the CD 105 antibody, we observed a sheath of positively stained cells, indicating that all the cells on the scaffold were of mesenchymal origin (Fig. 3.4D). In contrast, on scaffolds seeded directly with bone marrow we observed on average, 2-3 clusters of 3-4 cells positive for CD45 in each of the scaffold sections stained for CD45 (Fig. 3.4A). CD31 positive cells, however, were not observed in all sections. In the sections stained for CD31, 0-2 CD31 positive cells were observed per section (Fig. 3.4B). However, the majority of the cells on the direct seeded scaffolds

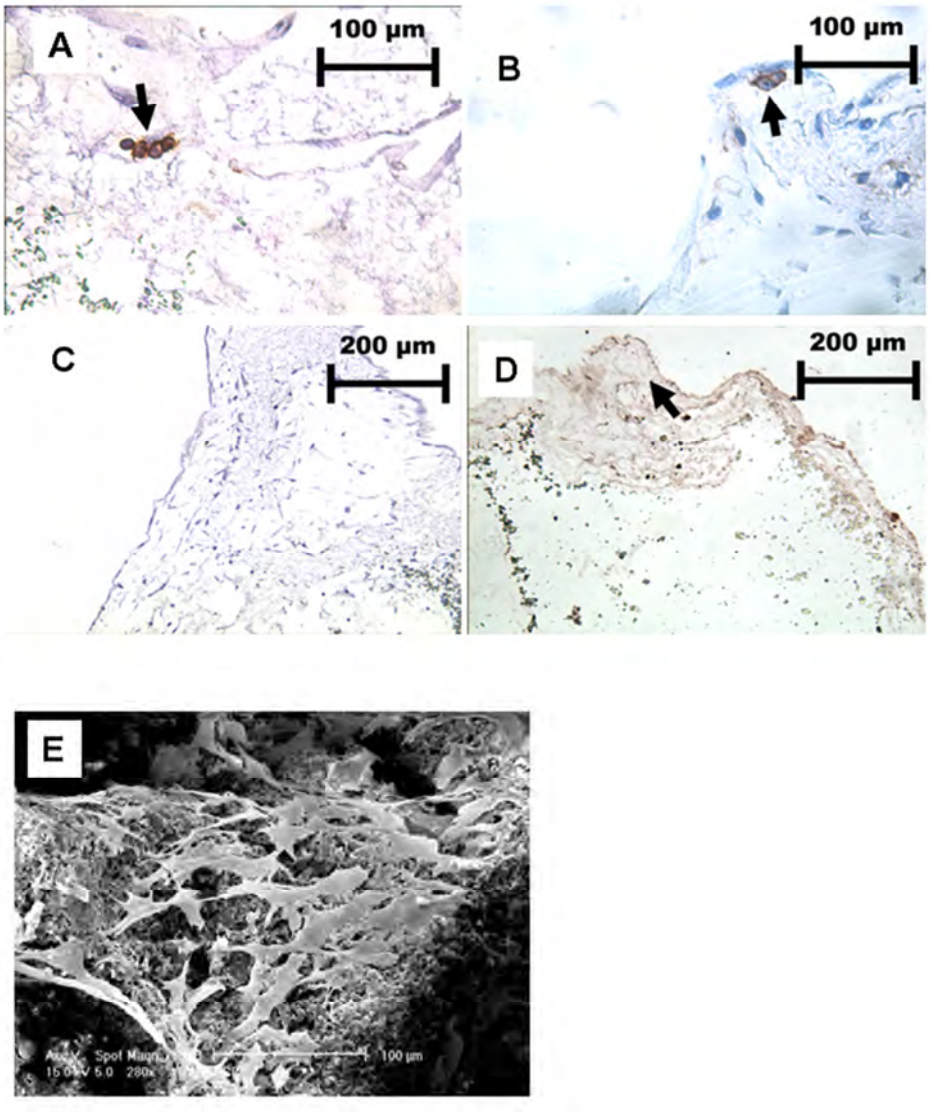

Figure 3.4. Characterization of cells on ceramic scaffolds

Immunostaining with CD45, CD31 and CD105 antibodies on ceramic scaffolds. Brown staining of membrane indicates cells positive for the antibody while blue dots represent the nucleus of the cells. Clusters of CD45 positive cells (A) and CD31 positive cells (B) on scaffolds seeded with unprocessed bone marrow. Both CD 45 and Cd 31antibody staining was negative on scaffolds seeded with 2D expanded cells (C). On staining with CD105, a positively stained cell sheath is noted on scaffolds seeded with unprocessed bone marrow (D). Similar results were observed with scaffolds seeded with 2D expanded cells. On scanning electron microscopy of scaffolds seeded with 2D expanded cells, all cells had a similar morphology indicating presence of cells of the same lineage (E).

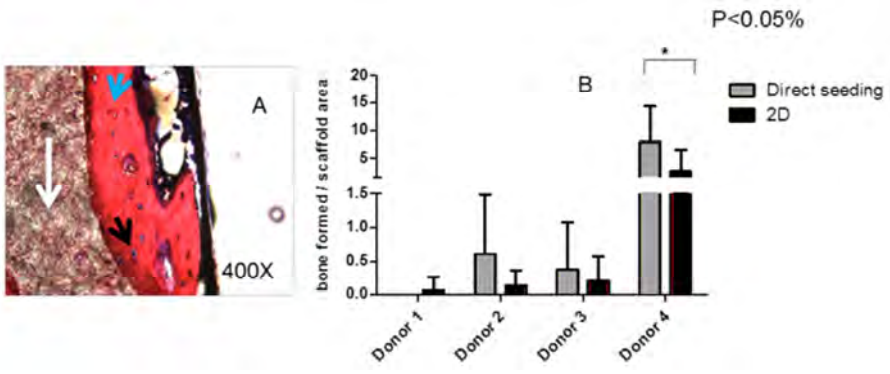

Figure 3.5. Bone formation on porous ceramic scaffolds
(A). Representative histological section of scaffolds directly seeded with bone marrow and implanted in vivo for 6 weeks prior to staining with basic fuschin and methylene blue. This representative image shows newly formed bone (red with blue arrow), osteocytes embedded in matrix (black arrow) and the scaffold (white arrow). (B). Bar graph comparing the amount of bone formed by scaffolds seeded directly or with 2D expanded cells from the bone marrow of 4 donors. The data was analysed using Students paired T test.

stained positive for CD105. In conclusion, as compared to scaffolds seeded with 2D expanded cells, directly seeded scaffolds have a more heterogeneous cell population. However, in spite of the heterogeneity, the majority of the cells on the directly seeded scaffolds are MSCs, confirming our hypothesis that despite the very low numbers of MSCs in unprocessed bone marrow, this population of cells directly adheres and expands on the scaffold surface.

3

3.3.4. In Vivo Bone Formation

To evaluate the bone forming capacity of the constructs, we implanted them for a period of 6 weeks in subcutaneous pockets in immune-deficient mice. Upon explantation, bone formation was analysed histomorphometrically. In the explanted samples from all 4 donors, except in those obtained from direct seeding of bone marrow from donor 1, histological examination revealed the presence of bone tissue, in which we observed osteocytes embedded in a mineralized extracellular matrix (Fig. 3.5A). As seen before, the amount of bone obtained differs between different donors [11]. In donor 2, the bone/ceramic surface ratio increased from 0.14% in the scaffolds seeded with 2D expanded cells to 0.6% in those seeded directly with bone marrow and in donor 3 from 0.20% in scaffolds seeded with 2D expanded cells to 0.37% in those seeded directly with bone marrow (Fig. 3.5B). However, the results were not statistically significant when compared with Student's paired t test ($p>0.05\%$). Donor 4 performed the best in terms of bone formation. In this donor, the bone/ceramic surface ratio significantly increased from 2.6% in the scaffolds seeded with 2D

expanded cells to 7.7% in the scaffolds directly seeded with bone marrow (Fig. 3.5B). This donor showed a significant benefit from the direct seeding approach ($p<0.05\%$ using Student's paired t test). When data from all the 4 donors was combined, the average percentage bone/ceramic surface ratio increased from 0.67 to 2.18. Although not statistically significant, the direct seeding method shows a trend toward increased bone formation in vivo compared to the 2D method. For practical purposes, the results indicate that the direct seeding method using unprocessed bone marrow is at least as efficient in generating bone tissue upon implantation.

3.4. Discussion

In the present study, we provide proof of principle on using pre-determined volumes of unprocessed bone marrow to generate grafts which have similar osteo-inductive potential as those produced within the same culture period, by seeding of MSCs derived from the same donor using the labour intensive 2D expansion.

In 1987, Friedenstein et al. demonstrated the osteogenic potential of bone marrow derived MSCs, which led to the idea that hMSCs can be used to regenerate bone defects. In spite of all the interest this idea generated, a quarter of a century later, hMSCs are still not available to the surgeons as a routine off the shelf treatment option [38]. One of the plausible reasons is that human bone marrow derived MSCs have very high donor to donor variability which cannot be predicted a priori. This translated into problems obtaining reproducible amounts of bone using MSCs, both in ectopic and orthotopic locations, thus limiting their use in clinical trials. The other limiting factor is the lack of standardized methods to generate grafts which, in addition to being cost efficient, are user friendly and do not require laboratory trained manpower and expensive equipment within the hospital set up. In this study we tried to address the second limiting factor. Proof of principle was found previously, when nucleated cell from minimally processed marrow was seeded onto scaffolds within a closed bioreactor with ectopic bone formation in vivo [23, 24]. Our approach of seeding scaffolds directly with unprocessed pre-determined volumes of bone marrow simplifies the process of generating osteogenic grafts even further. A similar approach of directly seeding bone marrow on scaffolds was also used by other researchers in the past to generate bone in a critical size defect with limited success [39] [40]. One of the main differences in their strategy as compared to ours was that in all these studies the scaffolds seeded with bone marrow were implanted within an hour (referred to as per-operative cell seeding). We have already demonstrated the poor performance of per-operative seeding even with 2D expanded cells [40]. Evidently expansion of MSCs prior to implantation is necessary. In this manuscript, MSC expansion occurred on scaffolds in a static environment in well plates whereas bioreactors were used by

previous researchers to culture the scaffolds in a dynamic environment. Although commercially viable, automated systems are still not available for routine hospital use [41], the direct seeding approach can be adapted for use within a bioreactor set up.

Previously researchers used nucleated cell counts as a guide to assess the population of CFU-Fs in the bone marrow. In our in vivo study, instead of using nucleated cell counts, an average volume of bone marrow was used in all the four donors as a practical readout. Although the nucleated counts were obtained, they were not taken into consideration when seeding the scaffold. In retrospect, we observe that donor 1 which had the lowest nucleated cell count (Table 3.1) performed worst in the in vivo setting and donor 4 with the highest nucleated cell count performed the best. In contrast, although donor 3 had a much higher nucleated cell count than donor 2, still directly seeded scaffolds in donor 2 gave more bone than donor 3. In general there was no definite correlation between the amount of bone formed and the initial number of nucleated cells in the bone marrow. This is in agreement with literature, which suggests that nucleated cell counts do not indicate CFU-Fs present in a particular amount of bone marrow [42]. One may argue that a pre- determined amount of bone marrow is also not a fool proof method of ensuring a cell scaffold construct with osteogenic potential thereby warranting additional quality control criteria in future studies. Markers such as STRO-1 and NGF currently available may prospectively determine the number of CFU-Fs in the bone marrow. Nevertheless, in our study using predetermined volumes of bone marrow as in the 8 donors with varying nucleated cell counts that we studied, we were able to obtain a complete coverage of the scaffolds in 3 weeks using 200 µl of bone marrow per 3 scaffolds, in all the donors.

It can be argued that using the 2D expansion method for greater than 2 weeks as used in this study, a larger number of cells could be obtained and this in turn could support generation of larger osteoinductive constructs. However it has been reported in literature that 2D expanded bone marrow derived hMSCs have a much lower differentiation capacity as compared to the MSCs found in fresh bone marrow. Repeated passaging of cells can eventually lead to their senescence [43-45]. Moreover, 20-40 ml of bone marrow can be obtained safely from patients [30]. This can generate as much as 8 to 13 cc of graft material which would be sufficient for most routine clinical applications. Further, here we chose a period of 3 weeks as the culture period as we wanted to compare our findings with scaffolds seeded with 2D expanded cells. However, depending on the application, the culture period and the amount of bone marrow used per scaffold can be modified.

Based on the results of this study and using this approach of direct seeding, we are now testing a prototype of a compact, closed, sterile system which is pre-packed with scaffolds and which could be used in the clinics without the need for trained personnel or special sterile work areas. This device in combination with the direct seeding approach can thus be utilised by a surgeon directly within the surgical theatre and then left for the culture duration in an incubator routinely present in most hospitals.

In conclusion, the direct seeding approach offers potential use in clinical situations. However, the osteogenicity of the grafts either using the conventional approach or the approach proposed in this work is not comparable as yet to the autologous bone grafts or that observed with MSCs derived from rat or goat. Further studies using supplementation of the medium with osteogenic factors should be considered to help address this issue.

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Chapter 4

Cell Aggregation Enhances Bone Formation by Human Mesenchymal Stromal Cells

The amount of bone generated using current tissue engineering approaches is not sufficient for most clinical applications. Previous in vitro studies suggest that culturing cells as 3D aggregates can enhance their osteogenic as well as chondrogenic potential. Here, we use agarose wells to generate uniform sized mesenchymal stromal cell aggregates. When combined with micrometre range calcium phosphate ceramic particles and a fibrin thrombin gel prepared from human platelet lysate, we generated a tissue engineered construct with significantly improved in vivo bone forming capacity as compared to the conventional system of using single cells seeded directly on the ceramic surface. In vivo testing with multiple donors confirmed the reliability of this system. In contrast, the in vivo performance of the constructs was significantly affected when unaggregated cells were used, indicating that cell aggregation is a potent trigger of in vivo bone formation by hMSCs.

4.1. Introduction

Large bone defects resulting from fractures, tumour resections, infections, metabolic disorders and abnormal skeletal development often necessitate surgical intervention. Autologous bone grafts are the preferred treatment choice because the patient's own bone lacks immunogenicity and is a good source of bone forming progenitor cells which are delivered directly to the defect site. However, due to problems associated with pain and morbidity at the donor site, alternative therapies are considered [1-3]. Bone void fillers made from natural or synthetic biomaterials with osteoconductive and/or osteoinductive properties are an option. We recently demonstrated that by controlling the physical and chemical properties of the biomaterial, we significantly improved its osteoinductive properties. This ultimately resulted in a biomaterial which, in an animal model could heal a large bone defect as efficiently as autologous bone grafts [4]. However, there are no studies in humans where the bare ceramics have been reported to heal a critical sized defect. [5-7]. Growth factors or stem cells are alternatives to further improve the osteoinductivity of bone graft substitutes. Currently, growth factors such as BMP2 and OP-1 are used to repair bone defects in the clinic [8, 9]. However, the supra-physiological doses required pose safety and economic issues. Therefore, researchers continue to search for alternative options [10-14]. In cell based bone tissue engineering, the osteoinductive properties of grafts are enhanced by combining scaffold materials with osteoprogenitor cells, prior to implanting them in vivo. Ideally these cells should be easily obtained from adult tissues without significant donor morbidity, should be able to differentiate efficiently into the osteogenic lineage, have no ethical implications and be safe for use in human patients[15]. Mesenchymal stromal cells (MSCs) can be easily isolated from a variety of adult tissues, cultured in a laboratory environment and differentiated into multiple lineages [16]. Since their discovery by Friedenstein et al. in 1966 from murine bone marrow[17], numerous animal studies and clinical trials have been performed to optimize the use of MSCs for bone tissue engineering. Using this approach, large bone defects have been safely and successfully repaired in animal models by using animal-derived MSCs [18-23]. However, models for ectopic bone formation as well as recent clinical trials demonstrate that bone formation by the majority of human MSC donors is still inadequate [24-26].

The low number of MSCs in native tissue often necessitates in vitro expansion prior to their use in vivo, which can lead to loss of multipotency. Osteogenic pre differentiation of the cells during the in vitro expansion phase is therefore often used by researchers as a strategy to boost the performance of the MSCs [27-29]. It is known from literature and our own research that the culture conditions during the in vitro phase such as the cell plating density, passaging densities, hypoxia, a 3-

dimensional culture environment or supplementation of the culture medium with various compounds can affect osteogenic differentiation of MSCs in vitro and their in vivo bone forming capacity [30-32].

Recently there have been a number of publications showing that compaction of hMSCs into 3D spheroids during the in vitro culture period is a technique to improve their in vitro osteogenic as well as chondrogenic potential [33-38]. During the natural course of fracture healing the first step in the healing process involves hematoma formation followed by infiltration of mesenchymal stromal cells into the fracture site. MSCs can differentiate into osteoblasts and chondrocytes and lay down an extracellular matrix which ultimately results in the formation of a soft callus which bridges the defect site. During the differentiation process, the mesenchymal stromal cells first undergo condensation [39], which is a pivotal stage in skeletal development[40]. We previously used condensation of embryonic cells to generate a cartilage template in vitro for endochondral bone tissue engineering [41]. Similarly, Scotti et al condensed hMSCs into small aggregates and cultured them in chondrogenic medium to form cartilage templates in vitro [42]. Recently, we observed very efficient bone formation by human MSCs donors when aggregates of hMSCs were implanted with ceramic micro particles in a collagen gel (see Figure 1 for an outline) [43]. This prompted us to further investigate in vitro cell aggregation as a strategy to enhance the in vivo bone formation.

The present study thus aims to test the in vivo bone forming capacity of the cell aggregate/micro particle/gel-based culture system (referred to as the cell aggregation system in this article) using MSCs from multiple human donors, and to make a direct comparison with the most commonly employed conventionally used technique of generating bone grafts i.e. by seeding a single cell suspension of MSCs directly onto the surface of ceramic particles [19, 26, 44, 45].

4.2. Materials and methods

4.2.1. Cell culture

Bone marrow aspirates (5-20ml) were obtained from healthy donors during hip replacement surgery with written informed consent. Alternatively, cryopreserved vials of hMSCs were purchased (Lonza group ltd). When isolated from fresh marrow aspirates, the aspirates were resuspended using 20G needles and plated at a density of 5X105 mononuclear cells/cm2 and cultured in proliferation medium (basic medium supplemented with 1 ng/mL basic fibroblast growth factor, bFGF, Instruchemie, The Netherlands). Basic medium was composed of α-minimal essential medium (α-MEM, Life Technologies), 10% foetal bovine serum (FBS, Cambrex), 0.2 mM ascorbic acid

(Asap, Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/mL penicillin (Life Technologies) and 10 mg/mL streptomycin (Life Technologies). Cells were grown at 37°C in a humid atmosphere with 5% CO2. Medium was refreshed twice a week and on reaching near confluence, cells were trypsinised and cryopreserved until further use. At the start of the experiments, cryovials of hMSCs were expanded further in proliferation medium until sufficient numbers for experiments were obtained.

4.2.2. Generation of cell aggregates

1400 wells, each measuring 400 μm in diameter and having a depth of 200 μm were patterned on every poly (dimethylsiloxane) (PDMS) stamp using etched silicon wafers [46] (Fig. 4.1A). After sterilising the PDMS stamps for 10 minutes in 70% ethanol, they were placed in the wells of a standard 6-well plate and completely covered with 3% agarose solution (Ultra-pure agarose, Invitrogen). Upon solidification, the agarose templates were demoulded and placed in a non-tissue culture treated 12 well plate. The agarose templates are henceforth referred to as chips. After wetting the chips with medium, a 1ml concentrated suspension of 1.5 million cells, unless otherwise stated, was uniformly dispersed over the wells on each chip. The chips were then centrifuged briefly at 1500 rpm to facilitate settling down of the cells (Fig. 4.1B). The cells were cultured on the chips for 24 hours using Exp. Medium. The Exp. medium comprised of Dulbecco's Modified Eagle Medium supplemented with 10-7M dexamethasone, 50 mg/ml ascorbic acid (Asap, Life Technologies), 40 mg/ml proline (Sigma-Aldrich), 100mg/ml sodium pyruvate 50 mg/ml and ITS 1 Premix (Becton-Dickinson). Cells spontaneously compacted to form uniform cell aggregates within 24 hours (Fig. 4.1C).

4.2.3. Platelet gel

After written consent, a standard thrombocyte apheresis procedure was performed on healthy donors using a Cobe Spectra/Trima apheresis unit at the Institut für Klinische Transfusionsmedizin, Braunschweig, Germany. The resulting single donor apheresis platelets were frozen at -80°C. At the time of the experiment, the bag was heated to 37°C which resulted in lysis of the platelets with subsequent release of the growth factors and formation of the platelet lysate. 235μl 1 M calcium chloride solution was added per 10 ml of platelet lysate and gently mixed on a shaker at 37°C for approximately 10 minutes. This resulted in the separation of the platelet lysate into a gel like component and a clear liquid. The clear liquid was the source of the thrombin component which, when added at a 1: 1 ratio at 37°C to the rest of the platelet lysate (fibrin source), activated the clotting pathway, thereby resulting in the formation of a platelet gel in 10-12 seconds.

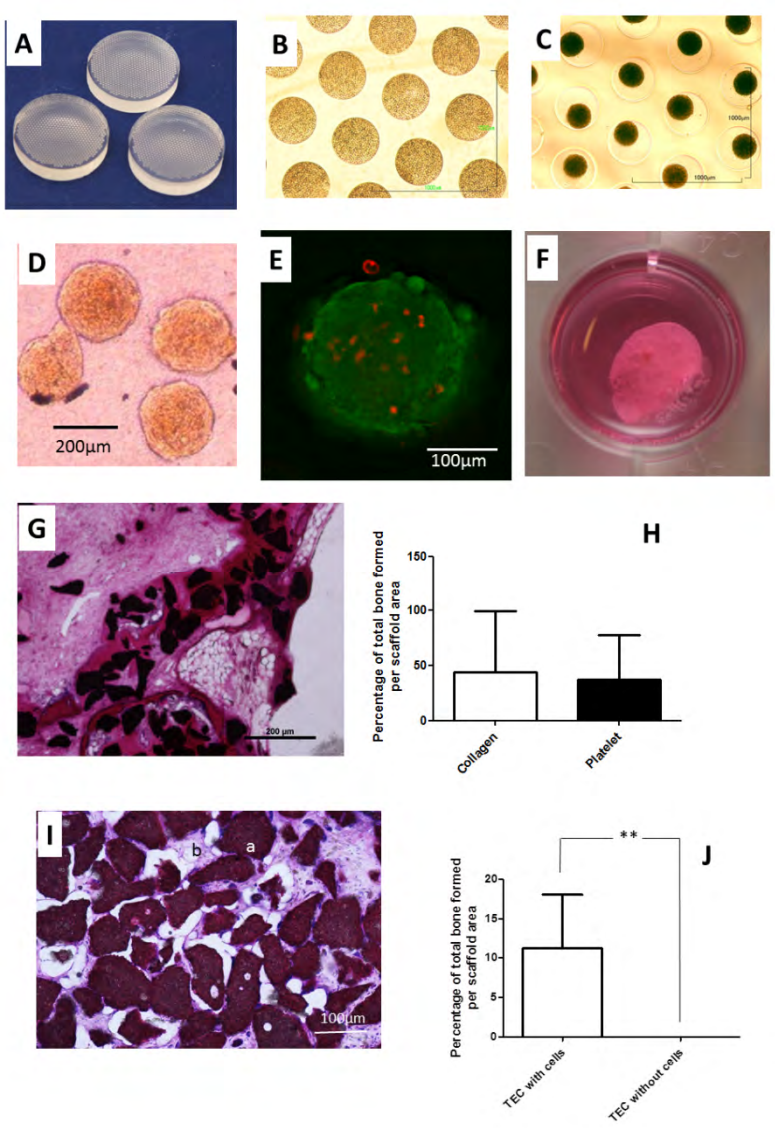

Figure 4.1. Formation of TECs using the cell aggregation system

(A) PDMS templates to generate agarose chips (B) On day 0, the cells occupy the entire well of the agarose chip. (C) after 24 hours, the cells condense into aggregates (D) Aggregates maintained size and shape after flushing (E) Live dead staining of the aggregates flushed from the chips. Live cells stain green while dead cells stain red.(F) final construct after combining the cell aggregates with micro ceramic particles and platelet gel (G) New bone generated using constructs made with collagen gel (H) No significant difference in the amount of bone formed in the aggregates using platelet gel or collagen gel (I) The constructs without cells do not generate bone in vivo (J) Quantification of the in vivo performance of constructs with and without cells implanted in the same animal. a. micro ceramic particles b. fibrous tissue.

4.2.4. Generation of tissue engineered constructs

Biphasic calcium phosphate ceramic particles, average size 53-63 μm and sintered at 1150 C, were produced according to the H2O2 method including naphthalene as described before [47]. Half a gram of ceramic micro particles was suspended in 4 ml of PBS and 200 μl of this suspension was then aliquoted into twenty 10 ml tubes, resulting in approximately 25 μg of particles per tube. Next, the cell aggregates were gently flushed from the agarose chips with Exp. Medium and transferred to a 10 ml tube. The chips were examined under to microscope to ensure complete flushing of the cell aggregates. After flushing, the aggregates maintained their shape and relative size (Fig. 4.1 D). Live dead staining of the cell aggregates was performed using the live dead assay (Invitrogen, Carlsbad, CA), according to the protocol suggested by the manufacturers. The results indicated that majority of the cells within the aggregates remained viable after flushing (Fig. 4.1E). The 10 ml tubes containing the cell aggregates and ceramics were centrifuged briefly to allow the aggregates to settle onto the ceramics. Then, 300 μl of the prepared thrombin solution was added to the cell aggregate scaffold mixture and finally 300 μl platelet lysate was added to the tube. The contents of the tube were aspirated into a pipette tip and then let out onto a non-tissue culture treated 25-well plate. The resulting constructs were maintained for 2 weeks in EXP. medium at 37°C. The cells and the scaffolds were rapidly encapsulated within the fibrin thrombin components of the platelet gel, resulting in an implantable TEC (Fig. 4.1F). Presence of viable cells was confirmed prior to implanting by incubating the constructs for 4 hours with a 1% solution of MTT (Sigma) (data not shown).

For generation of constructs with the collagen gel, the aggregates and ceramic scaffold mixture was incorporated in 600 μl of 2mg/ml rat tail collagen gel (Sigma) as opposed to fibrin thrombin gel. The rest of the procedure was identical to that of the TECs generated using the platelet gel.

For unaggregated cell constructs, a single cell suspension of 1.5 million cells was directly added to each 10 ml tube, pre-loaded with BCP particles, without the intermediate step of cell aggregation on the chips. The resulting TECs were cultured similar to the TECs generated using aggregated MSCs.

For the conventional system, a single cell suspension of 600,000 hMSCs were seeded over 3 BCP scaffolds, sintered at 1150°C, with chemical characteristics identical to the micro ceramics used for the cell aggregation system. However, the size of the particles used in the conventional system was 1-2 mm. The cell ceramic construct was cultured for 2 weeks in osteogenic medium (basic medium supplemented with 10^{-7}M dexamethasone, Sigma). Platelet gel was not used in this system. Prior to implanting

into the animals, the presence of viable cells on these ceramics was verified by incubating the TECs for 4 hours with a 1% MTT solution (data not shown).

Schematic representation of generation of the different types of constructs mentioned above is provided in Fig. 4.2.

4.2.5. Cell quantification

TECs generated via the conventional or the cell aggregation system were transferred to a tube containing CyQUANT cell lysis buffer and then left at -80°C for 24 hrs. After 24 hours, the contents of the tubes were thawed to room temperature. Then using an ultrasound sonicator, the TECs were broken into small fragments. The freeze thaw cycle followed by the sonication of the TECs, lysed the cells releasing their DNA into the supernatant. Quantification of total DNA was performed on the supernatant with the CyQuant DNA kit, according to the manufacturer's protocol (Molecular Probes, Eugene, Oregon, USA), using a fluorescent plate reader (emission: 520 nm; excitation: 480 nm, Perkin-Elmer, Victor 3, USA). The standard curve for DNA analysis was generated with λ DNA included in the kit.

4.2.6. Gene expression analysis

Aggregated and unaggregated constructs were cultured for 14 days in Exp. Medium, washed with PBS and lysed using Trizol reagent (Invitrogen, Carlsbad, CA). After five minutes, the samples were stored at -80 °C prior to RNA isolation. After addition of chloroform and phase separation by centrifugation, the aqueous phase containing the RNA was collected, mixed with an equal volume of 75% ethanol and loaded onto the RNA binding column of the Nucleospin RNA II kit (Bioke). Subsequent steps were in accordance with the manufacturer's protocol. The RNA yields were determined by spectrophotometry using the Nanodrop2000 (ND-1000 Spectrophotometer, Isogen LifeScience). Subsequently, cDNA was synthesized using iScript (BioRad) according to the manufacturer's protocol. One µl of undiluted cDNA was used for subsequent analysis. For quantitative PCR, a master mix containing distilled water, forward primer, reverse primer (Sigma Genosys), BSA and SYBR green I mix (Invitrogen) was prepared. Real-time qPCR was performed, for the osteogenic genes, on a Light-Cycler Real Time PCR machine (Roche) and for the chondrogenic genes on a MyIQ single colour Real-time PCR detection system (BioRad). Gene expression was normalized to the expression of the beta-2 microglobulin gene (B2-M). Light-Cycler data was analysed using the fit points method of Light-Cycler software. The baseline was set at the lower log-linear part above baseline noise and the crossing temperature (Ct value) was determined. MyIQ data was analysed using iQtm5 optical system software

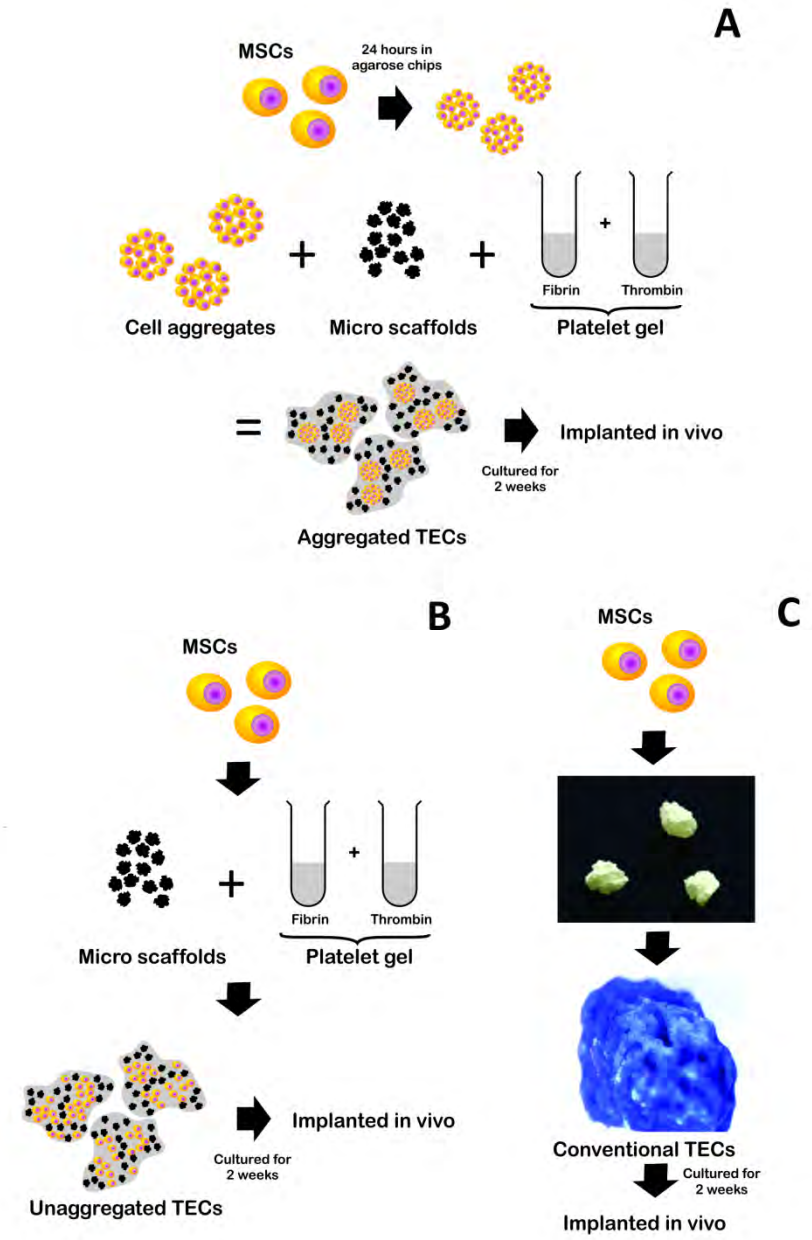

Figure 4.2. Schematic representation of generation of constructs

(A) Aggregated TECS (B) Unaggregated TECS (C) the Conventional system of generating constructs

(BioRad). Ct values were normalized to the B2M housekeeping gene and comparative ΔCt method (Ct control - Ct sample) was used to calculate the fold inductions. Primer sequences are listed in Table 1.

Table 1. Primer sequences

Gene	*Primer sequence*	*Tl (bp)*	*Ta (°C)*
B2M	5'-GACTTGTCTTTCAGCAAGGA-3' 5'-ACAAAGTCACATGGTTCACA-3'	106	60
Aggrecan	5'-AGAATCCACCACCACCAG-3' 5'-ATGCTGGTGCTGATGACA-3'	136	60
Collagen 2	5'-CGTCCAGATGACCTTCCTACG-3' 5'-TGAGCAGGGCCTTCTTGAG-3'	122	60
Collagen 10	5'-GCAACTAAGGGCCTCAATGG-3' 5'-CTCAGGCATGACTGCTTGAC-3'	129	56
Sox 9	5'-TGGGCAAGCTCTGGAGACTTC-3' 5'-ATCCGGGTGGTCCTTCTTGTG-3'	98	60
Alkaline Phosphatase	5'-GACCCTTGACCCCCACAAT-3' 5'-GCTCGTACTGCATGTCCCCT-3'	70	60
Osteopontin	5'-CCAAGTAAGTCCAACGAAAG-3 5'-GGTGATGTCCTCGTCTGTA-3'	348	58
BMP-2	Commercially bought (SABiosciences)	140	60

4.2.7. In vivo studies

Multiple in vivo studies were designed in ectopic locations in immune deficient mice, a model widely used for assessing bone forming capacity of hMSCs [26, 48-50]. Ten immune deficient male mice (Hsd-cpb: NMRI-nu, Harlan) were used for each of the

experiments except in the time course study, when 6 animals were killed at each of the three time points (2 weeks, 4 weeks and 8 weeks). The mice were anesthetized by inhalation of isoflurane and carbon dioxide. Four subcutaneous pockets were made on the dorsal aspect of each mouse. The incisions were closed using a vicryl 5-0 suture. The experiments were approved by the local animal experimental committee. The animals were sacrificed using carbon monoxide and samples were explanted.

4.2.8. Bone quantification

The explanted samples were fixed in 4% paraformaldehyde (Merck) and embedded in methacrylate for sectioning. Approximately 300 μm-thick, undecalcified sections were processed on a histological diamond saw (Leica saw microtome cutting system). At least 4 sections were made from each sample and scanned using a PathScan Enabler IV Histology Slide Scanner. The sections were stained with basic fuchsin and methylene blue to visualize new bone formation. The newly formed mineralized bone stains red with basic fuchsin, the unmineralized osteoid stains light pink while all other cellular tissues stain light blue with methylene blue, and the ceramic material remains black and unstained by both the dyes. Cartilage formation was visualized by 0.04% thionin (Sigma) in 0.1 M sodium acetate (Merck), which stains cells blue and glycosaminoglycan pink. Histological sections were qualitatively analysed by light microscopy (Leica), and each histological section was scored either positive or negative for bone formation. In addition, quantitative histomorphometry was performed. Briefly, high-resolution digital photographs were made of three randomly selected sections from each tissue-engineered graft. Before histomorphometrical analysis, bone and ceramic material were manually pseudocoloured green and red respectively using Photoshop CS2 (Adobe Systems). A custom-made Matlab script was used to measure the bone/ceramic surface ratios.

Statistical analysis was performed using One-way Anova followed by Tukey's multiple comparison test ($P < 0.05$) when more than two groups were compared. In cases where comparisons were made between 2 groups, a Student's paired t-test was performed. Again a p value less than 0.05 was considered as significant.

4.3. Results

4.3.1. Effect of replacement of collagen gel with platelet gel

To confirm our previous results using the cell aggregation system, cell aggregates of commercially obtained MSCs, micro ceramic particles and collagen gel were mixed together to generate 10 TECs. The resulting constructs were cultured for 2 weeks in the Exp. medium and implanted subcutaneously in 10 immune-deficient mice.

Histological analysis after six weeks demonstrated widespread bone formation (Fig1G). To test the possibility of replacing the collagen gel with platelet gel, we next combined cell aggregates from the same commercial donor and micro ceramics with either platelet or collagen gel. On in vivo implantation in 10 immune deficient mice, no significant difference in the total amount of bone formed was observed within constructs generated with either platelet or collagen gel (Fig. 4.1H). These data indicate that platelet gel is a suitable replacement for collagen gel.

4.3.2. Requirement of cells in the system

The platelet gel is considered to be a storehouse of multiple growth factors [51, 52]. Since osteoinductive growth factors such as BMP-2 or even osteo-inductive ceramics can induce bone formation in ectopic sites [14], we hypothesized that the platelet gel may in combination with the ceramic particles, be intrinsically osteo-inductive. To test this, we omitted the cells from the system and cultured the micro ceramics and the platelet gel under the same conditions as our positive control, the complete constructs with cell aggregates. After 2 weeks in vitro culture, both types of constructs were implanted subcutaneously in 10 nude mice. While abundant bone was observed in the cell-laden construct, no evidence of bone was observed in the constructs lacking the cells (Fig. 4.1 I, J). This shows that the growth factors in the platelet gel were not able to initiate ectopic osteoinduction in nude mice.

4.3.3. In vivo reproducibility of bone formation in multiple donors using the cell aggregation system

To test the reproducibility of the cell aggregation system, passage 2 hMSCs from three different donors (donors 236, 240 and 267) from our bone marrow bank were used to generate a total of 30 constructs (10 from each donor) via the cell aggregation system. As a control, a set of 10 constructs was prepared using cells obtained from the commercial donor. On histological evaluation of the explants after 6 weeks, we observed that all the donors reproducibly generated bone. 22.04, 28.7%, 8.6% and 20.52% of the total scaffold area was filled with bone in the case of donor 240, 236, 267 and commercially purchased donor respectively (Fig. 4.3A). Interestingly, with the constructs generated using the commercial donor, we occasionally observed areas of mature bone aligning areas of tissue resembling hypertrophic cartilage (Fig. 4.3B). With the three other donors, while there were areas of mature bone aligning areas of osteoid, no evidence of hypertrophic cartilage was observed. In all donors, areas suggestive of bone marrow were observed in some sections, indicating a functional bone organ (Fig. 4.3C).

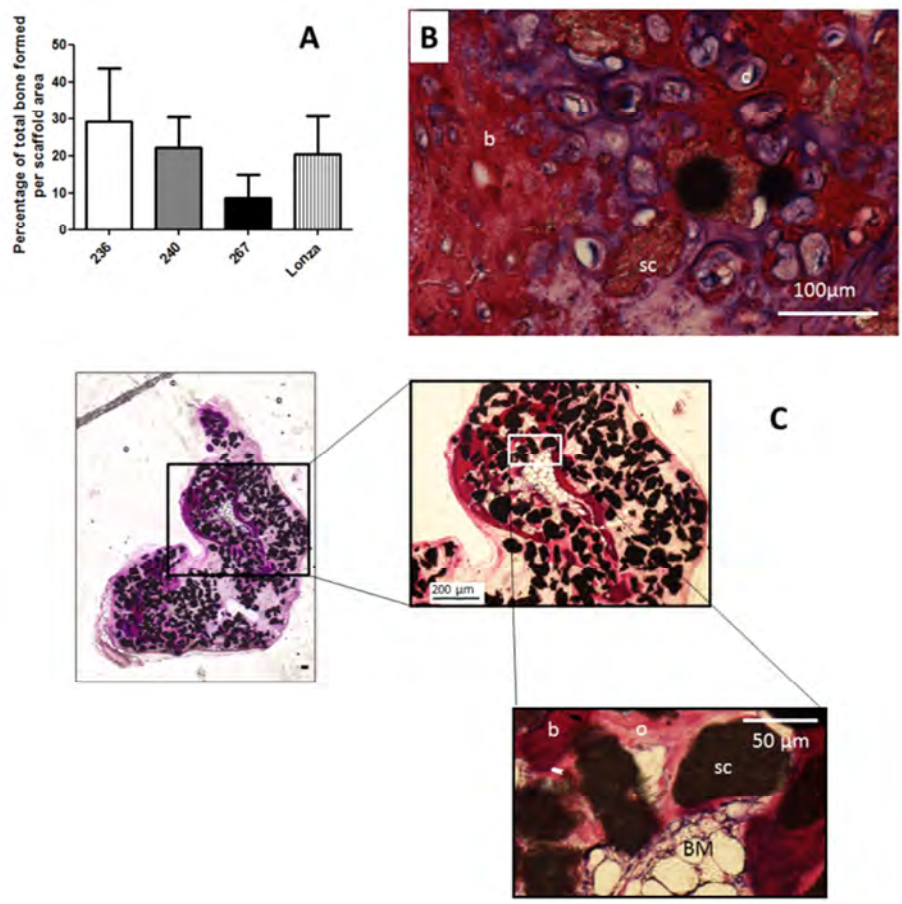

Figure 4.3. In vivo reproducibility with respect to bone formation of TECs generated via the cell aggregation system

(A) Quantification of the total amount of bone per scaffold area for four different donors (B). Cells from the commercial donor generated bone through the endochondral pathway. Methylene blue and basic fuschin stained sections displayed round cells in a lacuna, morphology indicative of hypertrophic cartilage. (C) On methylene blue and basic fushin staining, the sections from all four donors demonstrated large areas of mature bone and osteoid. The mature bone tissue stained red with basic fuschin and showed the presence of bone lining cells (indicated by white arrow) and embedded osteocytes (indicated by black arrow). The osteoid stained a lighter pink due to the unmineralized nature of the matrix. In some histological sections, bone marrow like structures was also observed. b, bone; sc, scaffold; o, osteoid; c, hypertrophic cartilage; BM, bone marrow.

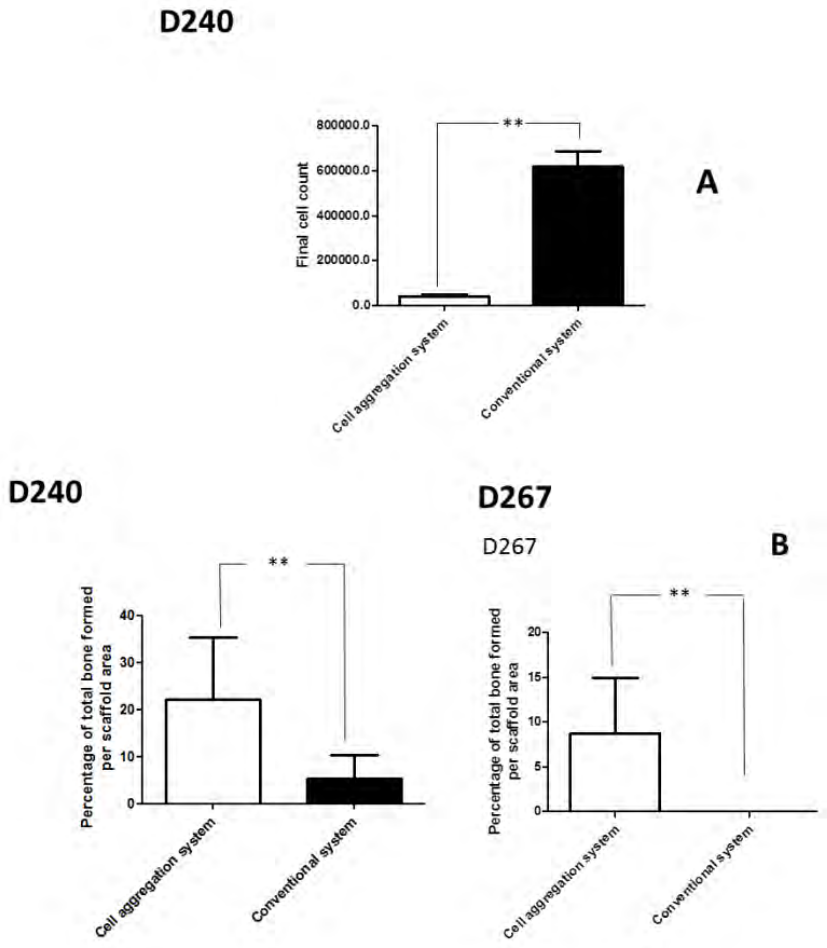

Figure 4.4. Comparison of the cell aggregation system and conventional system
*(A). Cell quantification analysis was performed in triplicate on tissue engineered constructs from a representative donor cultured for 2 weeks in vitro. There were a significantly increased number of cells on the scaffolds generated via the conventional system as compared to those generated via the cell aggregation system. (B).Tissue engineered constructs using 600,000 cells from the same donor were generated either via the conventional system or the cell aggregation system and cultured in vitro for 2 weeks prior to implanting in vivo for 6 weeks. Two donors were tested. On histomorphometric analysis of the stained sections, a significant increase was observed in the amount of bone formed via the cell aggregation system in both the donors. The amount of bone is expressed as the percentage of total bone formed over the total scaffold area. Statistical analysis was performed using Students paired t-test *= P<0.05%, **= p<0.005%*

4.3.4. Comparison of the in vivo bone formation in the cell aggregation system versus the conventional system

To compare the bone forming capacity of the cell aggregation system with the conventional system comprising single cells seeded on 1-2mm ceramic particles; both sets of tissue engineered constructs were created using cells from two donors (donors 267 and 240) and implanted subcutaneously in the same animals for 6 weeks. Prior to implantation, we verified the presence of viable cells by methylene blue staining (data not shown). Moreover, a cell quantification analysis was performed on samples from both the conventional and the cell aggregation system, from one representative donor, to rule out a potential effect of cell number on the amount of bone formed. We observed that the number of cells on the scaffolds in the conventional system after a 2 week culture period was significantly higher than that in the cell aggregation system (Fig. 4.4A). It is known from literature that cell aggregation suppresses proliferation [38], which could explain the difference in cell count. However, despite the lower number of cells in the final construct generated using the cell aggregation system as compared to the conventional system, after 6 weeks, the amount of bone formed in both the donors was significantly greater in the cell aggregation system (Fig. 4.4B). With donor 240, the amount of bone occupying the total scaffold area increased from 5% for the conventional system to 22% for the cell aggregation system. The advantage of the cell aggregation system was even more obvious when one of the donors (donor 267) which did not generate any bone with the conventional system, had 8.6 % of the total scaffold area filled with newly formed bone in the cell aggregation system. These data indicate that the culturing system using cell aggregation offers significant improvement in the in vivo performance of the constructs as compared to the conventional system.

4.3.5. Effect of pre aggregation of cells on their proliferation, in vivo bone formation and in vitro gene expression

The first step in our in vitro culturing system involves aggregation of the cells using non-adherent agarose microchips. The cell aggregates are then combined with scaffolds and gel to form an implantable tissue engineered construct. However, we observed that when unaggregated cells are mixed with the scaffold gel combination, the resulting construct rapidly compacts over the 2 week period into a tissue engineered construct similar to that obtained with aggregated cells. Therefore, we investigated the necessity of the additional step of pre aggregating the hMSCs. To determine if the cell aggregation affected the proliferation capacity of the individual cells, we performed a cell quantification assay on one of the donors (D236). Tissue engineered constructs were generated using either 1.5 million aggregated cells or the same number of unaggregated cells and cell numbers were determined after 2 weeks

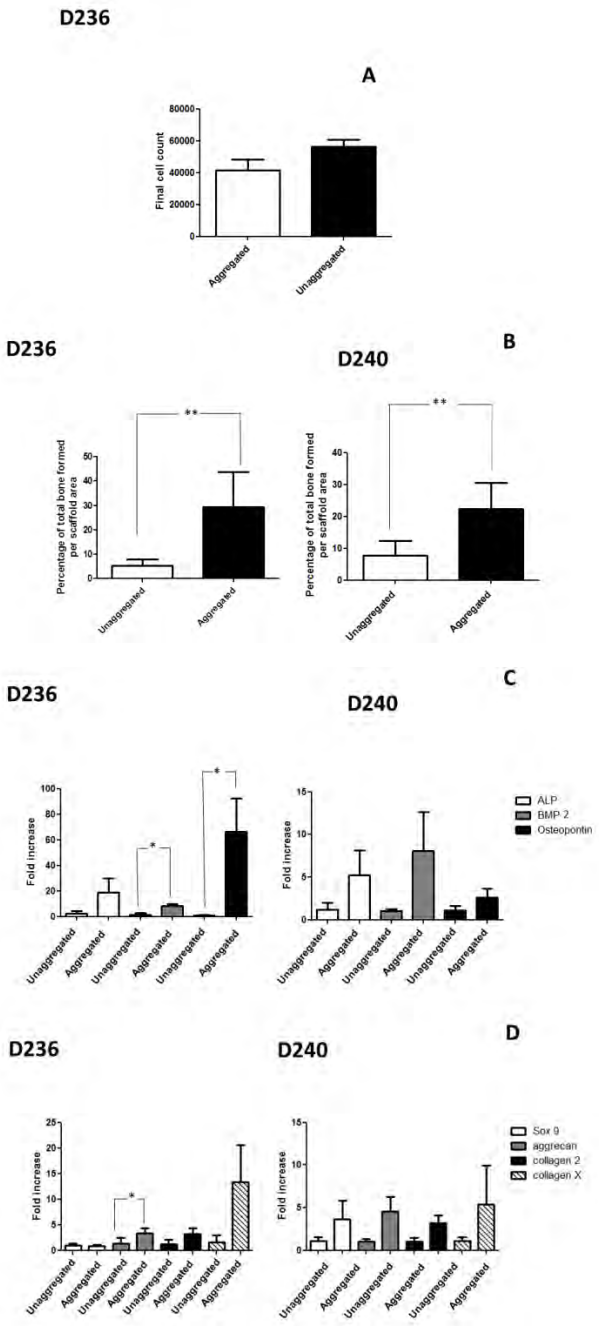

Figure 4.5. Effect of cell aggregation on cell proliferation and chondrogenic and osteogenic differentiation in vitro and bone formation in vivo

*hMSCs from the same donor were either aggregated into spheroids or left unaggregated.They were then combined with micro ceramic scaffolds and platelet gel. The resulting constructs were cultured in vitro for 2 weeks. (A) There was no significant difference in the number of cells, at the time of implantation, between the aggregated and unaggregated constructs. (B) The constructs using aggregated and unaggregated cells were implanted ectopically in nude mice for 6 weeks. After explantation, bone was quantified histologically. This study was repeated for 2 donors. In both the donors, there was a significant increase in the amount of bone formed in the constructs with aggregated cells. (C) The effect of aggregation on osteogenic and chondrogenic gene expression was analysed using qPCR. Two donors were analysed. The results indicate that aggregation has a positive effect on the expression of osteogenic and chondrogenic genes. The differences in gene expression were however significant for only BMP-2, osteopontin and aggrecan in one of the donors (D236). *P<0.05, **P<0.005.*

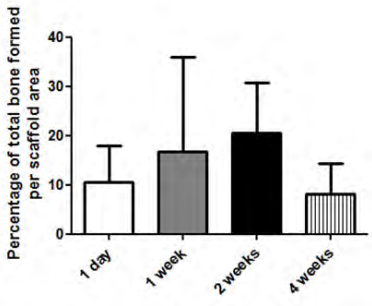

Figure 4.6. Effect of in vitro culture time of tissue engineered constructs generated using cell aggregation system on in vivo bone formation

hMSCs aggregated into spheroids were combined with micro scaffolds and platelet gel. These constructs were then either not cultured at all in vitro or cultured for 1, 2 or 4 weeks. They were then implanted in ectopic locations in nude mice for 6 weeks. Histomorphometric analysis of the explanted samples demonstrated that the in vitro culture time did not have a significant effect in the amount of bone formed. The significance was computed using one way Anova. P<0.05% was considered significant.

of culturing. Our results indicated that there was no significant difference in the number of cells (Fig. 4.5A). Both types of constructs were also implanted ectopically in immune deficient nude mice for 6 weeks. Histological examination revealed that even samples with unaggregated cells resulted in formation of bone tissue characterized by presence of a mineralized matrix with embedded osteocytes. However, the amount of bone formed in the constructs with aggregated cells was significantly higher with both donors than that with a single cell suspension (Fig. 4.5B). More specifically, the total amount of bone increased from 5.3% to 29.1% in donor 236 and from 7.6% to 22.2% in donor 240 for samples with aggregated and single cells respectively. We conclude that the aggregation step improves in vivo bone forming capacity of the hMSCs. Finally, to determine if the cell aggregation also enhances the in vitro expression of the osteogenic and chondrogenic genes, we performed qPCR using a panel of commonly used osteogenic and chondrogenic genes on the two types of constructs after 2 weeks of in vitro culture in the serum free Exp. Medium. The panel of osteogenic genes included ALP, osteopontin and BMP2 and the panel of chondrogenic genes included aggrecan, sox 9, collagen 2 and collagen X. There was a consistent increase in expression of all the osteogenic and chondrogenic genes in the aggregate culture compared to the constructs with unaggregated cells indicating that the beneficial effects of the aggregation in vitro parallel our in vivo results. This difference was statistically significant for the expression of BMP2, osteopontin and aggrecan in the constructs from donor 236 (Fig. 4.5C and 4.5D).

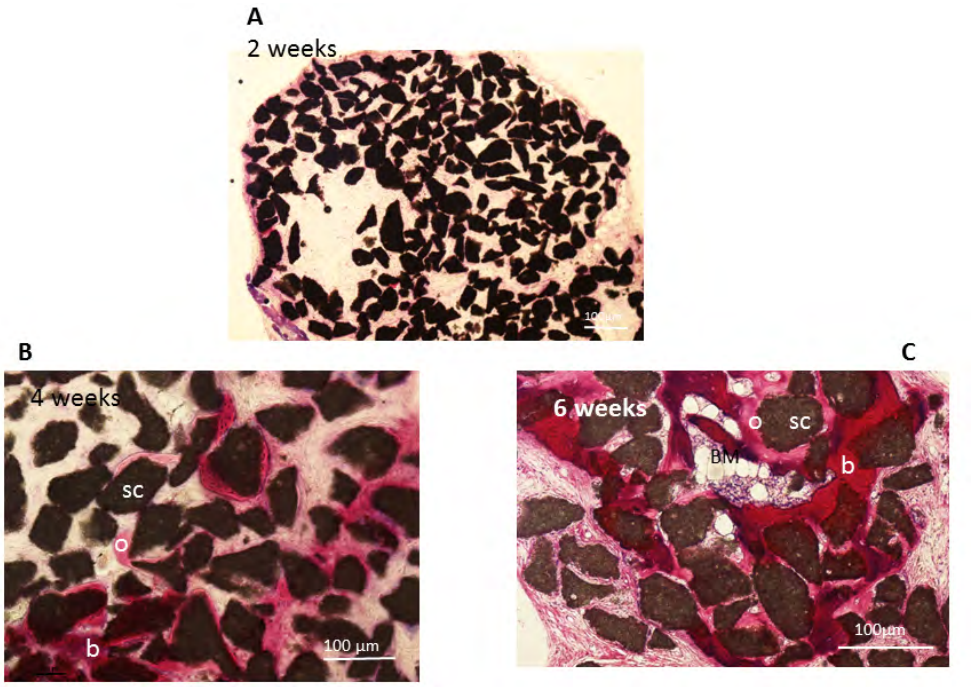

Figure 4.7. Time course study to determine the route to in vivo bone formation in constructs generated through cell aggregation system

Representative images of histological sections of tissue engineered constructs formed from 2 different donors (commercially purchased donor and donor 240) generated via the cell aggregation based culture system were analysed after 2, 4 and 8 weeks of in vivo implantation. No evidence of hypertrophic chondrocytes was observed at any of the time points. This indicates that the major route for bone formation in these constructs was intramembranous. Note also the increasing amount of mature bone from 2 weeks to 8 weeks and evidence of bone marrow, suggestive of a functional bone organ by 8 weeks B, bone: sc, scaffolds, o, osteoid; BM, bone marrow.

4.3.6. Effect of varying the in vitro culture times on the amount of bone formed

In all our previous studies, we employed a two week culture period after cell seeding to obtain bone in vivo [31]. However, to streamline the process for clinical applications, a shorter in vitro culture time is desirable. Thus, we decided to investigate the effect of in vitro culture time on the in vivo bone formation within the cell aggregation system. Tissue engineered constructs were prepared as described above using cell aggregates, micro ceramics and platelet gel. These constructs were either not cultured at all in vitro or cultured in the Exp. medium for 1, 2 or 4 weeks. Following 6 weeks of in vivo implantation, the constructs were explanted and histological analysis was performed. The results indicated that irrespective of the in

vitro culture time, all conditions generated osteoid as well as mature bone (Fig. 4.6). The amount of bone occupying the total scaffold area was 10.4%, 16.7%, 20.52% and 8.07% respectively for constructs either not cultured in vitro at all or cultured in vitro for a period of 1, 2 or 4 weeks, suggesting that in vitro culture is beneficial up to two weeks. However, statistical evaluation using one way Anova showed no significant difference between these values.

4.3.7. A time course study to determine the route of bone formation by aggregated hMSCs

Next, we investigated the process of bone formation in vivo by the tissue engineered constructs generated in vitro using the cell aggregation system. As described above the qPCR data showed an increased expression in the aggregates of chondrogenic genes including the collagen X gene which is indicative of hypertrophic cartilage formation. Moreover, after a 6 week implantation period, the cells from the commercial donor cultured in the cell aggregation system showed areas of round cells in large lacunae aligning areas of newly formed bone (Fig. 4.3B). This was strongly indicative of endochondral ossification. Therefore, we analysed the constructs from this donor along with another donor cultured in vitro for 2 weeks after 2, 4 and 8 weeks of in vivo implantation. After 2 weeks, we found no evidence of cartilage or mature bone although very small areas reminiscent of osteoid were observed (Fig. 4.7A). Four weeks after implantation, there were areas of mature bone lined with areas of lighter stained osteoid tissue. This indicated areas of intramembranous ossification. However, there was no evidence of any cartilage tissue at this point (Fig. 4.7B). At 8 weeks, there was an even greater increase in the amount of mature bone as well as osteoid tissue. In addition areas suggestive of bone marrow were also observed (Fig. 4.7C). No evidence of hypertrophic cartilage was seen at any time point. The results indicate that bone formation in this system follows the intramembranous route.

4.4. Discussion

The clinical application of human MSCs in the field of bone tissue engineering is currently limited. This can be partly attributed to the suboptimal amount of bone generated using conventional methods and the huge donor variation in the ability of the isolated cells to form bone in vivo. [53]. In this study, we describe a clinically applicable in vitro culture system that programmes the human MSCs to reproducibly generate significantly greater amounts of bone than that obtained via the conventional system. Exemplary for the in vivo efficiency of this system is a donor whose cells when employed in the generation of constructs using the cell aggregation system had

8% of the total scaffold area filled with bone while failing to generate any bone in the conventional system.

The low number of MSCs present in the bone marrow coupled with the high numbers required for most therapeutic applications often necessitates their in vitro expansion prior to their use in the clinic [54]. The mechanical and chemical cues provided to the MSCs during their in vitro culturing phase have been shown by a number of studies to determine their lineage commitment [31, 55, 56]. Thus, a lot of research in the bone tissue regeneration field is focussed on strategies which enhance osteogenic differentiation of the cells in vitro as a means to enhance bone formation in vivo. In our study, we conclude that an in vitro pre aggregation step is a simple, safe and cost effective approach to improve performance of the cells in vivo. The beneficial effects of cell aggregation observed in vivo was in accordance with the enhanced in vitro expression of osteogenic and chondrogenic genes observed in this study as well as that reported in a number of recent publications [35] [33, 34] [57]. While to our knowledge, this is the first publication that demonstrates the beneficial effects of culturing human MSCs as aggregates for bone tissue engineering in vivo, the use of cell aggregates for chondrogenesis is a routine practice [58] [59] [60]. Cell aggregates are also commonly used in other disciplines such as cancer research as well as in pharmaceutical industries in order to test the potential in vivo effects of drug therapies [61, 62]. Aggregation of cells into spheroids provides a three dimensional environment that more closely mimics the behaviour of the cells in the in vivo environment than the environment of a 2D polystyrene surface. Moreover, literature evidence suggests that better maintenance of the intercellular contacts probably results in integrin specific signalling that influences the proliferation and differentiation behaviour of the MSCs [63, 64]. In addition to being beneficial to the cell-cell signalling, culturing cells as aggregates also improves the interaction of the cells with the extra cellular matrix (ECM) which in addition to functioning as an adhesive substrate also acts as a reservoir of growth factors which play a role in maintenance of the differentiation potential of the cells [65].

There have been a number of possible techniques described in literature to generate cell aggregates. These include static techniques such as hanging drop cultures [66], use of trans well constructs [42] as well as generation of a cell sheet followed by cutting it into fragments of multicellular aggregates [33] and dynamic techniques such as spinner flasks and rotating vessel wall bioreactors (RWV) [34]. The dynamic methods can be useful to culture large tissue constructs because they provide constant mixing of the culture system, thus improving the flow of oxygen and nutrients to the cells which in turn can improve the viability of the cells. However, it is more difficult to control the size of the individual aggregates in the dynamic system as compared to the static

systems. The agarose templates used in this study to generate uniform cell aggregates is a straightforward method because the protocol needs no special training and uniformly sized cell aggregates can be generated within a day. Furthermore, the material to produce the moulds, PDMS, is very cheap. Once generated, the PDMS moulds can be re used to generate the agarose templates. The ease of handling coupled with the low costs make the system suitable for use in a hospital set up.

The cell aggregation based in vitro system described in this study involves combining the aggregates with micro ceramics and platelet gel. Although the platelet gel is known to be rich in growth factors, the beneficial effects of the growth factors on in vivo bone formation are not observed in our current study. However, as the platelet gel is an autologous product which is liquid at room temperature and gels at 37*o*C, i.e. the physiological body temperature, it is the preferred choice as a delivery vehicle for the cell ceramic mix.

There have been studies in literature where the cell aggregates without ceramics were matured into cartilage templates in vitro for varying time periods to generate bone in vivo. However, in such systems it is essential to culture the constructs in vitro for at least 2 weeks prior to in vivo implantation to ensure their retrieval at the time of explantation [42]. This delays the time required to generate a tissue engineered construct and thus may not be optimal for clinical settings. In contrast, the cell aggregation system described in this study enables generation of constructs with in vivo bone forming capacity with no requirement of in vitro culturing of the cell aggregates with the ceramics and the platelet gel. Further, our preliminary data suggests that in addition to easy retrievability, ceramics themselves actively contribute to the bone formation.

One of the benefits of using a scaffold free system involves the ease of adapting such systems to fill an irregular shaped defect [34]. This is indeed a reason for concern when larger ceramics blocks or granules are used as is the practice in conventional bone tissue engineering. Moreover, block based transplants demonstrate poor bone formation in their interior because the cell sheath on the surface interferes with nutrient delivery in the core areas of the block. However, particle based transplants have been shown previously to have abundant bone formation throughout their cross section [67]. Finally, there have been reports in literature suggesting that the increased surface area offered by smaller granules may potentially aid the release of greater amounts of osteoinductive calcium ions per time than larger granules, thereby influencing the in vivo outcome [68]. Based on these considerations, micro ceramic particles were used in our study, in combination with the platelet gel. Such constructs

can be adapted for injectable purposes as well as easily tailored to fit irregular shaped defects.

Under physiological conditions, bone repair in long bones follows the process of endochondral ossification [42]. However, in majority of tissue engineered approaches, the bone formed in vivo is via intramembranous ossification. The composition and metabolism of the bone formed via endochondral ossification is different from that formed via intramembranous ossification. Since it is desirable to recapitulate as closely as possible the physiological bone repair processes, it is appealing to generate tissue grafts which when implanted in vivo follow the endochondral route. Thus, when we observed hypertrophic cartilage aligned by mature bone in samples from one donor generated using the cell aggregation culture technique in two separate studies, we investigated it further with the same donor as well another independent donor. We performed a time course study to follow in vivo the path of bone formation in the two donors. Unfortunately, although we did observe extensive bone formation after 8 weeks of in vivo implantation, the earlier time points did not demonstrate any evidence of hypertrophic cartilage formation in either of the donors. Our in vitro results demonstrated that culturing the cells as aggregates enhanced expression not only of the osteogenic genes but also the chondrogenic genes, including collagen X which is a marker of hypertrophic cartilage. Thus, it appears that the culture system employing cell aggregation does offer a possibility to recapitulate the endochondral bone formation in vivo. However, yet unidentified factors, in vitro as well as in vivo probably influence the final behaviour of the cells which in turn determines the path taken by them to generate bone in vivo.

4.5. Conclusion and future directions

In conclusion, we outline in this study a technique for culturing cells in vitro to enhance their bone forming capacity in vivo. Although the origin of the newly formed bone was not determined, i.e. host or donor, the results convincingly indicate that the combination of cell aggregates with micro ceramics and platelet gel reliably generate significant amounts of bone in vivo. Moreover, this technique is of clinical relevance as the cell aggregation is performed in a serum free culture medium with a short in vitro culture duration and use of autologous platelet gel as the delivery vehicle. Further, future investigations toward a clinical implementation of the developed system including scaling up of the constructs implanted, use of different compositions of scaffolding materials, orthotopic implantation in an immunocompetent animal

model and studies to determine the contribution of the host cells in the observed process, could help better translate this system into the clinic.

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Chapter 5

A minimally invasive approach to engineering new bone in vivo using human bone marrow derived mesenchymal stromal cells

Currently, there is a rise in the popularity of arthroscopic procedures in orthopedics. However, the majority of cell based bone tissue engineered constructs are developed using solid pre formed scaffolding materials, which require large incisions and extensive dissections for placement at the defect site. They are thus not suitable for minimally invasive techniques. Here we describe a clinically relevant, minimally invasive, bone tissue engineering strategy, employing an in-situ forming autologous fibrin thrombin gel as a carrier for human mesenchymal stromal cell (hMSC) aggregates and calcium phosphate micro particles. The absence of the need for culturing MSCs on the scaffolds prior to implantation significantly reduces the time needed for generation of the constructs. The reproducibility of the system is confirmed using mesenchymal stromal cells from three different human donors. Aggregation of hMSCs proved to be a necessary step in this system as unaggregated MSCs did not generate bone. In this system, the MSCs were aggregated for 24 hours in a serum free medium. Our results indicate that while unaggregated cells generated no bone, a longer in vitro culturing of the aggregates resulted in a decreased viability of the cells within them and provided no beneficial effect on in vitro expression of the osteogenic genes as well as in vivo bone formation.

5.1. Introduction

The repair of bone non-unions and defects remains a significant clinical problem. The currently preferred treatment option is an autologous bone graft. However, the supply of suitable autologous bone is limited and its collection associated with a risk of infection, hemorrhage, chronic pain, cosmetic disability, nerve damage and loss of function at the donor site [1]. An alternative option available to the surgeons is the use of natural or synthetic biomaterials that promote the migration, proliferation and differentiation of bone cells from the vicinity of the defect site [1, 2]. However, the success of such materials in repairing critical sized defects is limited as they lack the osteogenic and osteoinductive properties of autologous bone grafts. Addition of bone morphogenetic proteins can improve the osteoinductivity of these materials. However, the high costs and potential for unwanted side effects is a deterrent [3]. Addition of cells having the potential to differentiate into the osteogenic lineage was suggested as another potential solution to improve the osteoinductive properties of the scaffold material [4]. Different types of cells such as osteoblasts, embryonic stem cells and mesenchymal stromal cells (MSCs) were examined as potential candidates [5, 6]. Ease of isolation, extensive expansion capabilities, a capacity to differentiate readily into multiple lineages and no demonstrated evidence of tumorigenic abilities, resulted in MSCs emerging as one of the most promising sources [6, 7]. Conventionally, for bone tissue engineering approaches, the MSCs harvested from the patient, are expanded in culture and combined with a pre formed scaffold to generate a tissue engineered construct (TEC) [8]. Such tissue engineered constructs have been shown to form bone convincingly in ectopic as well as orthotopic locations in many animal models as well as in a few human studies [9-16].

However, it is often difficult to fit a preformed scaffold into a defect with a complex geometric shape as can be the case following trauma or tumor resections. In such cases, the surgeon needs to either fabricate ex vivo complicated scaffold geometries or carve the defect site. The former is technically very challenging while the latter leads to greater manipulation of the surrounding tissue and increase in bone and blood loss, trauma and surgical time [17].

Minimally invasive approaches such as laparoscopy, thoracoscopy, and arthroscopy have revolutionized surgical care in the past two decades. Arthroscopic procedures are also becoming increasing popular in orthopedics. Preformed scaffolds are not amenable to arthroscopic techniques. The development of injectable tissue engineered formulations that can act as cell carriers can result in immediate clinical, benefits both in terms of convenience for the surgeon and faster recovery time for the patient[18].

An in situ forming gel or cement can be used either as the scaffold material itself or as a carrier for micro particle sized scaffolds. Such materials can conform to any shape and can be deposited at the target site via a simple syringe, facilitating patient comfort and compliance. A number of in situ forming materials have been proposed including calcium phosphate cements (CPC), polymeric gels, agarose, hyaluronate, cellulose, fibrinogen, collagen and pluronic acid [19-21]. While the CPCs provide good biomechanical stability, the exothermic reactions associated with their setting process prove to be a deterrent in combining them with cells. One of the approaches reported to overcome this drawback involves the use of self-dissolving alginate beads which serve as a protective capsule for the cells during setting of the injectable cements. Though an interesting approach, the results have not yet been verified in in-vivo settings [15] [22]. The agarose gel does not support vessel ingrowth and thus ideal as a delivery system for cartilage tissue engineering but not for bone tissue engineering. The degradation products of pluronic acid are toxic for the cells while the curing of hyaluronic acid from solution to a gel form is difficult making it unsuitable for routine clinical use. Many of the polymeric gels result in inflammatory reactions [20]. Collagen, alginate and fibrinogen are biocompatible and thus suitable for use with cells. However, of these three in situ gel forming compounds, fibrinogen offers distinct advantages as it gels at body temperature and can be prepared from the patient's own plasma. Mankani et al used fibrinogen as an in situ forming gel in combination with a single cell suspension of hMSCs and HA/TCP particles. This formulation when injected into ectopic and ortho topic sites in mice generated bone [18]. This provided a proof of principle on the possible use of ceramics and MSCs with fibrinogen as an injectable formulation for generating bone in vivo.

Recently, there has been a lot of focus on culturing cells as aggregates to improve their in vivo bone forming capacity[23-25] . Within our laboratory, we observed that greater amounts of bone were obtained in vivo when a single cell suspension of hMSCs conventionally used in bone tissue engineered constructs was replaced by hMSCs aggregates and cultured in a system comprising a fibrin thrombin based gel (manuscript submitted). In the present study we aimed to adapt this system to generate a formulation that could be delivered at the defect site using a minimally invasive technique without compromising its in vivo bone forming efficiency.

5.2. Materials and methods

5.2.1. Cell culture

Bone marrow aspirates (5-20ml) were obtained from healthy donors during hip replacement surgery with written informed consent. Alternatively cryopreserved vials

of hMSCs were purchased (Lonza group ltd). When isolated from fresh marrow aspirates, the aspirates were resuspended using 20G needles and plated at a density of 5X105/cm2 and cultured in basic medium supplemented with 1 ng/mL basic fibroblast growth factor (bFGF, Instruchemie, The Netherlands). This medium is henceforth referred to as proliferation medium. Basic medium was made of a-minimal essential medium (a-MEM, Life Technologies), 10% foetal bovine serum (FBS, Cambrex), 0.2 mM ascorbic acid (Asap, Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/mL penicillin (Life Technologies) and 10 mg/mL streptomycin (Life Technologies). Cells were grown at 37°C in a humid atmosphere with 5% CO2. Medium was refreshed twice a week and on reaching near confluence, cells were trypsinised and cryopreserved till further use. At the start of the experiments, the cryovials were expanded further in the proliferation medium until numbers sufficient for experiments were obtained.

5.2.2. Generation of cell aggregates

400 μm diameter wells with a depth of 200 μm were patterned on poly(dimethylsiloxane) (PDMS) stamps using etched silicon wafers according to established protocols [26]. After sterilising the PDMS stamps for 10 minutes in 70% ethanol, they were placed in the wells of a standard 6 well plate and completely covered with 3% agarose solution (Ultra-pure agarose, Invitrogen). Upon solidification, the chips were demoulded and the agarose templates were placed in a non-tissue culture treated 12 well plate. After wetting the chips with medium, a concentrated suspension (1ml) of 1.5 million cells was uniformly dispersed over the wells on each agarose chip. Agarose chips were then centrifuged briefly at 1500 rpm to facilitate the cells to settle down on the chips. For culturing on the chips, the medium used (termed Exp. Medium) was serum free and composed of Dulbecco's Modified Eagle's Medium supplemented with 10-7M dexamethasone, 50 mg/ml ascorbic acid (Asap, Life Technologies), 40 mg/ml proline ((Sigma-Aldrich), 100mg/ml sodium pyruvate 50 mg/ml ITS 1 Premix (Becton-Dickinson). 1.5 ml of the Exp. Medium was added to each chip. Cells spontaneously compacted to form aggregates within 24 hours. For the study comparing the viability of the cells after 1 day, 1 week and 2 weeks of in vitro culture, the aggregates were left in the agarose chips for 1 day, 1 week and 2 weeks respectively. Medium was changed every alternate day during culture in the agarose chips

5.2.3. Platelet gel

After written informed consent, venous blood was withdrawn from healthy blood donors and passed through an apharesis unit at the Institut für Klinische Transfusionsmedizin, Braunschweig. Platelets were isolated and immediately frozen at

-80 °C. At the time of the experiment, the bag was thawed at 37°C. This freeze thaw cycle resulted in lysis of the platelets with subsequent release of the growth factors and formation of the platelet lysate. 235µl 1 M calcium chloride solution was added per 10 ml of platelet lysate and gently mixed on a shaker at 37°C for approximately 10 minutes. This resulted in the separation of the platelet lysate into a gel like component and a clear liquid. The clear liquid was the source of the thrombin component which when added at a 1: 1 ratio at 37°C to the rest of the platelet lysate (fibrin source), activated the clotting pathway, thereby resulting in the formation of a platelet gel in 10-12 seconds.

5.2.4. Calcium phosphate micro ceramics

Biphasic calcium phosphate micro ceramics of size 53-63 um were provided by Xpand Biotechnology BV, The Netherlands. The particles contain 20±5% beta-tricalcium phosphate and 80±5% hydroxyapatite. The particles were autoclaved at 121ºC for 30 minutes before use.

5.2.5. Generation of constructs for in vivo implantation

In the minimally invasive approach, the cell aggregates were flushed out of the microchips and immediately mixed with micro ceramics and fibrin and thrombin components (1:1 ratio) of the platelet gel just prior to the introduction in the animal. The mix was then directly introduced into the defect through a small pocket, just enough to hold the tip of the pipette. The pipette tips were pre cooled and the cell ceramic constructs were also kept on ice to slow the gelling time. The contents were very slowly introduced into the defect and the edges of the pocket were held up to ensure that the contents did not leak out from the side. The injected material gellified within 30-45 seconds after injection. The pocket was then closed using 5-0 vicryl sutures. As a control, we implanted in the same animals, with an open 2 mm incision, the construct generated after the cells were mixed with scaffolds, fibrin and thrombin and then left to gel at 37 degrees for 15 minutes prior to implantation.

5.2.6. Cell viability assays

The viability of the cell aggregates within the constructs generated using cell aggregates, micro ceramics and platelet gel and deposited using a pipette tip in the bottom of a 6 well plate was checked using an MTT assay. The constructs were incubated with 1ml Exp. Medium and 20µl MTT solution (5mg/ml; Gibco) per well for 2 hours at 37° C in a 5% CO2 atmosphere incubator. Images were captured using a phase contrast microscope and MATRIX Vision SRGB 32 Bit software.

The viability of cells in the aggregates cultured for 1 day, 1 week and 2 weeks within the micro wells of the agarose chips was checked using a live dead assay (Invitrogen, Carlsbad, CA). The two components of the kit, ethidium homodimer 1 and calcein were mixed in a ratio of 4:1 and diluted in PBS as per the protocol recommended by the manufacturers. The constructs were incubated in the resulting mixture for 30 minutes in the dark. The fluorescence from the calcein dye was observed using a filter for the FITC 488 dye while the fluorescence for ethidium homodimer was observed using filters for Texas red dye. The images were captured using a colour camera (Nikon FDX-35) and QCapture software.

5.2.7. Gene expression analysis

Table 5.1. Primer sequence

Tl: transcript length, Ta: annealing temperature

Gene	*Primer sequence*	*Tl (bp)*	*Ta (°C)*
B2M	5'-GACTTGTCTTTCAGCAAGGA-3' 5'-ACAAAGTCACATGGTTCACA-3'	106	60
Collagen 1	5'-AGGGCCAAGACGAAGACATC-3' 5'-AGATCACGTCATCGCACAACA-3'	138	60
Alkaline Phosphatase(ALP)	5'-GACCCTTGACCCCCACAAT-3' 5'-GCTCGTACTGCATGTCCCCT-3'	70	60
Osteocalcin	5'-GGCAGCGAGGTAGTGAAGAG3' 5'-GATGTGGTCAGCCAACTCGT-3'	138	62
BMP-2	Commercially bought (SABiosciences)	140	60

Cells aggregated in the microwells for 1 day, 1 week or 2 weeks were washed briefly with PBS and lysed using Trizol reagent (Invitrogen, Carlsbad, CA). The samples were stored for at least 12 hours at -80°C for RNA isolation. After chloroform addition and phase separation by centrifugation, the aqueous phase containing the RNA was collected, mixed with equal volume of 75% ethanol and loaded onto the RNA binding column of a Nucleospin RNA II kit (Bioke). Subsequent steps were in accordance with the manufacturer's protocol. The RNA yields were determined by spectrophotometry using the Nanodrop1000 (ND-1000 spectrophotometer, Isogen Life Science). Subsequently cDNA was synthesized using iScript (BioRad) according to the manufacturer's recommendations. Three μl of undiluted cDNA was used for subsequent analysis. For quantitative PCR, a master mix, containing distilled water, forward primer, reverse primer (Sigma Genosys), BSA and SYBR green I mix (Invitrogen) was prepared. Real-time qPCR was performed, for the osteogenic genes, on a MyIQ single colour real-time PCR detection system (BioRad). Gene expression was normalized to the expression of Beta-2 microglobulin (B_2M) gene. My IQ data was analysed using iQ^{tm}5 optical system software (Biorad). Ct values were normalized to the B2M housekeeping gene and comparative ΔCt method (Ct control - Ct sample) was used to calculate the fold inductions. Primer sequences are listed in Table 1.

5.2.8. In vivo studies

An immune deficient mouse model was used for assessing the bone forming capacity of the hMSCs. TECs were introduced into their subcutaneous pockets either by injection or by an open approach. Ten immune deficient male mice (Hsd-cpb: NMRI-nu, Harlan) was used for each of the experiments. The mice were anesthetized by the inhalation of isofluorane and carbon dioxide. For the open approach, a 2mm incision was made on the dorsal aspect of each mouse while for the injectable approach, a hole was made using an 18 gauge needle and then enlarged to fit the pipette tip. The openings in either case were closed using a vicryl 5-0 suture. The constructs were left in the mice for 6 weeks at the end of which the mice were killed using carbon monoxide and the samples were explanted. All the experiments were approved by the local animal experimental committee.

5.2.9. Bone Quantification

The explanted samples were fixed in 4% paraformaldehyde (Merck) and embedded in methacrylate for sectioning. Approximately 300μm-thick, undecalcified sections were processed on a histological diamond saw (Leica saw microtome cutting system). At least 4 sections were cut per sample. Each section was stained with basic fuchsin and methylene blue to visualize new bone formation. The newly formed mineralized bone stains red with basic fuchsin, the unmineralized osteoid stains light pink while all other

cellular material stains blue with methylene blue. The ceramic material remains black and unstained by both the dyes. For quantitative histomorphometric analysis, three randomly selected slides were first scanned using a PathScan Enabler IV histology slide scanner. Mature and immature bone was manually pseudocoloured green while the scaffold material was pseudocoloured red using Photoshop CS2 (Adobe Systems). A custom-made Matlab script was used to measure the amount of bone per total scaffold area.

5.2.10. Statistics

All in vitro experiments were performed in triplicate. Statistical analysis was performed using One-way Anova followed by Tukey's multiple comparison test ($P<0.05$) when more than 2 groups were compared. When the statistical analysis was performed between 2 groups, a Student's paired t-test was used. As in the case of Anova, a p value less than 0.05 was considered as significant.

5.3. Results

5.3.1. Cell viability and cohesion of the cell aggregates after injection

To investigate if the shear force experienced by the cell-ceramic-fibrin thrombin construct, during passage through the pipette tip causes the cell aggregates to dissociate, the cell aggregates were mixed with the micro ceramic particles as well as fibrin and thrombin just prior to aspirating the mixture into the pipette tip and immediately let out on a clear bottom 6 well plate. The plate was kept at 37ºC for a minute prior to visualizing under a light microscope. Cell aggregates were seen interspersed with the micro ceramic particles indicating that the majority of the aggregates remained intact even after the injection process (Fig. 5.1A). To check for the effect of the injection forces on the viability of the cells within the aggregates, the constructs on the 6 well plate were then stained with a MTT solution for 30 minutes and observed under a phase contrast microscope. The dark purple colour of the aggregates indicated that the cells survive within the aggregates after injection (Fig. 5.1B).

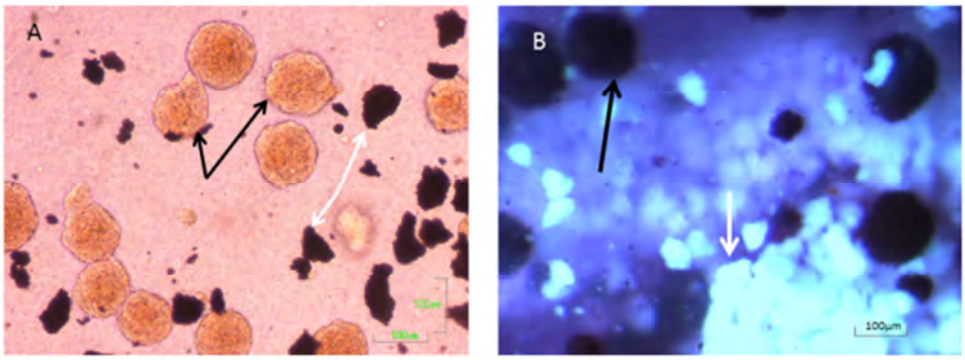

Figure 5.1. The viability and the morphology of the cell aggregates are not affected by the injection process

The cell aggregates mixed with ceramic micro particles and fibrin thrombin gel were passed through a pipette tip and released on the bottom of a 6 well plate. (a)The ejected construct was visualized under a light microscope. The cells persisted as aggregates even after passage through the pipette tip. (b) The viability of the aggregates was determined immediately after being released on the 6 well plate using a MTT assay. The dark purple colour of the aggregates on incubation with the MTT solution indicated that the cells also remained viable after passage through the pipette tip. The black arrow points to the aggregates while the white arrow points to the ceramic scaffolds.

5.3.2. In vivo bone forming capacity of the tissue engineered construct delivered to the defect site via a minimally invasive approach

To determine if a minimally invasive approach to introduce a tissue engineered construct could be a viable option to generate bone in vivo, 1.5 million cells were aggregated in agarose microchips for 24 hours. Just prior to introducing the cell aggregates into the defect site, they were flushed out of the agarose chips and mixed with ceramic micro particles and equal amounts of fibrin and thrombin. Fibrin and thrombin remained liquid at room temperature and served as delivery vehicles for the cell aggregates and micro particles. The whole mixture was aspirated into a pipette tip and introduced into the subcutaneous pocket on the dorsum of nude mice via a small opening just wide enough to hold the pipette tip. As the mixture of fibrin and thrombin gellifies at 37°C, the whole mixture introduced into the animal was localized within seconds at the injection site. All the implants were left in vivo for a period of 6 weeks. Histological analysis of constructs after 6 weeks demonstrated abundant bone formation (Fig. 5.2A). More specifically, 9.5% of the total scaffold area was occupied with newly formed bone in the constructs using the commercially obtained MSCs (Fig. 5.2B). To test if the results obtained using the minimally invasive approach were reproducible with other human MSC donors, we repeated the study using hMSCs

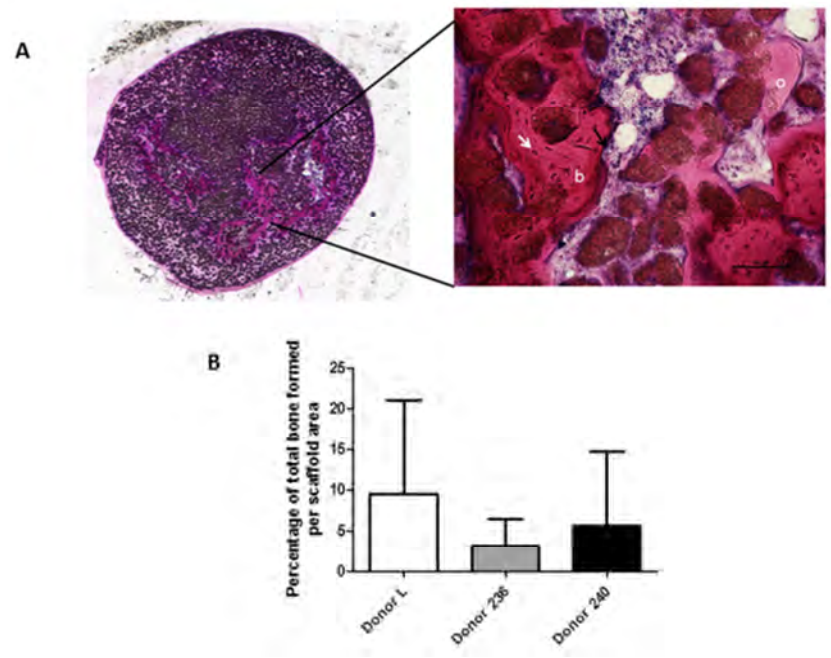

Figure 5.2. In vivo bone forming capacity of the constructs introduced into the subcutaneous pocket of the nude mice via the minimally invasive approach

Cell aggregates mixed with micro ceramic particles and fibrin thrombin gel were immediately introduced in the subcutaneous space in the nude mice via an opening which fits the tip of a 1ml pipette. (A) On histological staining with methylene blue and basic fuschin after 6 weeks of in vivo implantation, these constructs demonstrated large areas of mature bone (B) and osteoid (O). The mature bone tissue stained red with basic fuschin and showed presence of bone lining cells (indicated by black arrow) and embedded osteocytes (indicated by white arrow). The osteoid stained a lighter pink due to the unmineralized nature of the matrix. (B) In vivo bone was obtained when the study was repeated using cells from a commercial donor (donor L) as well as two other donors (D 236 and D 240) from our donor bank.

from two different donors (donor 236 and donor 240) from our donor bank. In the constructs employing cells from D 236, 3.1% of the total scaffold area was filled with bone while for the constructs using cells from D 240, newly formed occupied 5.6% of the total scaffold area (Fig. 5.2B). These data indicate that cell aggregates and micro ceramics can be reliably employed for bone tissue engineering purposes via a minimally invasive approach.

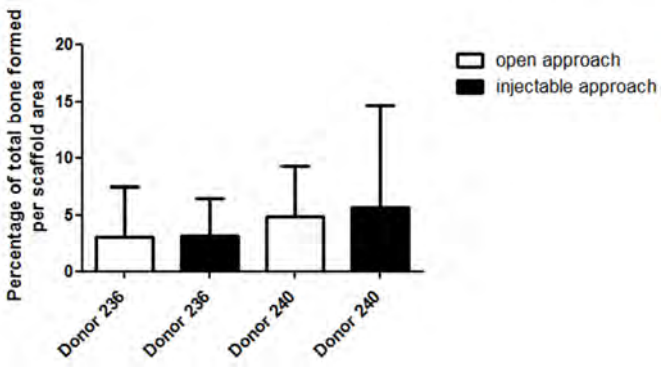

Figure 5.3. Comparison of the conventional open approach for introducing constructs into a defect with the minimally invasive approach

Cell aggregates mixed with micro ceramic particles and fibrin thrombin gel were either generated ex vivo and introduced into the nude mice through an open incision or directly delivered into the subcutaneous pocket through a pipette tip. Both sets of constructs were implanted in parallel in the same animals. The study was repeated with 2 separate donors (D 236 and D 240). In both the donors tested, no significant difference was observed in the amount of bone obtained using the open incision approach versus the minimally invasive approach of construct delivery via the pipette tip.

5.3.3. Comparison of the injectable system versus the invasive system in terms of in vivo bone formation

Conventionally tissue engineered constructs are introduced into a defect site via an open incision. Our results indicate that in our system, a minimally invasive approach also results in new bone formation in vivo. To investigate if there are significant differences in the amounts of bone obtained when the constructs are gelled ex vivo at 37° for 15 minutes and then inserted via an open incision of around 2 mm or introduced directly into the subcutaneous space of the animal using an opening, big enough to just fit the pipette tip and allowed to gel in vivo, we implanted both groups in parallel within the same immune-deficient mouse. The study was performed with cells from 2 human MSC donors (donor 236 and donor 240). In the open approach, the constructs comprising the MSC aggregates, micro ceramic particles and fibrin thrombin gel were generated in vitro on 6 well plates prior to introducing them into the subcutaneous pocket via a 2 mm incision. In the minimally invasive approach, the components of the construct were directly introduced into the subcutaneous pocket via a small hole, just enough to fit the pipette tip. On histological evaluation of the two types of samples after 6 weeks, the constructs employing cells from donor 236 had 3.09% and 3.1% of the scaffold area filled with newly formed bone in the case of an open and injectable approach respectively. In the case of constructs employing cells from Donor 240, 4.8% of the scaffold area was covered with bone in the open

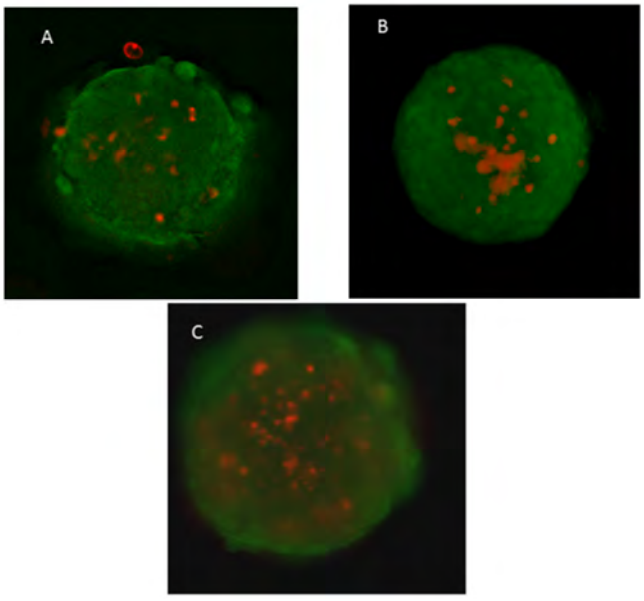

Figure 5.4. Effect of varying periods of in vitro culturing of cell aggregates on the cell viability

Similar cell numbers from the same donor were aggregated in agarose microchips and cultured for 1 day, 1 week or 2 weeks in vitro. The medium was refreshed every alternate day. At the end of 1 day (A), 1 week (B) and 2 weeks (C), the viability of the cells within the aggregates were analyzed using a live dead assay. An increase in the number of red fluorescent dots in aggregates cultured for longer periods in vitro indicated that longer periods of culturing as aggregates within the microchips compromised the viability of the cells.

approach versus 5.6% for the injectable approach. In both the donors, the differences were not statistically significant, (Fig. 5.3) indicating that in our system, the minimally invasive approach is a viable alternative to the open approach for the purpose of bone tissue engineering.

5.3.4. Effect of a prolonged in vitro culture time of cell aggregates on their in vivo bone formation, in vitro gene expression and cell viability

Previously in our lab, on culturing unaggregated MSCs with ceramics and gel for 2 weeks in vitro, we obtained bone in vivo, although the amounts were significantly lower compared to constructs employing aggregated cells and cultured for the same amount of time in vitro (manuscript submitted). To determine whether aggregation is necessary for in vivo bone formation and whether the system can be improved by prolonged in vitro culturing of the cell aggregates, the MSCs were cultured as aggregates in the agarose microchips for 1 day, 1 week and 2 weeks or left unaggregated. In all cases, the medium was refreshed every alternate day. The

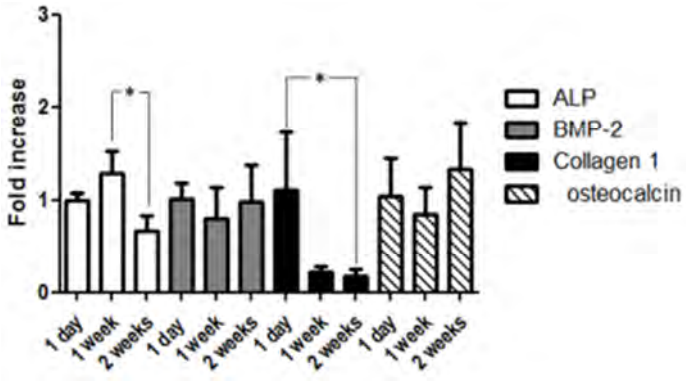

Figure 5.5. Effect of varying periods of in vitro culturing of cell aggregates on the expression of osteogenic genes

*Expression of a panel of genes in hMSC aggregates of donor 240, relative to unaggregated MSCs. Results were analysed using one way Anova. *P<0.05, **P<0.005.*

unaggregated cells as well as the cell aggregates were then mixed with the micro ceramics and the fibrin-thrombin gel and introduced into the subcutaneous pocket of nude mice using the minimally invasive approach. Prior to implantation, we assessed cell viability using a life/dead staining and we observed an increased number of red fluorescence in 2 week group compared the 24 hours group. This indicates that cell viability is compromised upon extended culture. (Fig. 5.4). Furthermore, we examined the expression of a panel of osteogenic genes using qPCR analysis. We observed that there was no significant difference in the expression of either BMP-2 or osteocalcin between different groups. ALP gene expression was highest at 1 week with a significant decrease at the 2 week time point. Collagen 1 expression was significantly lower after 2 and 3 weeks of culture. (Fig. 5.5). Next, we implanted the TECs and after 6 weeks of in vivo implantations, the amount of bone was determined histomorphometrically. In the unaggregated group, no bone formation was observed. The percentage of bone generated for the total scaffold area varied from 7.62, 5.32 and 5.36 % for 1 day old aggregates, 1 week old aggregates and 2 week old aggregates (Fig. 5.6). Statistical evaluation indicated that the differences were not significant.

5.4. Discussion

Minimally invasive techniques for delivery of cell based tissue engineered constructs are limited as most cell based constructs rely on the use of a solid preformed matrix that require an open procedure for delivery at the defect site. This is associated with significant manipulation of the soft tissues with consequent risk of infections and longer intra- operative times [27]. This is of special concern in case of staged surgical interventions, where multiple invasive surgeries may be associated with increased

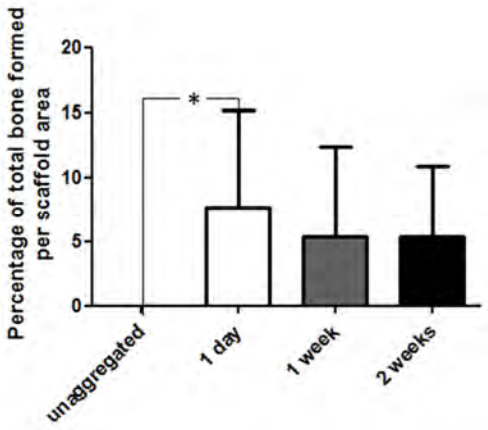

Figure 5.6. Optimization of the amount of bone formed in vivo via the minimally invasive approach

*To optimize the amount of bone obtained in vivo using the minimally invasive approach, the cell aggregates obtained using cells from the commercial donor (donor L) were cultured in the agarose microchips for 1 day, 1 week or 2 weeks. Prolonged in vitro culturing of the cell aggregates did not significantly affect the amount of bone formed in vivo. However, constructs employing unaggregated cells from the same donor did not generate any bone in vivo indicating that in this system, the cell aggregation was a pre requisite for in vivo bone formation. Results analyzed using one way Anova *P<0.05, **P<0.005.*

patient morbidity and negative aesthetic effects. Here we describe a technique to introduce cell and ceramic based tissue engineered constructs through an in situ forming fibrin thrombin based gel, into the defect site via a minimally invasive route. Such techniques can have immediate clinical significance in the treatment of conditions such as mandibular atrophy, scaphoid non unions or spinal disease where endoscopic efforts at placing conventional bone grafts are already under investigation or to fill complex contours in craniofacial defects without need for pre surgical modeling [28-31].

Currently available injectable materials for regeneration of bone rely on ceramic pastes and cements and polymer based formulations. Some of these materials have been used in the past in combination with demineralized bone matrix, or growth factors as the osteoinductive agent [24-26]. However, there are only a few growth factors approved for clinical use and supraphysiological doses are needed which raises concerns about the possibility of unwanted side effects [32-34]. Demineralized bone matrix is considered a reprocessed human tissue and not a medical device by the FDA and is thus not subjected to the rigorous testing of its efficacy. Several studies demonstrated varying levels of growth factors within different batches of the same DBM product and a highly variable amount of BMPs [35, 36]. Mesenchymal stromal cells are a safe, more physiological approach to improving the osteoinductivity of the tissue

engineered constructs, though the variation in the bone forming capacity of different donors, suggest that they alone may not be an ideal candidate [37]. Thus "polytherapy" with a combination of a matrix in combination with MSCs and growth factors maybe the most promising alternative to autologous grafts. However, the exothermic reactions during the setting process, poor degradability hampering bone ingrowth, need for toxic chemical initiators for the crosslinking process, potential to elicit strong inflammatory responses and the possible toxic effects of the degradation products explains the relatively few reports in literature employing human bone marrow derived MSCs in formulations suitable for an injectable approach to bone formation in vivo[19, 38-41]. An approach proposed by two independent researchers, Zhao et al and Sang-Hyug Park to overcome the possible detrimental effect on the cells due to the heat generated by the calcium phosphate cement (CPC) during the setting process involved encapsulating the MSCs in self-dissolving alginate hydrogels prior to mixing them with the CPC. Though potentially promising, no in vivo proof of its performance was provided [15] [22]. Further, the in situ polymerization and cross-linking of some polymers is mediated via the use of radical initiators which employ photo polymerization techniques. Though such polymers can be used with cells, the necessity of an open defect to allow for penetration of light to the material can be a potential drawback in the clinical setting [42].

In the current study we have employed a fibrin thrombin based gel derived from human platelets. The gelling in addition to being temperature dependent only occurs after the two components, i.e. fibrin and thrombin are mixed together, giving the surgeon time and flexibility during the surgical procedure. Tisseel® (Baxter Biosciences, USA) is a commercially available preparation based on the same principle and is already approved for clinical use as an adhesive sealant in controlling bleeding during surgical procedures[43] . However, the commercially available product unlike that used in this study, is not suitable for tissue engineering applications as the high crosslinking density of the fibrin network prevents cell migration within the construct[44-46]. Moreover, the fibrin thrombin gel used in the current study can be an autologous preparation, which can be obtained easily and at no extra cost as well introduced into the defect during a single surgery, without any risk or concerns about disease transmission and immunogeneic reactions associated with allogeneic or xenogeneic preparations giving it a distinct advantage over commercial preparations. Further, unlike the commercially available fibrin glue, the fibrin thrombin gel is derived by activating the release of the native concentration of fibrin within the platelets with calcium chloride and thrombin. During the activation of the platelets, a myriad of growth factors are released which can potentially enhance bone regeneration and vascularization of the construct.

A study by Yamada et al involved the use of a similar autologously derived fibrin thrombin gel in conjunction with canine MSCs for repair of critical sized defects in dogs [47]. However, in their study, the gel in addition to being the delivery vehicle also doubled up as the scaffolding material. According to studies reported in literature, a pre formed solid matrix helps in better retention of the cells at the defect site, provides the cells with a substrate for bone deposition, acts as a filler at the defect site and in many cases, the osteoconductive and osteoinductive properties of the scaffold promote faster bone healing[18-21]. In fact, we have preliminary results which indicate that the calcium phosphate ceramics are necessary to generate bone in our system. In addition, reports in literature suggest that the use of micro particles in place of bigger granules increases the surface area available for cell attachments and calcium release, both of which can influence in vivo bone formation [48]. The micro particles can also easily adapt to the contour of the defect without compromising the use of the minimally invasive delivery route for the graft placement. Finally, as the gel is very soft, the ceramics aid in increasing the mechanical strength of the tissue engineered graft.

It can be argued that use of even smaller sized particles can further aid the placement of constructs via the minimally invasive approach. Though we have not tested within this system, according to literature evidence, at least in case of HA and TCP particles, 44 microns represents the lowest threshold below which bone marrow derived hMSC-ceramic combinations do not yield any bone in subcutaneous locations in nude mice at the two time points tested, i.e. 4 weeks and 10 weeks. However, though this provides an indication, it would be interesting to confirm the validity of these findings using particles of different sizes within our system [48, 49].

A study by Mankani et al described the use of single cell suspension of hMSCs in combination with 0.5-1mm HA/TCP particles and a mouse fibrinogen and thrombin mixture to generate bone in vivo [18]. Our study differed from this study with respect to the use of fibrin thrombin gel of human origin and smaller ceramics which aided the delivery of the construct into the defect site. Another salient feature of our study was the use of cell aggregates instead of single cells. We observed in the past that in vitro aggregation of the cells prior to in vivo administration, significantly improved the performance of the cells. Within this study, we observed no bone formation when unaggregated cells from the same donor were combined with the ceramics and gel and implanted in vivo. These results are in accordance with studies from other groups that have reported the beneficial effects of cell aggregation on the expression of osteogenic genes [23-25]. It is believed that the behavior of the cells within the aggregate can be influenced by the improved integrin specific signaling as a result of the better cross talk of the cells within the aggregate [50, 51]. In addition to the cell-cell signaling,

culturing cells as aggregates also improves the interaction of the cells with the extra cellular matrix (ECM) which in addition to functioning as an adhesive substrate also acts as a reservoir of growth factors which play a role in maintaining the differentiation potential of the cells [52]. Surprisingly, our results indicate that although the initial aggregation was beneficial for in vivo bone formation, neither the expression of the osteogenic genes nor the amount of bone obtained rose proportionately to an increase in the in vitro culture duration of the cell aggregates. One of the possible explanations was the increase cell death within the aggregates over time. This can be improved via either generation of smaller aggregates or via use of dynamic culturing techniques which will ensure a better nutrient supply to the cells in the core of the aggregates.

In conclusion, we describe here a clinically applicable system using human MSCs for generation of tissue engineered constructs for delivery via a minimally invasive route. Future studies aimed at scaling up the size of the implants, implantations at orthotopic sites in immunocompetent animals and investigations into the mechanical properties of the newly deposited bone in the minimally invasive approach compared to the conventional surgically placed implants can all prove beneficial in clinical translation of this technique. Possibilities to replace the need for aggregation of expanded MSCs with aggregation of the mononuclear fraction obtained directly from a fresh bone marrow or fat tissue may be of interest to further streamline the technique for clinical applications.

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Chapter 6

Suppression of the immune system as a critical step towards allogeneic bone tissue engineering

The surface marker profile of MSCs suggests that they can escape detection by the immune system of an allogeneic host. Thus, potentially allogeneic MSCs can be used without any immunosuppressant therapy, as an off-the-shelf replacement for autologous MSCs in bone tissue engineering applications. However, in our study we demonstrate, using immunohistological techniques that allogeneic MSCs mount a T and B cell mediated immune response resulting in absence of in vivo bone formation. Suppression of the host immune response using daily administration of an immunosuppressant FK506 is effective in preventing the immune attack on the allogeneic cells. In the immunosuppressed environment, the allogeneic MSCs are capable of generating bone in amounts similar to that of isogenic cells. However, in one of the allogeneic donors tested, the newly deposited bone was attacked by the host immune system, in spite of the continued administration of the immunosuppressant. This suggests that though using an immunosuppressant can potentially suppress the immune attack on the allogeneic MSCs, optimizing the dose of the immunosuppressant may be crucial to ensure MSC driven bone formation within the allogeneic environment.

6.1. Introduction

Bone tissue has a very good regenerative capacity and thus often bone defects heal without intervention [1, 2]. However, when the remodeling capacity of the bone is affected, as is the case in metabolic disorders, during infections or in case of large bone defects caused by severe trauma or surgery due to malformations or congenital disorders, surgical interventions are required [3, 4]. More than 2 million bone graft surgeries are performed every year [5]. Surgeons typically reconstruct these defects with autografts or allografts. Autografts are the gold standards as they provide the best clinical outcomes. However, limited amounts of tissue that can be harvested coupled with problems of morbidity at the donor site and increased medical costs associated with an additional surgery to obtain the bone graft are factors that make the search for alternative therapies necessary [6]. Allografts are more easily available, but are associated with a risk of disease transmission [7, 8]. Moreover, large structural allografts are poorly remodeled by the host and are consequently prone to fractures [9]. Surgeons attempt to overcome these problems by using scaffolds of synthetic or natural materials, which promote migration, proliferation and differentiation of cells. However, the success of such materials in repairing bone defects is limited as they lack the osteoinductive and osteogenic properties present in an autograft [10]. It has been suggested that the bone regenerative capacities of these materials can be improved by combining them with stem cells [11].The challenges that exist with the use of embryonic stem cells (ESCs) such as ethical concerns surrounding the derivation of the cells, tumorigenicity of the cells in vivo as well as the immune rejection of the cells following transplantation have led researchers to focus their attention on stem cells from adult sources such as the bone marrow [12] [13]. The propensity of these cells to adhere to tissue culture plastic permits their easy isolation from other marrow cells. They can differentiate into osteoblasts, chondrocytes, adipocytes, tenocytes, myoblasts as well as neural cells and are commonly referred to as multipotent mesenchymal stromal cells (MSCs) [6, 14]. They have extensive proliferative capacities and a small volume of bone marrow aspirate (10 to 20 ml) can be expanded in culture to generate billions of cells. It has been demonstrated that the MSCs retain their functionality after culture expansion and cryopreservation, thus increasing the possibility to use these cells in tissue engineering therapies [15]. Studies have already demonstrated the beneficial effects on both the quantity and quality of bone formation using MSCs in combination with scaffolds as compared to using the scaffolds by themselves [16].

However, providing an autologous mesenchymal stem cell construct in the clinical setting involves an invasive procedure for aspiration of bone marrow from the patient followed by several weeks for expansion and quality control testing prior to generation of the tissue engineered construct that can finally be re-implanted into the patient.

Moreover, it may sometimes be difficult to obtain a sufficient marrow sample as the cell yield from the marrow may be compromised due to factors such as chemotherapy, myelofibrosis or lipid storage diseases [17]. Another bottleneck in using autologous MSCs for bone tissue engineering approaches is the variation of the bone forming capacity of the MSCs isolated from different donors [18]. Currently there are no in vitro markers that can reliably predict the in vivo bone forming capacity of a particular donor [19]. These problems associated with using autologous MSCs for generation of tissue engineered constructs (TECs) opens up the possibility of replacing the autologous MSCs with allogeneic MSCs. It would be extremely convenient for both the patient and the surgeon if bone marrow could be collected from another patient, MSCs isolated and tested and then cryopreserved for later use as an off-the-shelf therapy. However for such therapies to be of clinical use it is necessary to determine the safety of allogeneic MSCs in recipients who are unrelated and thus HLA mismatched.

MSCs express low levels of MHC class I and lack MHC class II, thereby having the potential to escape detection by the host immune system. In addition, MSCs do not express the CD40, CD80 or CD86 co-stimulatory molecules that are required to stimulate the T cell response. MSCs are also known to suppress lymphocyte alloreactivity in vivo, by directly inhibiting CD3+CD4+ T cell proliferation, and secretion of TH1 lymphokines, such as IL-2 and IFN-γ [20-22]. Taken together these features suggest that MSCs are poorly immunogenic and can be transplanted in an allogeneic host without rejection and without the need for additional immunosuppressive therapy [23, 24]. These immunomodulatory and immunosuppressive properties probably contributed to the success in using allogeneic MSCs from third party HLA mismatched donors for treatment of acute graft-versus-host-disease (GVHD) [25-27]. Similarly allogeneic MSCs were used from HLA identical siblings for treatment of osteogenesis imperfecta [28], metachromatic leukodystrophy and Hurler syndrome [29] with therapeutic benefits and no adverse reactions.

It is worth noting that in all the studies where allogeneic MSCs were successfully used to obtain therapeutic benefits, non-differentiated MSCs were used. Bone tissue engineering involves directing the MSCs into the osteogenic lineage to heal bone defects. Thus, one of the concerns about using MSCs in bone tissue engineering is whether the immunogenicity of the cells changes during the process of bone formation through osteogenic differentiation in vivo [30]. While there are only a few reports in literature where allogeneic MSCs were employed to heal bone defects, the results, both with respect to inducing an immunogenic response and generating bone are conflicting. The allogeneic MSCs were shown to be immunogenic in goats, mice

and rats while non-immunogenic in dogs. Interestingly, in the goat study, the severity of the immune response was even reported to correlate with bone formation [9, 31-33]. Further, though MSCs of animal origin have superior bone forming capacities in vivo, in vitro differentiation using compounds known to stimulate osteogenic differentiation has often been used by researchers as a strategy to overcome the poor bone forming capacity of human MSCs in vivo [34]. In spite of that, in none of the reported studies using allogeneic MSCs for bone tissue engineering, the cells were exposed to compounds known to induce osteogenic differentiation prior to implantation. The osteogenic differentiation during pre-culture could potentially make the MSCs immunogenic at an earlier stage, thereby preventing them from generating bone.

The knowledge regarding the immunogenic potential of osteogenically differentiated allogeneic MSCs, irrespective of whether the differentiation is initiated in vitro or in vivo, is very crucial as it may affect the feasibility of subsequent revision surgeries using tissue engineered constructs employing allogeneic MSCs. Therefore, the purpose of the present study was to determine whether allogeneic MSCs cultured in osteogenic differentiation medium can escape detection by the immune system and induce bone formation. A rat ectopic implantation model was chosen, in which we monitored T and B cell presence in the constructs. After having demonstrated that allogeneic MSCs activated both the T and B cells, leading to a widespread immune response with no bone formation, we studied the effectivity of employing an immunosuppressant, FK-506, currently also used in organ transplantations, in prevention of in vivo rejection of the osteogenically differentiated allogeneic MSC and on subsequent bone formation. FK-506 inhibits the production of T cell derived soluble mediators such as interleukin 2 and 3 as well as interferon gamma. Further it blocks mast cell, basophil, neutrophil as well cytotoxic T lymphocyte degranulation, thus providing potent humoral and cellular immunity [32]

6.2. Materials and methods

6.2.1. Isolation and culture of mesenchymal stromal cells from the rat bone marrow

Marrow cells were obtained from the femoral shafts of 6 week old male inbred Fischer 344 (F344/NCrHsd) (F1 and F2) or Wistar rats (HsdOla: WI) (W1 and W2). As the Fischer 344 rats are inbred, the major histocompatibility complex of all Fischer 344 rats is RT11v1. Thus, cells from one Fischer 344 rat can be used as syngeneic cells in another F344 rat. The HsdOla: WI is an outbred Wistar strain. The genetic constitution of the outbred rats is not known. This permits the study of allogeneic

cells implanted in a wide range of possible histocompatibility mismatches. Further, MSCs from both Wistar and Fischer 344 rats have been demonstrated to have in vivo bone forming capacities [35, 36]. To isolate the bone marrow from the rats, both ends of the rat femurs were cut at the epiphysis and the marrow was flushed out using 10 ml of culture medium expelled from a syringe through a 20 gauge needle according to a previously described protocol by Maniatopoulos et al [37]. The cells from each femur were collected in two T-75 flasks and cultured in rat proliferation medium consisting of α-minimal essential medium (αMEM, Gibco) supplemented with 15% fetal bovine serum (FBS; Lonza), 0.2 mM ascorbic acid (Sigma), 2 mM L-glutamine (Gibco) and 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 1 ng/ml basic fibroblast growth factor (bFGF, Instruchemie, The Netherlands). Cultures were maintained in a humidified atmosphere at 37ºC with 5% CO2. The medium was changed after 72 hours to remove the non-adherent cells. Subsequently the medium was refreshed twice weekly. On reaching near confluency, the cells were trypsinised and used for further sub-culturing or cryopreserved for future use.

6.2.2. Mineralization and adipogenesis

To test the in vitro multilineage differentiation potential of the rat MSCs, the cells were subjected to osteogenic and adipogenic differentiation. Experiments were performed in triplicate using rat proliferation medium without addition of basic fibroblast growth factor (basic medium) as the negative control. For mineralization, von Kossa staining was performed. Briefly, hMSCs were seeded at a density of 5000 cells/cm2 in T25 flasks for 21 days and cultured in mineralization medium consisting of basic medium supplemented with 0.01 M β-glycerol phosphate (Sigma, 50020) and10-8 M dexamethasone (Sigma, D8893). After 21 days, the cells were fixed with 4% paraformaldehyde (Merck, 104005) for four hours at RT, rinsed with tap water and stained with 5% AgNO3 (Sigma, S-6506) in demineralized water for about 15 seconds and finally exposed to mild UV light for 10 seconds.

For adipogenesis, cells were seeded at 10,000 cells/cm2 and cultured in adipogenic medium for 21 days. The adipogenic medium consisted of DMEM (Gibco, 41-965-062), supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco, 15140-122), 10% fetal bovine serum (Lonza), 0.2 mM indomethacin (Sigma, 57413), 0.5 mM IBMX (Sigma, I5879), 10-6 M dexamethasone (Sigma, D8893) and 10 mg/ml insulin (human, Sigma, I9278). Medium was refreshed twice weekly and lipid formation was visualized using Oil Red O staining (Sigma). This involved fixing the cells in 4% paraformaldehyde (Merck, 104005) for 4 hours at room temperature, followed by incubation for 5 min in 60% isopropanol and staining with a freshly

filtered Oil Red O solution (stock: 500 mg Oil Red O (Sigma, O0625), 99 ml isopropanol, 1 ml water; stain: 42 ml stock + 28 ml water).

6.2.3. Generation of the syngeneic and allogeneic constructs for in vivo implantation

For the in vivo implantations in rats, 6 week old inbred Fischer 344 rats (F344/NCrHsd) were used as recipients. Thus, the constructs generated using the inbred Fischer 344 marrow cells and the outbred Wistar marrow cells were termed isografts and allografts respectively. To generate the constructs, 200,000 expanded rat MSCs were gently dispersed over each calcium phosphate scaffolds (kindly provided by Dr Huipin Yuan, University of Twente, The Netherlands). A total of 3 scaffolds were used per condition. After 4 h, 2 ml osteogenic medium was slowly added to each set of three scaffolds. The osteogenic medium consisted of α-MEM (Gibco) supplemented with 15% fetal bovine serum (FBS; Lonza), 0.2 mM ascorbic acid (Sigma), 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 10−8 M dexamethasone (Sigma). The cell scaffold constructs were cultured for a total period of 4 days in a humidified atmosphere at 37ºC with 5% CO2. The BCP scaffolds used for generating the constructs were produced according to the H2O2 method, including naphthalene as described previously [38].The material was sintered at 1300 ◦C. The average size of the granules was 2–3 mm, with the specific surface area being 0.2 m2/g. The composition of the particles was 20TCP/80HA. The microporosity (volume percentage of micropores <10 μm within the ceramic) was 8.7%, while the calcium release was 4.2 ± 0.4 ppm.

6.2.4. In vivo studies

To assess the bone forming capacity of the bone marrow derived rat mesenchymal stromal cells (MSCs), MSC ceramic constructs were generated using F1, F2, W1 and W2 cells and implanted in parallel in the subcutaneous pockets on the dorsum of 6 nude mice ((Hsd-cpb: NMRI-nu, Harlan) for a total period of 6 weeks. After confirming the bone forming capacity of the F1, F2, W1 and W2 cells, constructs using the same cells were then implanted in subcutaneous pockets on the dorsum of immunocompetent Fisher 344 rats.

In the first study, 24 immunocompetent Fisher 344 rats (F344/NCrHsd) were used for the constructs generated using the F1 and W1 donors. Of these 24 rats, 12 rats received an immunosuppressant FK 506 (Tacrolimus 5 mg/ml , (Astellas Pharma B.V, Leiderdorp, The Netherlands) in a dose of 1 mg/kg daily intramuscularly (i.m.) for the entire duration of implantation while the other 12 received the same amount of saline via i.m. injections. The dose of 1 mg/kg was chosen based on organ transplantation

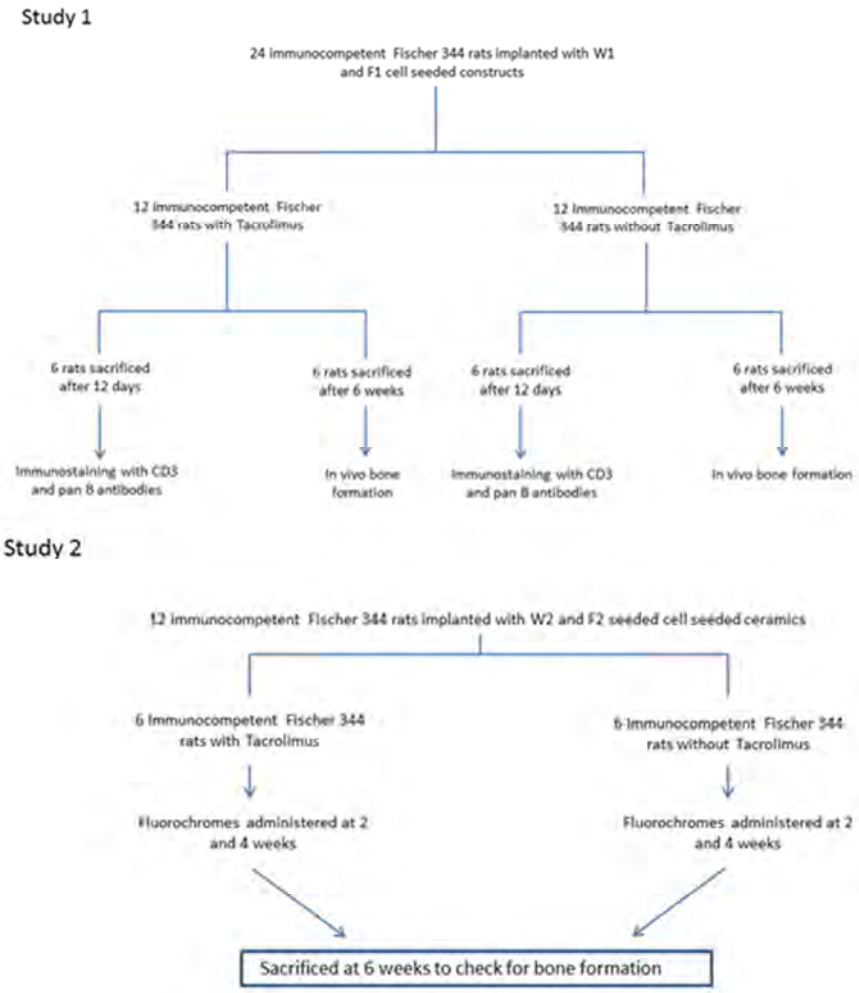

Figure 6.1. Schematic representation of the in vivo set up
A schematic representation of the implantation scheme.

studies in rats showing no side effects and positive therapeutic outcome [32, 39, 40]. Of these 24 rats, 6 from the immunosuppressed group and 6 from the saline group were sacrificed after 12 days to analyze the immune response while the remaining 12 rats were sacrificed after 6 weeks to measure bone formation. Two randomly selected rats from the immunosuppressed and non-immunosuppressed groups also received cell-free control ceramics.

In the second study, constructs generated using MSCs from F2 and W2 were implanted subcutaneously in 12 immunocompetent Fischer 344 rats. In this study, the dynamics of bone apposition between the syngeneic and the allogeneic constructs was

determined using sequential fluorochrome labels infused at 2 weeks (Calceine green in 2% NaHCO3, 10 mg/kg s.c., Sigma Aldrich, Zwijndrecht, The Netherlands) and 4 weeks (Xylenol orange in 1% NaHCO3, 100 mg/kg i.v. ,Sigma Aldrich, Zwijndrecht, The Netherlands). Immunosuppression was induced in 6 of the 12 rats using FK 506 (Astellas Pharma B.V, Leiderdorp, The Netherlands) at the dose of 1 mg/kg daily i.m. for 6 weeks. The other 6 rats received i.m. saline injections of the same volume. All these 12 rats were sacrificed after 6 weeks.

Both mice and rats were anesthetized by inhalation of isoflurane. The incisions were closed in both animals using a vicryl 5-0 suture. The experiments were approved by the local animal experimental committee. The animals were sacrificed using carbon dioxide and samples were explanted.

A flowchart representing the scheme of the in vivo implantation is described in Fig. 6.1.

6.2.5. Characterization of the immune response

All implants retrieved after 12 days in vivo were fixed in 4% paraformaldehyde, embedded in agarose, decalcified using 12.5% EDTA and dehydrated using sequential ethanol series [70%, 80%, 90%, 96%, and 100% (v/v), 1 hour in each]. Once dehydrated, they were incubated in butanol overnight and then in a solution containing butanol and paraffin (50:50) for 8 hours. Ultimately, the scaffolds were embedded in paraffin and 3 μm thick sections were cut using a microtome. The samples were then either subjected to immunohistochemistry using a T cell (CD3 Polyclonal rabbit Anti-Human, Dako), and B-cells (pan) (Monoclonal mouse anti rat B-Cells, clone Ki-B1R, Acris) antibody or stained with eosin and haematoxylin (Sigma-Aldrich). Rat spleen and staining with secondary antibody were used as controls. For staining with CD3, antigen retrieval was performed using a 1:50 dilution of the Target Retrieval Solution (TRS), pH 9 (EnVisionTMFlex, Dako) as per the protocol recommended by the manufacturer. No antigen retrieval was required for the pan B cell antibody. The endogenous peroxidase activity for all stainings was blocked using hydrogen peroxide (EnVisionTMFlex, Dako) for 5 minutes. Primary antibody incubation was performed for 20 minutes with 1:200 dilution of the CD 3 antibody and 1:50 dilution of the pan B cell antibody in PBS. This was followed by sequential incubations with the contents of EnVisionTMFlex mini kit (Dako), as per the manufacturer's recommendations. For the Pan B antibody EnVision™ FLEX+ Mouse (LINKER) from Dako was used for optimal signal amplification. Finally

haematoxylin (EnVisionTMFlex, Dako) was used to counterstain the slides. The slides were visualized with a light microscope (Leica).

6.2.6. Histology and histomorphometry of the explanted samples

All implants retrieved after 6 weeks in vivo were fixed in 4% paraformaldehyde (Merck chemicals, The Netherlands), dehydrated within an industrial microwave using JFC solution (Leica Microsystems, Rijswijk, The Netherlands) and transferred into a methylmethacrylate (MMA) solution (L.T.I, Bilthoven, The Netherlands) that polymerized at 37ºC within 3 days. Sections having an approximate thickness of 10 to 15 μm were made by using the modified interlocked diamond saw (Leica Microtome, Nussloch, Germany). Sections were stained either with 1% methylene blue and 0.3% basic fuchsin after etching with HCl/ethanol mixture for routine histology and histomorphometry or left unstained for epifluorescence microscopy with a light microscope (LM; E600, Nikon, Japan) equipped with a quadruple filter block (XF57, dichroic mirror 400, 485, 558 and 640 nm, Omega Filters, Didam, The Netherlands).

Histological sections from MMA embedded samples were qualitatively analyzed by light microscopy (Leica), and each histological section was scored either positive or negative for bone formation. In addition, quantitative histomorphometry was performed. Briefly, high-resolution digital photographs were made from three randomly selected sections from each tissue-engineered graft. Before histomorphometrical analysis, newly formed bone as well as the material were manually pseudocoloured green and red, respectively using Photoshop CS2 (Adobe Systems). A custom-made Matlab script was used to measure the ratio of bone to the scaffold area.

6.2.7. Statistics

Statistical analysis for implants tested within the same rodent was performed using a Student's paired t-test. For samples that were tested in different rodents, an unpaired t test was used. Finally, in the study when the amount of bone formed in one group was compared to another group which generated no bone, a one sample t-test was used to determine if the column means varied significantly from a hypothetical value which was considered as 0 in our case. In all conditions, a p value less than 0.05 was considered significant.

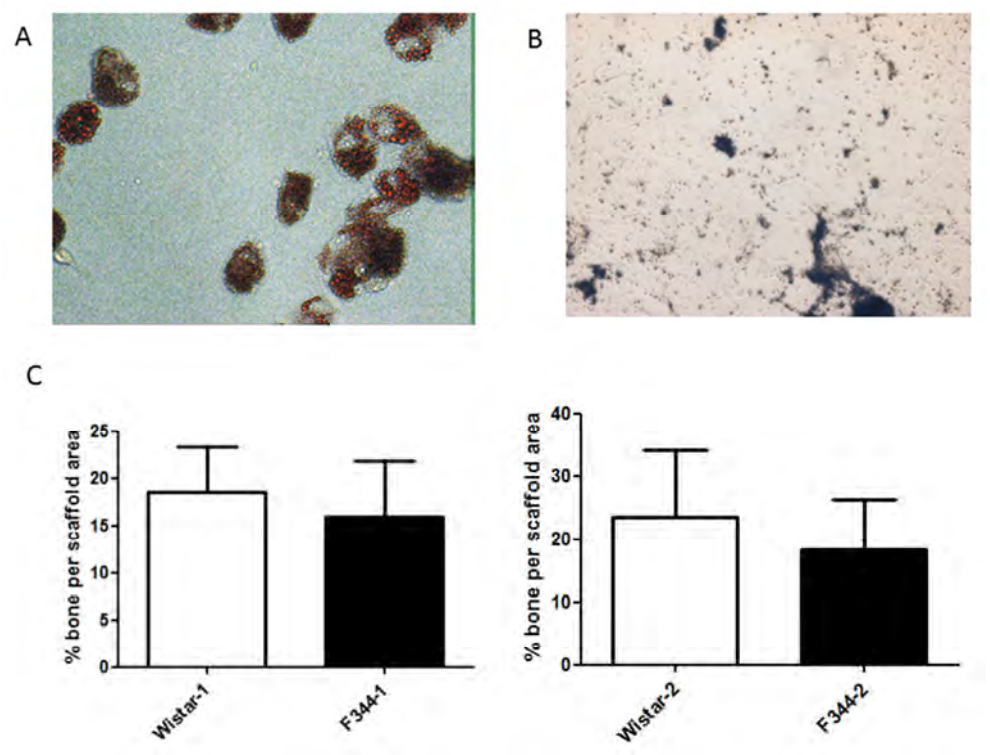

Figure 6.2. In vitro and in vivo quality assessment of the rat MSCs

In vitro multilineage differentiation potential of the rat MSCs was confirmed by subjecting them to mineralization and adipogenic assays. Staining with silver nitrate solution (von Kossa staining) revealed presence of black mineralized nodules (Fig 6.2B) while the Oil Red O stained the lipid droplets within the cells, which is suggestive of adipogenic differentiation (Fig 6.2A) (Magnification used 20X). In vivo bone formation by the MSCs from the Wistar and Fischer 344 rats (Fig 2C). Statistical significance was calculated using Student's paired T-Test. P<0.05% was considered significant.

6.3. Results

6.3.1. In vitro and in vivo testing of the MSCs isolated from the Wistar and Fischer rats

The multilineage differentiation capacity of the rat MSCs isolated from the bone marrow of 2 in-bred Fischer 344 (F1 and F2) and 2 outbred Wistar rats (W1 and W2) was tested using mineralization (Fig. 6.2A) and adipogenic assays (Fig. 6.2B). Further, the in vivo bone forming capacity of the cells was determined by seeding the cells on ceramic particles and culturing them for 5 days in an osteogenic medium prior to implanting them in the subcutaneous space on the dorsum of nude mice. Bone tissue

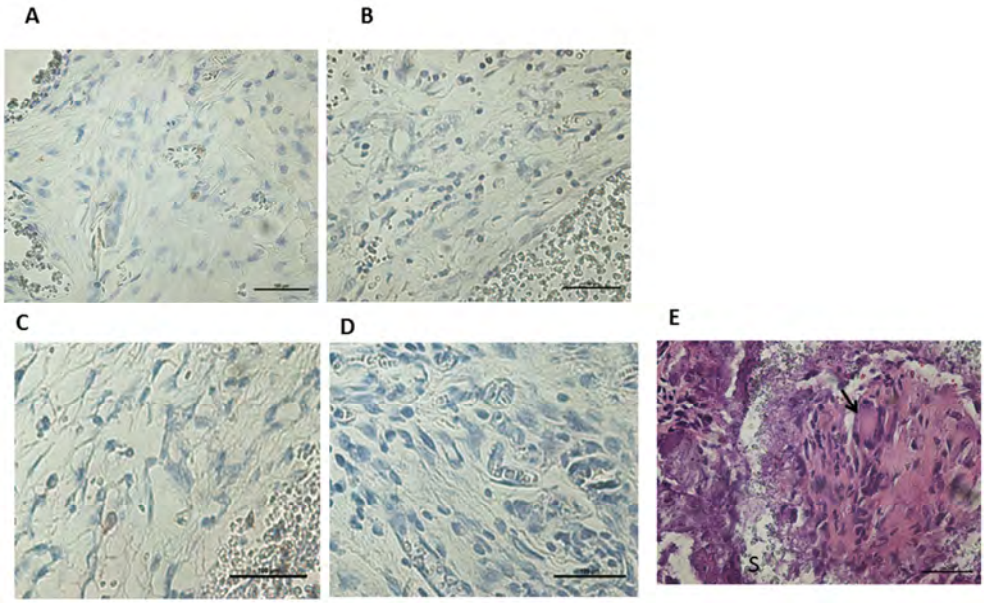

Figure 6.3. Immunohistological staining in cell-free ceramics after 12 days in the host rats with and without immunosuppressants

No evidence of a T or B cell mediated immune response was noted in the empty ceramics after 12 days, irrespective of the administration of immunosuppressants. CD3 staining in rats without immunosuppressant (A), pan B staining in rats without immunosuppressant (B), CD3 staining in rats with immunosuppressant (C) and Pan B staining in rats with immunosuppressant (D). Giant cells (marked with a black arrow) were observed with cell free ceramics, suggestive of an inflammatory reaction to the ceramics (E) (original magnification 20X).

was observed in the ceramic pores of all the 4 donors, in all the animals tested, after 6 weeks of in vivo implantation. The Wistar rats from both donors generated greater amounts of bone than the Fischer rats, though in both cases, the difference was not significant (Fig. 6.2C). More specifically, 18.5 and 23.5% of the total scaffold area was covered with newly formed bone with the MSCs from the first and second Wistar rat respectively while the corresponding values with the Fischer 344 rats was 15.9% and 18.4% respectively. This indicated that in an immunocompromised environment, bone marrow derived MSCs isolated from the both rat strains have a comparable potential to generate new bone.

6.3.2. Empty ceramics do not induce a T and B cell mediated immune response

To determine the immune response elicited by the ceramic component of the tissue engineered constructs, BCP ceramics without cells were cultured in osteogenic medium for 5 days and then implanted into the subcutaneous pocket of two randomly

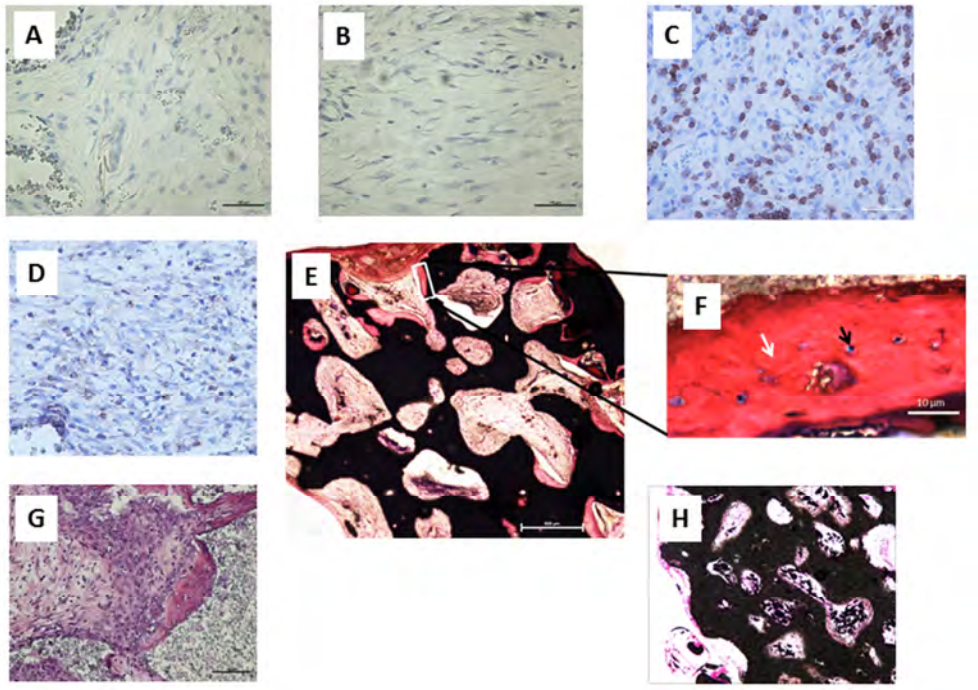

Figure 6.4. Immunohistological staining in vivo in an immunocompetent host
Absence of positively stained cells with CD3 (A) and pan B antibody (B) within the syngeneic constructs generated using the F1 cells. Presence of brown positive staining with CD3 (C) and pan B antibody (D) within the constructs seeded with allogeneic MSCs (W1) (magnification 20X). Methylene blue and basic fuschin staining of the syngeneic constructs (F1) after 6 weeks of in vivo implantation (E) (magnification 20X). A 40X magnified view demonstrates the presence of osteocytes (black arrow) embedded with the matrix (white arrow) (F). A few bony areas were already observed in the H&E stained sections, after 12 days of in vivo implantation. The white arrow points to the newly deposited matrix while S indicates the ghost of the ceramic, which was removed by decalcification (G). No evidence of bone was observed in the allogeneic constructs (seeded with W2 cells) even after 6 weeks of in vivo implantation (H).

selected immunosuppressed and non-immunosuppressed Fischer 344 rat. No evidence of T and B cell mediated immune response or new bone deposition was observed in the ceramics from either the immunosuppressed (Fig. 6.3A, 3B) or non-immunosuppressed animals (Fig. 6.3C, 3D). However multinucleated giant cells were observed in the H&E stained sections suggesting that the empty ceramics induce a foreign body reaction within the recipient animals (Fig. 6.3E).

6.3.3. MSCs elicit an immune response in an immunocompetent allogeneic host

Rat MSCs from the bone marrow of Wistar (W1) and Fischer 344 rats (F1) were seeded on the ceramics, cultured for 5 days in osteogenic medium and implanted in

subcutaneous pockets of immunocompetent Fischer 344 rats for 12 days to compare the immune response elicited by the tissue engineered constructs in a syngeneic or allogeneic setting. The constructs generated using Wistar cells and implanted into the Fischer 344 rats were the allogeneic constructs while those generated using Fischer 344 cells and implanted in the Fischer 344 rats were considered as syngeneic constructs. After 12 days of in vivo implantation, the tissue engineered constructs were decalcified, embedded in paraffin and sectioned. The sections were subjected to immunostaining with an anti-CD3 antibody which recognises its epitope on all mature T cells that play a role in cell mediated immunity and a pan B cell antibody which is present on all B cells and plasma cells, which in turn are mediators of humoral immunity. While the syngeneic constructs generated using the F1 cells did not stain positive for either CD3 antibody (Fig. 6.4A) or the pan B cell antibody (Fig. 6.4B), the allogeneic constructs generated using W1 cells showed a strongly positive staining with CD3 (Fig. 6.4C) and pan B antibodies (Fig. 6.4D). This indicated that the allogeneic MSCs are recognised as foreign by the host immune system, resulting in a T and B cell mediated immune response.

6.3.4. Immune response is associated with absence of bone in vivo

MSCs from two Wistar (W1 and W2) and two Fischer 344 rats (F1 and F2) seeded on BCP ceramics and cultured in osteogenic medium for 5 days were implanted in the subcutaneous pockets of Fischer 344 rats. All syngeneic constructs generated using the F1 and F2 donors generated bone after 6 weeks of in vivo implantation (Fig. 6.4E, F). This indicated that the syngeneic MSCs were capable of in vivo bone formation. In many of the constructs, bone formation was already observed after 12 days of in vivo implantation (Fig. 6.4G). However, no evidence of any new bone formation was observed in the constructs generated using the allogeneic W1 and W2 MSCs even after 6 weeks (Fig. 6.4H). As noted in section 3.2, the constructs using the allogeneic MSCs had elicited a T and B cell mediated immune response. These results thus indicate that the allogeneic MSCs are recognised and attacked by the host immune system, prior to the deposition of bone by these cells, resulting in absence of in vivo bone formation.

6.3.5. Administration of immunosuppressant effectively blocks the T and B cell recruitment

To determine the possibility of suppressing the immune response elicited by the allogeneic MSCs, FK 506, an immunosuppressant widely used in organ transplantations, was administered daily via the intramuscular route to the Fischer 344 rats. After 12 days of in vivo implantation, the constructs generated using the Wistar

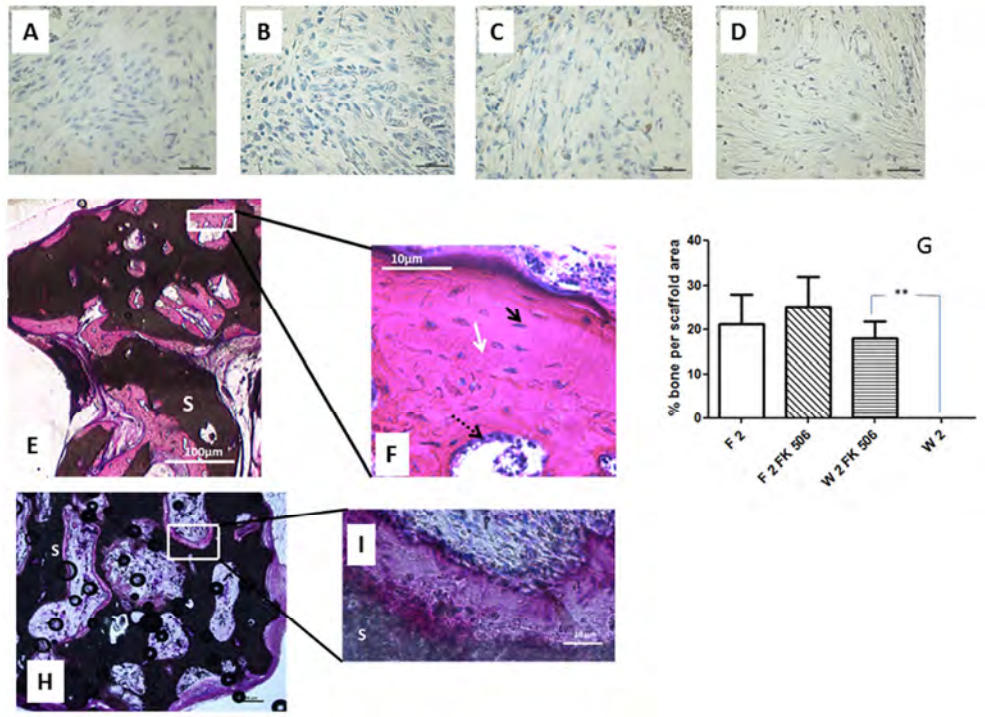

Figure 6.5. Immunohistological staining in host rats receiving daily doses of the immunosuppressant FK-506

Absence of cells positively stained with CD3 and pan B antibody within the syngeneic constructs generated with F1 cells and the constructs generated using the allogeneic W1 MSCs shown in (A), (B), (C) and (D) respectively (magnification 20X). Methylene blue and basic fuschin staining of the constructs implanted with the syngeneic Fischer 344 cells (F1 cells). (E) A 40x magnified representative image of the bone formed, demonstrates the osteocytes (black arrow) embedded in the matrix (white arrow) while the dotted black arrow represents the bone lining cells (F). Comparison of the amount of bone formed by the syngeneic and allogeneic MSCs in the presence or absence of immunosuppressant (G) Methylene blue and basic fuschin staining of the tissue engineered constructs seeded with the allogeneic W1 cells (H) On 40X magnification, no evidence of osteocytes or bone lining cells are seen (I). S represents the scaffold material in all the images. Statistical significance compared using unpaired t-test. P< 0.05% considered significant.

(W1) as well as Fischer 344 cells (F1) were explanted and analysed for the presence of the immune response using the CD3 and pan B cell antibodies. As observed previously, no positive staining was observed with the CD3 (Fig. 6.5A) or pan B marker (Fig. 6.5B) in the constructs generated using the F1 cells. However, a drastic reduction or complete absence of both the T (Fig. 6.5C) and B cell (Fig. 6.5D) response in the constructs generated using the W1 cells and explanted from all the Fischer 344 hosts suggested that the immunosuppressant did effectively block the immune response associated with the allogeneic cells.

6.3.6. Allogeneic MSCs can generate bone within an immunosuppressed milieu

To determine if the suppression of the cellular and adaptive immunity was a possible intervention to permit the use of allogeneic MSCs for bone tissue engineering purposes, the immunosuppressant FK 506 was administered i.m. daily for 6 weeks to the rats receiving the constructs generated using the syngeneic (F1 and F2 cells) as well as the allogeneic cells (W1 and W2 cells). The ceramic constructs seeded with the syngeneic cells from both the Fischer 344 donors (F1 and F2) generated new bone (Fig. 6.5E, F). Further, the administration of immunosuppressant also proved to be a successful strategy to permit good bone formation by the allogeneic constructs generated using the W2 cells. While no bone was generated using allogeneic cells in the absence of FK 506, an average of 17.9% bone was observed when these allogeneic constructs were implanted in the presence of immunosuppressant. On statistical evaluation this difference was found to be significant (Fig. 6.5G). The syngeneic constructs generated using F2 cells implanted in the same recipient F344 rats generated an average of 25% new bone. On statistical evaluation, the difference in the amount of bone generated using the allogeneic and syngeneic MSCs was insignificant (Fig. 6.5G). Further, a statistically comparable amount of bone was generated using the syngeneic cells in the presence or absence of FK 506. More specifically, the average amounts of bone in the constructs with no exposure to FK 506 was 21%, while 24% of the constructs was on an average filled with bone in the rats that were not exposed to the drug (Fig. 6.5G). These results suggest that FK 506 does not directly influence bone formation.

In contrast to the findings of the allogeneic constructs generated using the W2 cells, in the allogeneic constructs generated using the W1 cells, a deposition was observed in close contact with the ceramic pores (Fig. 6.5H). Though the location as well as the gross appearance of the deposit was suggestive of bone, closer histological examination, under higher magnification, revealed the absence of bone lining cells, osteoblasts and osteoclasts (Fig. 6.5I). We propose that an immune attack on the newly formed bone has occurred resulting in destruction of the cellular component, and a cell free mineralized matrix as a consequence.

6.3.7. Dynamics of bone deposition between allogeneic and isogeneic constructs in immunosuppressed rats

To determine if the initiation and progression of bone deposition varied between the syngeneic and allogeneic constructs in the immunosuppressed animals, we performed a fluorochrome study, a well-accepted technique to study bone deposition and the

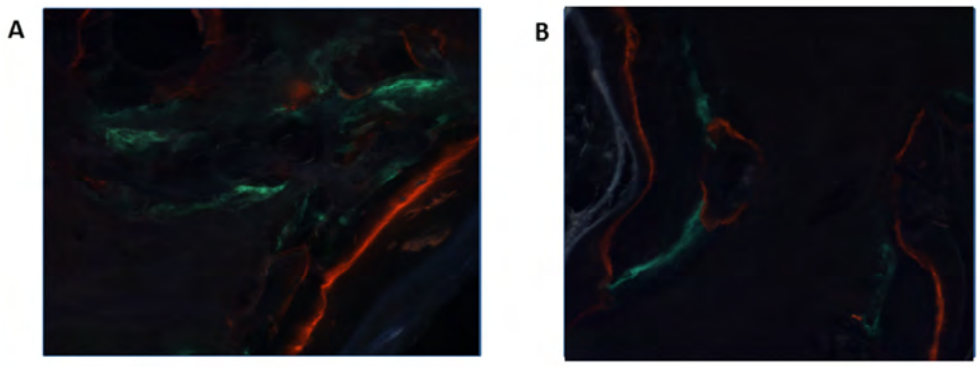

Figure 6.6. Fluorochrome administration at 2 weeks and 4 weeks in the immunocompetent rat implanted with syngeneic and allogeneic grafts

Calcein green in a dose of 10 mg/kg and xylenol orange in a dose of 100 mg/kg were administered subcutaneously to the immunocompetent rats in the second study, at 2 weeks and 4 weeks respectively. Presence of the green label as well as the orange label in the allogeneic grafts (W2) ***(A)*** *as well as syngeneic grafts (F2)* ***(B)*** *suggest that in both cases bone formation commenced before 2 weeks and continued after 4 weeks (magnification 10X).*

dynamics of bone remodelling [41]. W2 and F2 cells were seeded and cultured on ceramics for 5 days in osteogenic medium. These constructs were implanted in the subcutaneous pockets of immunocompetent Fischer 344 rats for a period of 6 weeks. The rats received daily injections of FK 506 for the entire 6 weeks duration. Calcein green was the fluorochrome marker which was administered as a single dose after 2 weeks while xylenol orange was the second fluorochrome administered as a single dose at 4 weeks after the start of the in vivo implantation. The results obtained on fluorochrome analysis demonstrated that in both syngeneic and allogeneic constructs, bone formation started before 2 weeks and continued even after 4 weeks (Fig. 6.6A, B). This indicated that the allogeneic environment did not affect the dynamics of bone deposition.

6.4. Discussion

Allogeneic MSCs have been reported to suppress MHC mismatched T cell responses in vitro [21, 42-44] and are considered largely non-immunogenic, given their low levels of MHC class I expression and a lack of MHC class II expression. Allogeneic MSCs have also been used successfully in animal models of multiple sclerosis, rheumatoid arthritis, autoimmune encephalitis and type I diabetes [45-47] as well as clinically to treat conditions such as graft-versus-host disease [26, 48]. However, there are also reports suggesting that MSCs are susceptible to immune rejection when administered to adult MHC mismatched recipients with intact immune systems [49-52]. One of the explanations put forth for these discrepancies is that allogeneic MSCs can be used for

the treatment of diseases involving activation of the T helper type 1 responses (Th1) but not for T helper type 2 (Th2) and B cell responses [53]. Further, other reports suggest that the site of administration of the MSCs is an important determinant of the allogeneic MSC immunogenicity. Sites such as intracranial, intracerebral and implantation into skin wounds appear to be non-immunogenic for MSCs while intraperitoneal, intravenous, subcutaneous and intramyocardial are associated with detectable anti donor immunity and sometimes even rejection [30]. In view of all these reports and findings, it appears that each disease setting is unique and use of allogeneic MSCs for a particular clinical conditions needs testing in in vivo systems to evaluate more accurately the ultimate impact of MSC administration in the context of that disease condition. In this study we demonstrate using histological means that for the purpose of bone tissue engineering, tissue engineered grafts generated using rat allogeneic MSCs elicit a host T and B cell mediated immune response, resulting in destruction of the implanted cells and absence of in vivo bone formation.

Allogeneic MSCs have been used in a limited number of studies for the purpose of bone tissue engineering. However, the data obtained from these studies also demonstrate conflicting results. Our results convincingly demonstrate a substantial T and B cell infiltration in the constructs generated using allogeneic MSCs. These results were comparable to that obtained by Sempuku et al in a rat study using allogeneic bone marrow in combination with hydroxyapatite discs [32]. No bone was obtained in these constructs while grafts generated using syngeneic bone marrow reproducibly generated bone. Though no tests to identify the nature of the immune response were performed, the results were strongly suggestive of an attack on the allogeneic cells by the host immune system before they could contribute to bone formation. Tasso et al implanted allogeneic MSCs obtained from green fluorescent protein (GFP) labelled transgenic mice and observed a rapid destruction of these cells as compared to the syngeneic cells. Further while the syngeneic cells generated bone, no bone was obtained using the allogeneic cells [33]. In contrast, Arinzeh et al loaded allogeneic MSCs on hydroxyapatite-tricalcium phosphate implants in an attempt to repair a critical-sized segmental defect in the canine femur [9]. Histologically, no lymphocytic infiltration occurred and no antibodies against allogeneic cells were detected in this study. Bone formation comparable to the constructs with autologous cells was detected 16 weeks after implantation. In another in vivo study performed in goats for bone tissue engineering purposes, we noted lymphoid clusters in constructs generated using goat allogeneic MSCs seeded on BCP scaffolds and implanted in the para-spinal muscles of unrelated goats of the same strain [31]. These clusters were absent in the constructs generated using autologous goat MSCs. Interestingly unlike our results, in this study, the inflammatory response induced by the allogeneic cells was positively

correlated to the amount of newly formed bone. It is worth noting that except in our study, the allogeneic MSCs were not subjected in vitro to compounds known to influence their differentiation potential. In an in vivo setting, the differentiated MSCs may have contributed to a stronger activation of the immune system, resulting in a faster clearance of the cells from the host system. As, unlike with MSCs from other mammals, human MSCs appear to have a lower efficacy for bone formation in vivo [3, 54, 55], for clinical applications, an in vitro pre-differentiation of the cells is often necessary to improve their in vivo bone forming potential [34, 56].

The effect of in vitro differentiation on the in vivo immunosuppressive potential has also been studied by other researchers [33, 57]. In an in vitro study performed by Le blanc et al, it was demonstrated that the immunoprevileged status of the MSCs are retained even after differentiation of the MSCs [58]. However, a recent study by Huang et al demonstrated that exposure of MSCs to compounds such as 5-azacytidine for myogenic differentiation, increased the major histocompatibility complex-Ia and -II (immunogenic) expression and reduced major histocompatibility complex-Ib (immunosuppressive) expression, in association with increased cytotoxicity in co-culture with allogeneic leukocytes. Further, upon direct implantation into the infarcted cardiac muscle, the allogeneic (but not syngeneic) cells were eliminated from the heart by 5 weeks after implantation, and their functional benefits were lost within 5 months [59].

The inflamed milieu in a bone defect can, via up regulation of the proinflammatory mediator interferon ᵞ (IFN-ᵞ), enhance the expression of both MHC class I and class II on cells, rendering them susceptible to rejection in an immunocompetent host [60, 61]. Rafei et al, in a mouse model of experimental autoimmune encephalitis reported that although the native allogeneic MSCS had the potential to decrease the severity of the disease, stimulation with IFN-ᵞ led to complete immune rejection of the MSCs [62]. As bone tissue engineering is a clinical condition which requires implantation of MSCs in a bony defect, where the levels of pro-inflammatory factors are high [55], it is important to bear in mind, when designing future experiments, the possible effects of inflammation on the performance of the bone forming cells.

Further, as it was clinically interesting to know whether immunosuppressive therapies currently prescribed in organ transplantation could be effectively used to prevent anti donor immune response of the allogeneic cells, we tested the possibility of generating bone by administering FK 506, a commonly used immunosuppressant in transplant medicine [63-66].. Our results suggest that although FK 506 successfully blocked the T and B cell recruitment, no bone was formed with the MSCs from one of the allogeneic donors, though the donor had shown good bone forming capacity in the

nude mice. Thus, it is likely that MSCs from some allogeneic donors elicit a stronger immune response necessitating a higher dose or a polytherapy with immunosuppressants. Further, it was interesting to note that while the cells from the other allogeneic donor (W 2) had performed better than the syngeneic cells (F2) in the nude mice, within the immunosuppressed allogeneic rat setting, the allogeneic W2 cells generated lesser amounts of bone (17.9%) bone as compared to the 25% obtained by the isogeneic F2 rats cells (Fig. 6.2C, 5H). Though not statistically significant, this reversal in pattern may be suggestive of a negative effect of the allogeneic environment on the MSCs. It could indicate that though the immunosuppressant suppresses the immune response mediated via the T and B cells, other pathways might still contribute in modifying the survival and thus the inherent osteogenic capabilities of the allogeneic cells.

In our study, we have used daily administration of the immunosuppressant for the entire duration of the experiment. This may be an unacceptable option in a clinical setting, as there are several severe side-effects reported. In heart, lung, liver and small intestine allografts, a short term treatment with FK-506 has been reported to be sufficient for engraftment in an allogeneic host [40, 67]. While this is a possibility for bone tissue engineering approaches using allogeneic MSCs, in a previous report, intermittent administration of the immunosuppressant in association with grafts generated using whole rat bone marrow, implanted in allogeneic hosts, reported evidence of empty lacunae surrounded by cellular infiltration, suggestive of an immune attack following the discontinuation of the immunosuppressant after 2 weeks [32]. Further our own results suggest that the currently used dose possibly represents the lower limits, below which some allogeneic donors are capable of mounting an immune response. Nonetheless, it is reported in literature that the implanted MSCs recruit the host MSCs and over time there is formation of an intermediate chimeric tissue formed by cells of both donor and host origin. This is eventually replaced by new bone which is entirely of host origin. Studies have also suggested that the formation of this chimeric tissue can start anytime between 1 to 12 weeks depending on the species origin of the MSCs [33, 68]. Thus, it is possible that the 2 weeks used in previous studies was too early a time point to stop the immunosuppressant while the 6 weeks of low dose therapy used in our study was also not optimal. Thus, it may be interesting to attempt future studies to test the ideal combination of doses and durations of immunosuppressants to protect the implanted allogeneic MSCs for a window period during which they can initiate the bone formation of the host cells via paracrine effects. This may provide an attractive possibility of avoiding a lifelong dependency on immunosuppressants for clinical cases implanted with allogeneic MSCs for bone formation.

6.5. Conclusion

In conclusion, our study demonstrates that in an allogeneic environment rat MSCs are not intrinsically immunoprevileged or immunosuppressive. Instead, the immune reaction generated to the constructs employing the allogeneic cells is probably detrimental to the eventual bone formation by the differentiated MSCs. However, under appropriate immunosuppressant therapy, the allogeneic MSCs can survive and generate bone in vivo. In view of the contradictory results obtained in vivo with the different animal studies employing allogeneic MSCs for therapeutic applications, we suggest that the severity of immune responses determines eventual outcome with respect to bone regeneration. There exists a strong possibility that MSCs from different species exhibit different degrees of immune suppression in vivo and this in turn may determine the rate of clearance of the MSCs from the allogeneic host. To determine the degree of immune response elicited by allogeneic hMSCs for a bone tissue engineering purpose within an immunocompetent human, it is necessary to perform human clinical trials under supervision and possibly under appropriate immunosuppressant treatment. Use of approaches to track the fate of the implanted MSCs can greatly aid eventual clinical translation. Once the safety and efficacy of the treatment has been determined, it is indeed an option to taper both the number of allogeneic MSCs used as well as the dose of the immunosuppressant to finally strike a balance for the survival of the allogeneic MSCs for a window period necessary to achieve a therapeutic efficacy of the cells. Further, as the in vitro culture conditions, degree of in vitro differentiation, delivery route and the clinical condition for which the allogeneic MSCs are being used, can all determine the in vivo effects on cells, studies to determine the optimal conditions necessary to generate therapeutically relevant cells for a specific clinical situation are required.

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Chapter 7

A histological study to compare the inflammatory response and the bone healing capacity of porous β-tricalcium phosphate and hydroxyapatite within a critical sized orthotopic defect

We previously produced hydroxyapatite (HA) and beta tricalcium phosphate (TCP) porous ceramics without and with osteoinductive properties, respectively, demonstrated in ectopic as well as spinal fusion studies. The relation of the osteo-inductive behavior of ceramic material in such sites to the bone forming potential in an actual orthotopic environment is of interest for clinical applications. In this study, HA and TCP cylinders were implanted in a critical sized rat femoral defect to compare their tissue response and bone healing potential. Histological and fluorochrome-based analysis at multiple time points were used to compare the progression of inflammation and initiation of bone formation between the two ceramics. We observed that in both the ceramics, at 2 weeks post implantation, there was a strong inflammatory response present. However, by 4 weeks, bone formation had been initiated in both the types of ceramics and by 6 weeks inflammation had subsided. Interestingly, the known osteoinductive effect of TCP at an ectopic site also translated to a significantly better bone healing capacity within the orthotopic defect.

7.1. Introduction

Autograft bone is currently the gold standard for treating bone defects. Despite its excellent success rate and low risk of rejection and transmission of diseases, autografts are only available in limited quantities, and post-operative complications frequently occur at the donor site [1]. Allogeneic and xenogenic grafts are viable alternatives. However, the risk of transmitting infections as well as the possibility of an immune response are factors limiting their routine use [2]. Bone graft substitutes such as ceramics, polymers, and metals have the potential to replace the natural graft materials while avoiding the problems associated with their use [3, 4]. However, providing these materials with osteoconductive and osteoinductive properties similar to the autografts is a challenge. Osteoconduction is defined as spreading of bone over the surface proceeded by ordered migration of differentiating osteogenic cells within the ceramic [5, 6]. This results in a tight bond with the pre- existing bone without intervening fibrous tissue deposition, thus improving the implant stability [7]. Calcium phosphate (CaP) based ceramics are generally considered to be osteoconductive as they permit the osteoblasts to attach, proliferate and differentiate on their surface. This in combination with the fact that CaP based ceramics have a very similar chemical composition to the bone mineral (bone mineral is calcium phosphate in the form of carbonated apatite) have resulted in many bone graft substitutes based on calcium salts being extensively used in the clinics. [7-9]. Osteoinductivity, on the other hand is defined as the induction of undifferentiated inducible osteoprogenitor cells that are not yet committed to the osteogenic lineage to form osteoprogenitor cells [10]. For instance, biological stimulii such as cells or growth factors like bone morphogenetic proteins (BMPs) are able to provide an osteoinductive potential to bone graft substitutes [11]. In 1960, Selye and coworkers implanted Pyrex® glass tubes, with a diameter of 30 mm and a length of 20 mm, the so-called tissue diaphragms, subcutaneously in rats. Histological analysis of tissue formed inside the diaphragms 60 days following implantation, revealed the presence of bone, cartilage and hematopoietic tissue [12]. A report by Winter and Simpson demonstrated bone formation using polyhydroxyethylmethacrylate under the skin of pigs in the absence of any additional osteoinductive signals [13]. These findings suggested that bone graft substitutes do not merely support bone formation but also actually induce bone formation. This opened avenues to use them as a ready off-the-shelf grafts to treat bone defects without addition of cells or growth factors. Over the past decades, many other investigators have provided convincing proof of the osteoinductive potential of several porous CaP biomaterials [14-18]. However, the degree of osteoinductivity varies between different bone graft substitutes and as the mechanism behind osteoinduction is yet unknown, it is difficult to improve the osteoinductive

performance using a defined approach. Yet, research by multiple investigators has suggested that different factors such as chemical compositions as well physical characteristics such as presence of macropores, microporosities as well as surface concavities that increase the surface area of the ceramic, can influence the osteoinductive capacity of the ceramics [19-21]. Taking these factors in consideration, in a recent study from our group, a family of porous CaP ceramics was developed and their osteoinductive capacities were compared within dogs in muscle tissue as well as in a spinal fusion model [22]. After 12 weeks, a 5 fold increase in the amount of bone was observed within the TCP as compared to the HA implants at the ectopic site as well in the spinal fusion regions. The aim of the current study is to determine if the superior osteoinductive performance of the TCP at the ectopic and spinal fusion sites as compared to the poorly osteoinductive, slow resorbing HA is relevant to the dynamics of new bone deposition and bone healing in a clinically relevant critical sized orthotopic defect. Further, in the canine model used in our previous study, we observed that TCP displayed the most pronounced dissolution and degradation behaviour in vivo while HA was at the opposite end of the spectrum of dissolution and degradation. While a stronger dissolution and degradation potential in vivo is linked to superior osteoinductive properties, its relation to post transplantation inflammatory changes has not been studied previously. Therefore, using histological analysis at different time points, this study also compared the inflammation elicited by the fast degrading TCP and the slow-resorbing HA ceramics at the orthotopic sites.

7.2. Materials and methods

7.2.1. Synthesis and characterization of the calcium phosphate ceramics

In this study two ceramic types were investigated: hydroxyapatite (HA) and tricalcium phosphate (TCP). The porous hydroxyapatite implants were prepared from HA powder (Merck) using the dual phase mixing method as described earlier[7]. The processing route employed in this method consisted of three steps. In the first step, HA slurry was prepared by mixing 2/3 wt% of calcined HA powder with 1/3 wt% water containing deflocculant (dolpix CE 64, Germany) and binder (carboxylmethyl cellulose, Pomosin BC, The Netherlands). In the second step, two immiscible phases were mixed: water-based HA slurry and polymethylmethacrylate (PMMA) resin with a volume ratio of 1:1. The PMMA resin consisted of PMMA powder, MMA monomer and naphthalene (<10% v/v %) as an additional fugitive pore maker. In the final step the mixture was polymerized, dried and pyrolized and sintered at 1250 ° C for 8 hr.

The TCP ceramics were fabricated by the H2O2 method as described previously using TCP powder purchased from Plasma Biototal [7]. The powder was mixed with 2% H2O2 solution (1.0g powder/1.20 ± 0.05ml solution) and naphthalene (Fluka Chemie, Zwijndrecht, The Netherlands) particles (710-1400 μm; 100g powder/30 gm particles) at 60ºC. The naphthalene was then evaporated at 80ºC and the porous bodies were dried, and sintered at 1050ºC.

For both ceramic types, a lathe was then used to produce the cylinders. Subsequently, the cylinders were cut into implants 6 mm in length and 3 mm in diameter. The implants were cleaned ultrasonically with acetone, 70% ethanol and demineralized water, dried at 80ºC, and sterilized by gamma irradiation prior to use.

7.2.2. Material characterization

The chemical composition of the ceramics was analyzed with X-ray diffraction ((XRD, Miniflex, Rigaku, Japan). The macropore size and porosity of the ceramics was measured by morphometric analysis on thin sections using a KS400 image system (Carl Zeiss, Germany) attached to a light microscope (Nikon, Japan, Objective, X10). Thin sections were made on a Leica diamond saw after embedding ceramics blocks in methylmethacrylate (MMA). The microstructure was evaluated as described previously using a scanning electron microscope (XL30, Environmental SEM-Field Emission Gun, Philips while the specific surface area of the different ceramics was analyzed with Mercury intrusion (Micromeritics Instrument, Inc) [22].

7.2.3. Animals and implantation

In 34 skeletally mature male Wistar rats, a 6-mm segmental femoral bone defect was grafted with either HA (n=14), TCP (n=14) or left empty (n=6) as control. The study was approved by the institution's Animal Ethics Committee (EUR2317) and all animals were housed according to National guidelines for the care and use of laboratory animals.

The surgical procedures were performed under general anesthesia (isoflurane 1-3.5% in air) under aseptic conditions. Before the start of the procedure each animal received a subcutaneous injection of buprenorphine (Temgesic, Schering-Plough B.V., Amstelveen) at a dose of 0.05mg/kg body weight and enrofloxacine (Bayer B.V., Mijdrecht) at a dose of 5mg/kg body weight. The rats were immobilized on a heated plate to remain body temperature. The right thigh was shaved and disinfected with polydine tincture. The femur shaft was identified by palpation and a longitudinal incision extending from the trochanter major to the lateral condyl was made. The shaft was exposed after dissection of the skin, superficial fascia and deep fascia lata

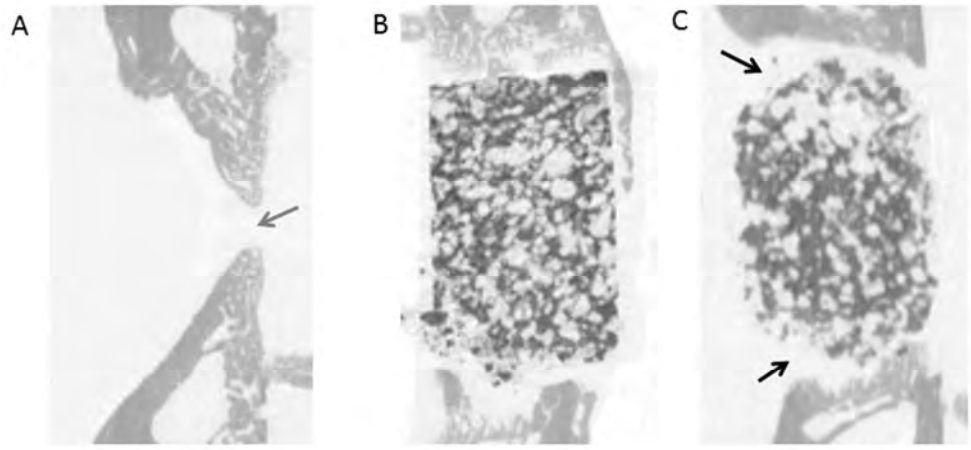

Figure 7.1. Micro CT findings after 8 weeks of in vivo implantation in the rat femoral defect

No evidence of bone bridging the entire defect was observed suggesting the critical nature of the defect (A). The grey arrow points to the unfilled defect area. The HA ceramic maintained its shape (B) while rounded and smooth edges (marked by black arrows) were observed in the TCP ceramic (C)

between the m. vastus lateralis and the m. biceps. Next, a 23mm PEEK plate (RatFix, RISystem, AO Foundation) was positioned onto the anterolateral side of the femur by three cortical and three distal screws. Two osteotomies were performed using a saw guide with a wire saw (Drill&Saw guide, RatFix, AO Foundation) to create a 6mm large segmental defect. Within the defect, depending on study group, one of the scaffolds was press fit or the defect was left empty (Fig 7.1). The wound was irrigated with sterile saline. Finally, both the fascia and the skin were closed with 5-0 Vicryl sutures.

Post-operative care consisted of administration of buprenorphine, (Temgesic 0.05mg/kg body weight) as analgesia twice a day for the first three days.

In order to visualize the dynamics of bone growth, the rats received sequential fluorochrome labels at 4 weeks (calceine green, 10mg/kg subcutaneously, Sigma Aldrich, Zwijndrecht, The Netherlands) and 6 weeks (xylenol orange, 100 mg/kg subcutaneously, Sigma Aldrich, Zwijndrecht, The Netherlands).

2 animals from each of the HA and TCP group were euthanized after 5 and 10 days while 1 animal from each of the HA and TCP groups were euthanized after 6 weeks. The remaining 9 animals from each of the HA and TCP groups and 6 animals from the empty control group were euthanized after 8 weeks of implantations. After killing

of the animal, the femur and surrounding tissue were explanted for micro-CT and histological analysis.

7.2.4. Micro-CT analysis

A high-resolution ex-vivo micro-CT scan was acquired of all samples to provide a qualitative overview of bone integration and resorption of both calcium phosphates and in order to confirm the critical size of the defect in the empty control group. Therefore the right femurs were collected and fixated in 4% formaldehyde. The samples were kept hydrated during the scanning process by wrapping them in foil. The micro-CT scan was acquired using a SkyScan 1076 (SkyScan, Kontich, Belgium) with an 18μm-resolution protocol (70kV energy, 100μA current, 1.0mm Al/0.25mm Cu filter, 0.5 degree rotation step, 3hrs scanning time). CT shadow projection images were converted into three dimensional (3D) reconstruction of cross-sectional images in bitmap files using volumetric reconstruction software NRecon version 1.5 (SkyScan, Kontich, Belgium).

7.2.5. Retrieval of the implants, histology and histomorphometry

After micro-CT scanning, the femur defect with the surrounding bone and some muscle tissue was removed and fixed overnight in 10% formalin, (Merck chemicals, The Netherlands), dehydrated within an industrial microwave using JFC solution (Leica Microsystems, Rijswijk, The Netherlands) and transferred into a methylmethacrylate (MMA) solution (L.T.I, Bilthoven, The Netherlands) that polymerized at 37ºC within 3 days. Sections having an approximate thickness of 10 to 15μm were made by using a modified interlocked diamond saw (Leica Microtome, Nussloch, Germany). Sections were stained either with 1% methylene blue (Sigma) and 0.3% basic fuchsin (Sigma) after etching with an HCl/ethanol mixture for routine histology and histomorphometry or left unstained for epifluorescence microscopy with a light microscope (LM; E600, Nikon, Japan) equipped with a quadruple filter block (XF57, dichroic mirror 400, 485, 558 and 640 nm, Omega Filters, Didam, The Netherlands).

Histological sections from MMA embedded samples were qualitatively analyzed by light microscopy (Leica), and each histological section was scored either positive or negative for bone formation. In addition, quantitative histomorphometry was performed. Briefly, high-resolution digital photographs were made from three randomly selected sections from each tissue-engineered graft. Before histomorphometrical analysis, the entire defect area was defined as the region of interest. The newly formed bone as well as the material were then manually pseudocoloured green and red, respectively using Photoshop CS2 (Adobe Systems). A custom-made Matlab script was used to measure the percentage of bone occupying

the total defect area (area% bone/scaffold). To get insight into the in the process of defect healing, histomorphometry was performed not just in the total implant area but also within the central area of the defect. The central area was determined as the area covered by a rectangle drawn along the points 1mm on either side of the horizontal line running through the center of the implant and 2mm on either side of the vertical line running through the center of the implant. For statistical analysis, two sided, paired Student's t-test was used to analyze the differences between TCP and HA.

7.3. Results

7.3.1. In vitro results

7.3.1.1. Characterization of the calcium phosphate ceramics in vitro

Porous HA and TCP ceramics with physical and chemical characteristics similar to those used in our previously performed canine study were used [22]. An overview of the material properties are briefly described below and also presented in Table 1. Using X ray diffraction analysis (XRD) analysis, it was observed that HA was phase pure while TCP had a trace of HA (<10% by weight). Image analysis on cross section showed no differences in the macrostructure among the different ceramics while the micropore size and the surface area varied. Pores having a volume smaller than 10 μm were considered as micropores. Using this criteria, TCP had high microporosity (±15%) while HA had a low microporosity (±5%). As a consequence, the specific surface area of the TCP, as determined by mercury intrusion was much higher than HA (<0.5m2/g for HA vs. >1.5m2/g for TCP).

Table 7.1. Ceramic characterization

	HA	TCP
Chemistry	HA	90%TCP and <10% HA
Sintering Temperatures	1250 C	1050 C
Macroporosity	±60%	±60%
Microporosity	Low (±5%)	High (±15%)
Surface area	Low (<0.5m2/g)	High (>1.5m2/g)

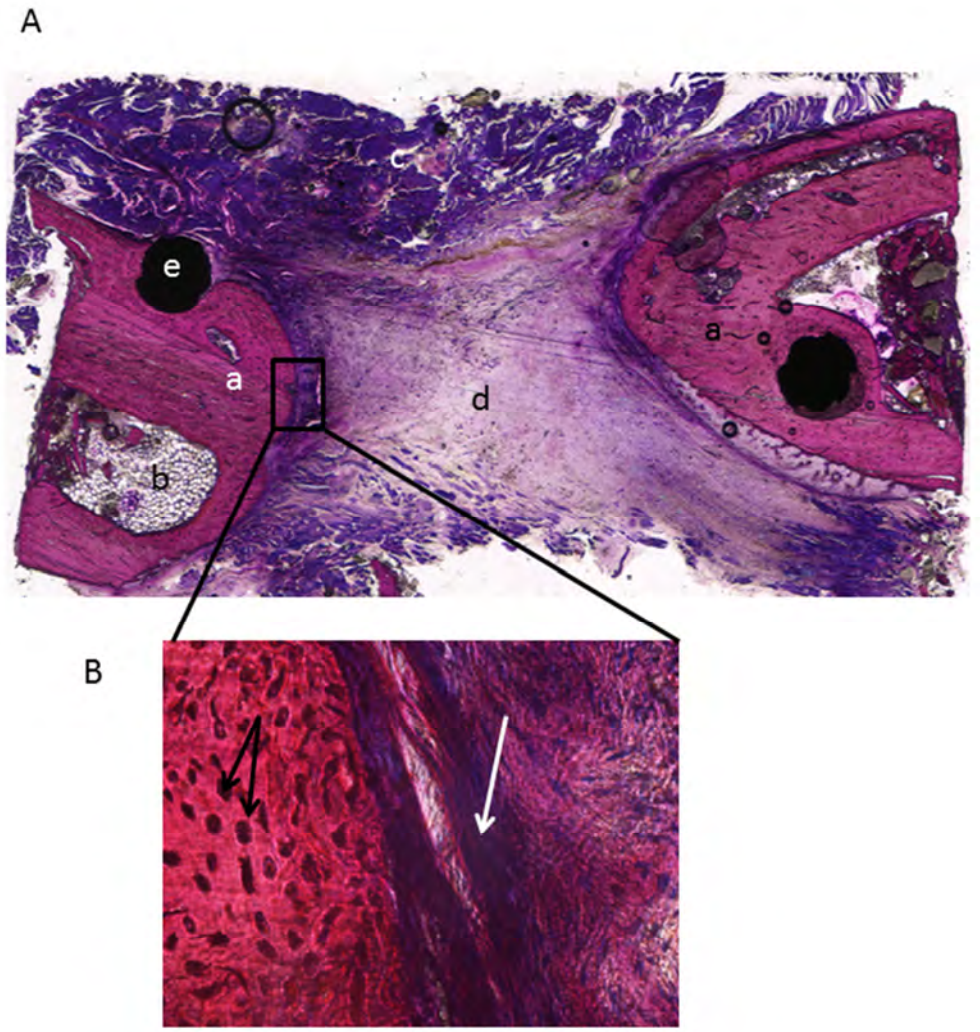

Figure 7.2. Histological findings in the empty rat femoral defect
The defect was filled with some loose fibrous tissue. No evidence of a bone bridge spanning the defect space was observed within 8 weeks of creating the defect. (a) the two ends of the bone (b) marrow cavity (c) muscle surrounding the bone and defect site (d) the actual defect (e) screws used to hold the PEEK plate in place over the defect. Black arrow points to osteocytes. While arrow points to the fibrous tissue occupying the defect site.

7.3.2. In vivo results

7.3.2.1. General findings at the time of explantation

There were no surgical complications, and all animals remained in good health during the course of the experiment. The rats could walk and bear weight on the operated

limb within 48 hours post-surgery. Adequate fixation was maintained in all the defects except in one femur fitted with HA ceramic and left in situ for 8 weeks. In this case a fracture was observed on the proximal end of the operated femur. Macroscopically, upon explantation, there was evidence of resorption on the corners of the TCP ceramics (rounded and smooth appearance of the corners) while the HA implants maintained their shape and size.

7.3.2.2. Micro-CT evaluation

In 34 animals, a 6mm defect was created in the right femur using a saw and drill guide and the defects were stabilized using a PEEK plate. 6 of the defects were left empty while 14 were fitted with HA implants while the others received TCP implants. All the femurs with the defect were subjected to micro CT post-explantation from the rats to check for implant positioning, any visible differences between the two different ceramic types as well to determine the bone bridging in the empty defects. The defects which were left empty (control group) did not show complete bridging confirming the critical nature of the defect. However, from both cortical sites, some bone formation could be observed (Fig 7.1A). Adequate fixation was maintained in 5 out of 6 cases, while in one case fixation failure had occurred.

The difference in resorption between HA and TCP was clearly seen in the micro-CT images. Whereas HA has kept its cylindrical shape over the eight weeks study period, the TCP implants clearly showed signs of degradation on the upper and lower edge at the periphery of the implant (Fig 7.1B,C).

7.3.2.3. Histological analysis of the explanted samples

To compare the bone forming capacity of the HA and TCP particles, cylinders of both materials were press fit into a 6 mm rat femoral defect and left in-situ for 5 days (n=2), 10 days (n=2), 6 weeks (n=1) or 8 weeks (n=9). The critical nature of the defect was confirmed by leaving the defect empty (n=6) and observing the degree of bridging observed within the defect after 8 weeks. After 8 weeks, within the empty defects, the defect site was filled by loosely organized fibrous tissue with no indication of bony bridge formation (Fig 7.2). These findings were similar to our micro CT findings.

We performed histological analysis of the samples explanted at different time points from the rat femoral defect in order to obtain an overview of the initiation and progression of inflammation and bone formation within the ceramics. We chose two early (5 days and 10 days) and two late time points (6 weeks and 8 weeks). Histological analysis at 5 and 10 days revealed the presence of round cells suggestive of cells of inflammatory nature such as monocytes or lymphocytes within the pores of both the

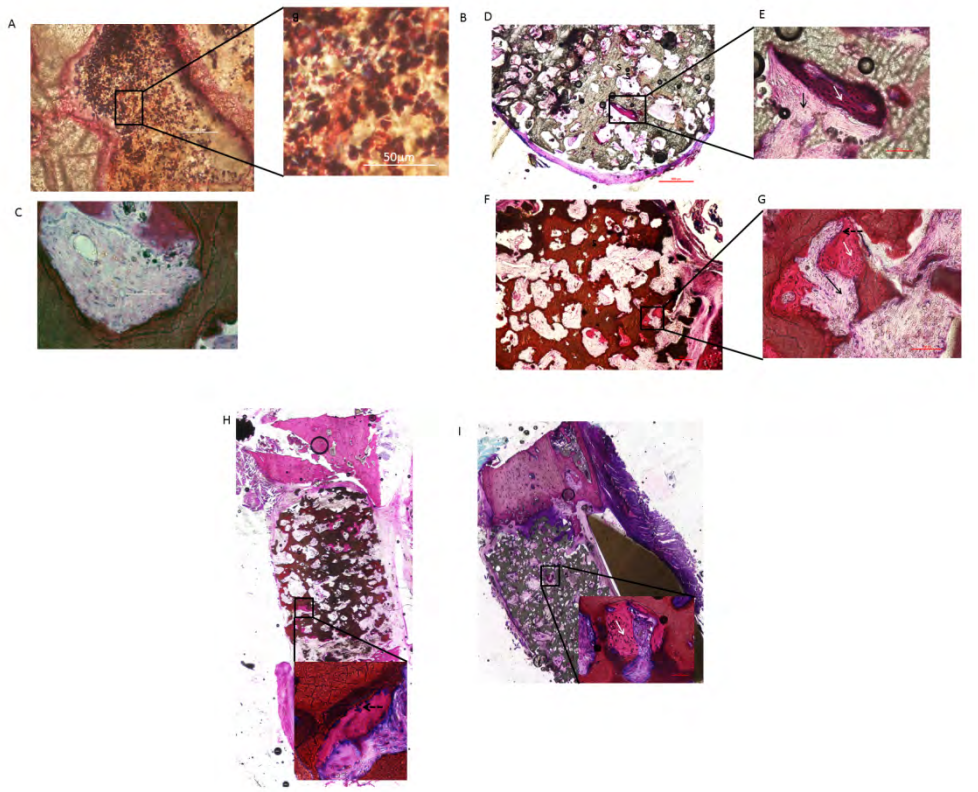

Figure 7.3. Histological overview of the TCP and HA ceramics after varying time points in vivo

Basic fuchsin stains the bone pinkish red while methylene blue stains the cell nucleus and fibrous tissue blue. Rounded cells, probably of inflammatory origin within the pores of the TCP ceramic after 10days (A). A 40X magnified view of the inflammatory cells (B). Absence of inflammatory cells with onset of new bone formation after 6 weeks (C). Similar results were also obtained in HA ceramics after 5 and 10 days. Small islands of bone in both HA (D) and TCP (F) after 6 weeks. Magnified view of bone formed within the pore of the HA (E) and TCP scaffold (G). Note the presence of osteocytes (white arrow) embedded in the pink matrix and the bone lining cells (dotted black arrow). Rest of the pore was filled by cells with elongated nuclei (Fibrous tissue marked by black arrow). After 8 weeks, again islands of new bone were observed in both TCP (H) and HA (I). The inset images show magnified views of the newly formed bone. Note the presence of osteocytes, bone lining cells and matrix.

HA and the TCP ceramics (Fig 7.3A,B). No obvious difference was observed between the two types of ceramics at either 5 or 10 days. Further, there was no obvious change in the cellular infiltration between the 5 and 10 day samples (data not shown). In both the ceramic types explanted after 6 weeks, no cells with morphology suggestive of an

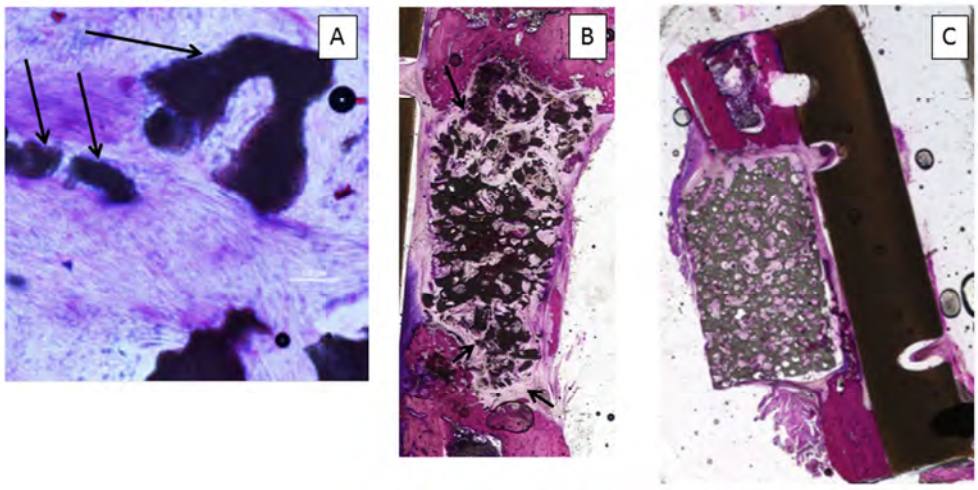

Figure 7.4. Degradation profiles of TCP and HA

The degradation products of the TCP ceramic particles (marked by a black arrow) within the muscle surrounding the defect (A). Note the rounded edges of the TCP ceramics after 8 weeks (marked by black arrow), suggestive of increased resorption at these sites (B). The HA ceramic maintained its cylindrical shape after 8 weeks (C)

inflammatory origin were observed (Fig 7.3C). Instead, small islands of new bone formation were seen in the pores of both HA (Fig 7.3 D, E) and TCP (Fig 7.3 F, G). These results suggested that the cellular infiltration which was probably of inflammatory origin subsided between 12 days and 6 weeks while the bone formation was initiated during this time. The presence of bone with a corresponding lack of inflammatory cells was also observed after 8 weeks in both HA and TCP (Fig 7.3 H, I). The insets in Fig 7.3 H and I demonstrate the histological features such as mineralized matrix, bone lining cells and embedded osteocytes which characterize bone. Further, another interesting observation from the histological slides was the material degradation behaviour. As illustrated in Fig 7.4A, in some TCP implants, debris was observed in the surrounding of the implant. Bone tissue, however was only confined to the region of the implant that was not fragmented. Moreover, there was no obvious associated increase in inflammatory reactions surrounding the TCP implants. In addition, we observed rounding of the edges and loss of structure in some of the TCP particles (Fig 7.4B), possibly as a result of increased resorbability of the material. These findings were not observed in the HA ceramics (Fig 7.4C).

After 8 weeks, bone was observed in 7 of the 9 HA implants while bone formation was observed in all the TCP implants (9 out of 9). Fig 7.5 illustrates the histomorphometrical comparison of the percentage of bone occupying the total (A)

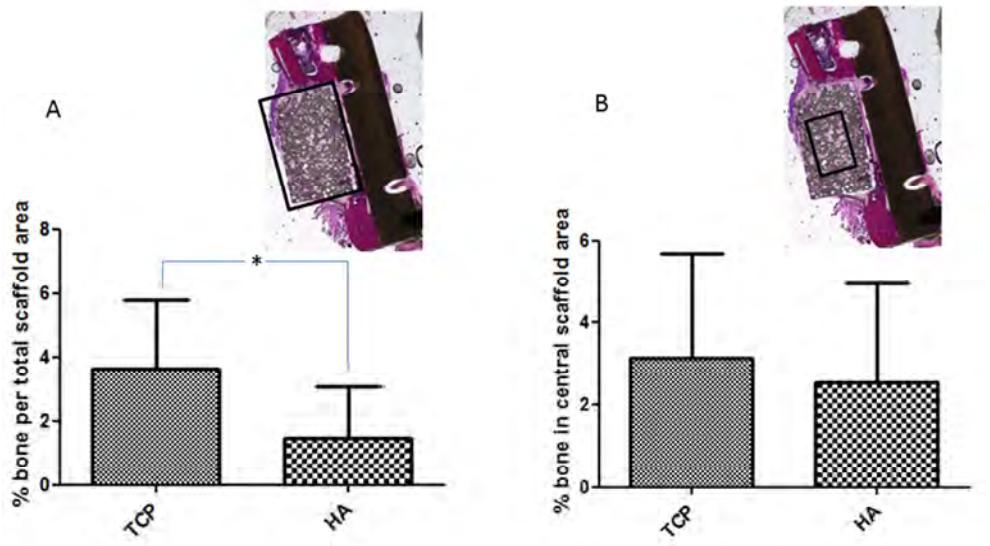

Figure 7.5. Quantification of bone formed within the pores of the HA and TCP cylinders

To compare the relative contribution of osteoinduction in the bone formation, the bone formed was determined in the pores of the total scaffold (A) and in the centre of the scaffold (B). The black rectangle in both the insets represents the area of the implant in which the new bone deposition was calculated. In both graphs, the error bars represent the standard deviation. An asterix (*) denotes statistical difference (Student's paired t test, $P < 0.05$).

and central (B) defect areas fitted with either TCP or HA. The amount of bone formed with the total TCP ceramics was significantly higher than that formed with HA ceramics (3.6 ± 2.12 % vs 1.4 ± 1.6 % respectively). However, it was interesting to note that there was no significant difference between the amounts of bone formed within the pores occupying the central area of the TCP or HA implants , where chances of bone ingrowth from the host bed was lowest. While 3.1 ± 2.53 % of the central pore area was filled with newly formed bone in TCP, the corresponding value for HA was 2.5 ± 2.38 % (Fig 7.5B). These results indicate that while even within the orthotopic sites, the overall bone healing capacity of the TCP ceramics were higher than that of HA, there was no significant difference in the ability of the two ceramics to bridge the central defect areas.

7.3.2.4. Analysis of the dynamics of bone deposition

To study the dynamics of bone deposition, all the rats received sequential fluorochrome labels at 4 weeks (calceine green) and 6 weeks (xylenol orange). Analysis of the presence of the fluorochrome markers indicated that in both ceramics, the bone

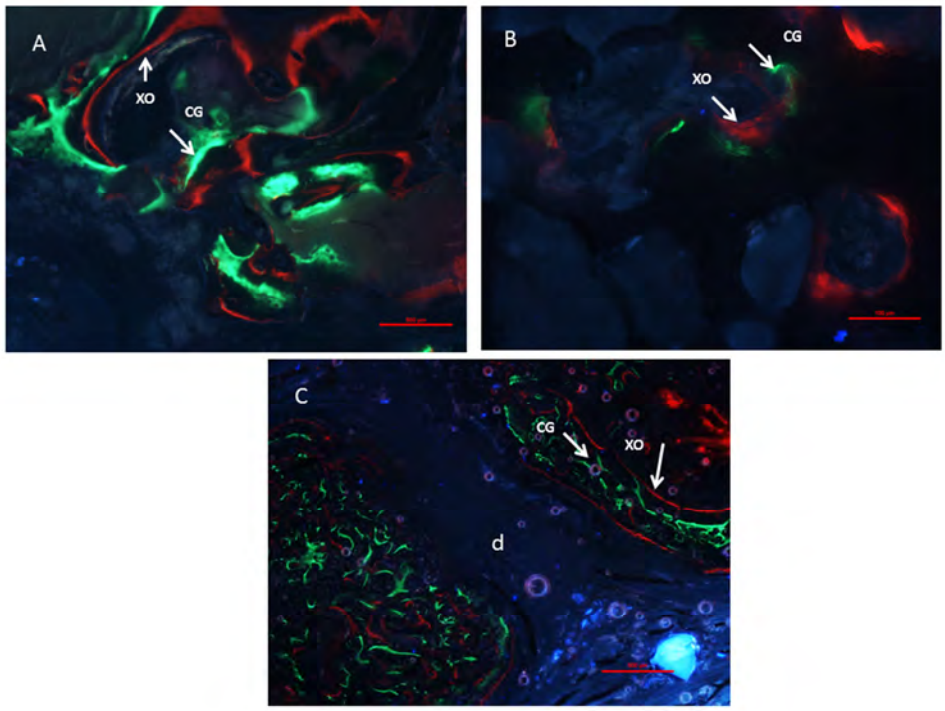

Figure 7.6. Light microscope photographs (original magnification 10X) of the fluorochrome markers in the center of the TCP (A), HA (B) ceramics

(C) represents a 4X time magnified image of the two ends of the femur with the intervening defect (d). CG, Calcein Green; XO, Xylenol orange. Both the markers were seen in the HA as well as the TCP ceramic suggesting that in both ceramics, bone formation had started in the ceramic center before the fourth week of implantation. However, the relatively greater amounts of CG in the TCP showed that a much greater amount of bone deposition had occurred by the fourth week in this ceramic as compared to the HA.

apposition started in the pores in the center of the ceramic. In both HA and TCP the green label was observed, indicating that bone deposition started in both ceramics within the first 4 weeks of implantation (Fig 7.6A, B). However, more calcein green label was observed in the TCP ceramics as compared to the HA, suggesting more early bone formation in TCP. No bone was observed bridging the empty defect. However, both fluorochrome markers were observed on the two ends of the defect, indicating that new bone was being deposited and remodeled as a part of the body's intrinsic healing process (Fig 7.6C).

7.4. Discussion

The TCP and HA ceramics chosen in this study differed not only in their chemistry but also in the grain size and microporosity. However, in spite of the difference between the materials in more than one parameter, they were chosen as TCP and HA were identified as being at the two extreme ends of the osteoinductive spectra in the study where the two materials were implanted at the ectopic and spinal fusion sites in dogs [21]. The interest in osteoinductive ceramics is based on the hypothesis that a material that is able to induce bone in an ectopic location will also perform better at an orthotopic site. To our knowledge, apart from this current study there are a only a few other studies that have performed comparisons of ectopically proven osteoinductive and non-inductive ceramics within critical sized, clinically relevant, orthotopic defects. A study performed by Gosain et al demonstrated that HA-based cement with higher osteoinductive potential showed superior performance in a critical-sized calvarial sheep model [23, 24]. Two other studies tested osteoinductivity of ceramics in ectopic locations and then compared the ectopic performance within a transverse process and spinal fusion model in large animals. The transverse process or spinal fusion models, though not ectopic are also not truly orthotopic critical sized defects. The first study, performed previously by our group, compared a family of 6 ceramics (4 BCP ceramics with different physical properties, one carbonated apatite based ceramic and one phase pure HA ceramic) within intramuscular and transverse spine locations[25]. Two BCP ceramics from this family of ceramics had been tested previously in an iliac wing defect in goats[19]. We had also performed another, in which the same materials tested in this study, i.e. HA and TCP were tested simultaneously in intramuscular pockets and spinal fusion sites in dogs[22]. In both these studies, the results indicated that the ceramic with superior osteoinductive performance at the ectopic site demonstrated an earlier initiation and greater amount of bone at the spinal site. Since all these studies used large animals to test the biological performance of the bone graft substitutes, and use of large animals may limit the number of formulations that can be tested, we used a true critical sized orthotopic defect in a rodent model to test the significance of the ectopic findings at the orthotopic site.

Similar to the other studies mentioned above, our current study within the rodents, also indicate that the TCP ceramic which had previously demonstrated a superior osteoinductivity as compared to the HA at the ectopic site, resulted in a better overall healing potential within a critical size orthotopic site. However, it does not necessarily mean that the improved healing at the orthotopic site is solely based on the osteoinductive potential of the implant material. It cannot be excluded that the presence of a higher microporosity resulting in a 12x greater surface area led to better osteoconduction. In fact, our finding of a significantly higher percentage of bone in

the total implant area with no significant difference within the central portion of the implant, points to the fact that the major difference between the bone formation in the two implants was on the peripheral areas of the implant. As osteoconduction as per definition, proceeds from the host bone bed, the bone in the periphery of the implant results from osteoconduction. On the other hand, the central area of the scaffold has no direct contact with the osteogenic cells present in the host bed. Thus, measurements of the amount of bone formed in this region can, in principle, predict the relative contribution of the material's osteoinductive properties. However, it can be argued that in the absence of studies that determine the origin of the bone, it cannot be stated definitively that the bone formed within the central pores of the implant is due to direct differentiation of the inducible osteoprogenitor cells present in the bone marrow or the surrounding muscle. Further the phenomenon of osteoinduction has mainly been observed in big animals like goats, sheep and dog. In small animals, particularly rodents, induction of bone by biomaterials is limited, if at all [18] [26]. However, in spite of these arguments, it is probable that quantification of the bone formed in the central pores of the implant, within the critical sized defect, is a measure of the osteoinductive capacity of the ceramic. In that case, the lack of significant difference in the amount of bone observed within the central area of the implant suggests that the superior osteoinductivity of the TCP at the ectopic site is not paralleled at the orthotopic site.

Our results confirmed the faster degradation profile of TCP as compared to HA, as reported by previous researchers. However, though some researchers have reported that released micro particles from fast degrading ceramics may induce an inflammatory reaction which may affect the survival of the osteoprogenitor cells in the vicinity[8, 27-29], in our study we observed no obvious differences in the degree of the cellular infiltration, at both early and late time points between the TCP and HA. Though it can be argued that no tests were done to identify the nature of the cells, the rounded morphology with the presence of a deep staining nuclei suggests that these cells are likely to be of inflammatory nature. The superior biological performance of the TCP taken together with the lack of an increased potential to generate inflammatory reaction as compared to HA, suggests that the TCP formulation used in this study is a good replacement to autologous bone. However, the highly micro-porous structure of the TCP probably compromises with its mechanical properties and this can be a concern to clinicians. In a previous study, We suggested incorporation of an ultra-thin layer of polylactic acid (PLA) within an osteoinductive BCP ceramic to improve its mechanical properties and facilitate easier handling. However, the incorporation of PLA blocked some of the micropores and decreased the specific surface area of the ceramics. This in turn affected the biological

performance of the ceramic. Nevertheless, such further studies to optimize the mechanical properties of the TCP without affecting the biological performance are needed.

In our previous study with goats, the TCP particles had shown a capacity comparable with autologous grafts in healing iliac defects after 12 weeks[22]. However, in our current study, though the amount of total bone obtained with TCP was significantly higher than that obtained with the HA ceramics, the overall quantity was quite limited. This could be attributed to a shorter implantation time, smaller volume of implants as well as difference in the animal species, all factors known to affect the amount of induced bone[19, 26]. However, use of MSCs maybe a potential strategy to improve the biological performance of the ceramic. The absence of a relevant increase in the amount of bone formation with cell seeded constructs as compared to cell free constructs at orthotopic sites was attributed to poor cell survival in a previous study by Kruyt et al[30]. The invasive nature of the procedure which is needed to implant the bone graft elicits an inflammatory reaction within the body. The inflammation at the fracture site is well documented within this study. The inflammatory milieu can be a potential cause of death for the implanted MSCs. In an opinion paper by Meijer et al. it was proposed that MSCs should be injected within the implant, a few days after the implant is placed at the defect site, in order to promote their survival[31]. This proposal was based on the hypothesis that few days are needed for the inflammatory processes to subside following the handling of the tissues during implant placement as well due to direct impact of the fracture itself. Further, in the natural course of bone healing, the precursor cells migrate to the defect site and start forming the bone matrix a few days following the actual fracture[31]. In this study, we demonstrate with fluorochrome administration that the bone formation in both TCP and HA started prior to 4 weeks. In addition, we had observed no histological evidence of bone in both HA and TCP for up to 12 days post implantation. In fact, the presence of abundant cells with morphologies similar to inflammatory cells suggested a strong inflammatory reaction till up to around 2 weeks post-implantation. Based on these findings, we suggest that the best time to introduce cells into the implant to avoid their destruction by the hostile inflamed milieu of the defect site is around 3 weeks. Further histological studies, comparing empty implants with implants in which cells have been injected around the 3rd week can help confirm this hypothesis.

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Chapter 8

Conclusion

8.1. General discussion

Throughout history, mankind has been attracted to the idea of replacing diseased limbs and organs. However, it was only in the twentieth century that organ transplantation became a real therapy option. Landmark discoveries such as the ABO blood system by Karl Landsteiner in 1900, discovery of penicillin by Alexander Fleming in 1928 followed by the description of the HLA typing system by Dauset in the 1950s, and then the discovery of immunosuppressive agents and treatments such as cyclosporine A, tacrolimus, prednisone, and irradiation all heralded a new era in our approach to treat failing organs. In 1979, the first report on the clinical use of cyclosporine A in renal allograft recipients was published [1]. From then on, patients had a good chance to live a longer life with transplanted organs. However, by the late 1980s, it became clear that organ transplantation options were plagued by a shortage of donor organs relative to an increasing number of people in need. This saw the emergence of a new field of research termed "Tissue Engineering" that focused on the development of biological substitutes to restore, maintain and improve tissue function via a comprehensive interdisciplinary approach.

For engineering any tissue, a number of single components need to be assembled such that the interplay between these components can dictate the functional properties of the final construct. In order to successfully assemble these single components, bone tissue engineering, similar to engineering other tissues and organs, requires integration of multiple disciplines such as cell biology, developmental and molecular biology, biomechanics, biomaterials science and immunology. Although each of these individual research areas have undergone huge advances in the last decade, till date, the translation to clinical care with a bone tissue engineered construct having a healing potential similar to the gold standard, i.e. "autologous bone" has remained a major challenge . The single U.S. Food and Drug Administration (FDA) approved product available for bone tissue engineering purposes involves the use of the genetically engineered rhBMP-2 on absorbable collagen sponge carrier and is limited for use in spinal fusion procedures in skeletally mature patients with degenerative disk disease at the lumbo-sacral vertebrae level and (ii) treatment of acute, open fractures and tibial shaft [2]. Though this product is commercially successful, judging by its use in more than 25% of spinal fusion surgeries [3], the significant complications resulting from the use of rhBMP2 use, including patient death, dysphagia, and airway compression in cervical spine fusion cases [4, 5], heterotopic bone formation in the spinal canal [6, 7] and retrograde ejaculation in patients undergoing anterior lumbar interbody spine fusion [8], indicate that there is a pressing need for other safer strategies.

Mesenchymal stromal cells (MSCs) provide a safe osteoinductive alternative to growth factors such as BMP2 without having any ethical drawbacks as observed with embryonic stem cells (ESCs). They were first identified in the bone marrow by Friedenstein and coworkers in 1966 [9]. Subsequently the multipotent nature of these cells were highlighted in the studies performed by Pittinger et al in 1999 [10]. Since then they have been used extensively for tissue engineering applications. However, the donor-donor variability in the amount of bone formed [11], the limited amounts of bone generated using human MSCs [12] as well dependence on highly skilled manpower to generate cell based tissue engineering strategies and stringent guidelines governing the introduction of cell based tissue engineered products in the market[2] have proved to be major hurdles in the clinical translation of these products. However, clinical translation is particularly urgent as our present day lifestyle options combined with an ageing population have made us vulnerable to a variety of unmet clinical needs such as large segmental defects, spinal problems and medically compromised conditions such as tumor removal and infection sites. Thus, as indicated in the introduction, the general aim of this thesis was to address the various aspects involved in improving the osteogenicity of a tissue engineered construct using bone marrow derived MSCs while keeping an eye on its applicability to a clinical setting, safety and commercial feasibility.

8.2. Conclusions

As a first step in identifying the bottlenecks in translating cell based tissue engineering into the clinics, we performed a systematic review, as described in chapter 2 of this thesis, to critically study and compare the clinical trials which employed non-genetically modified, bone marrow derived MSCs for bone tissue engineering applications. We concluded that comparing the different clinical studies was difficult in view of the lack of internationally recognized standards for isolating and culturing cells, small sample size employed in the studies, lack of internal controls using autologous bone grafts, short follow up periods, different methods to determine the outcome of the study as well different areas of application of the constructs. However, we identified the need to improve the in vivo bone forming capacity of the MSCs, improve the delivery of the MSCs at the defect site as well as well streamline the generation of grafts for clinical use as areas that needed attention, in order to facilitate the translation of MSC based bone tissue engineering into the clinics.

We observed that the process of generation of bone grafts currently used in most laboratories, besides being unphysiological to the MSCs, is very labour, time and space intensive. We hypothesized that directly culturing pre-determined volumes of bone marrow on the ceramics can bypass the unphysiological expansion of MSCs on tissue

culture plastic while saving resources. We concluded in chapter 3 that, the direct seeding of bone marrow on ceramics generated grafts which did not differ in their in vivo bone forming capacity from grafts generated using the conventional 2D expanded cells. Further, based on literature evidence, we concluded that without compromising on the safety of the patient, one could obtain up to 40 cc of bone marrow. According to the approach outlined in chapter 3, this amount can generate approximately 13cc of graft material, which is sufficient for most clinical applications. However, in certain conditions such as hereditary bone disease, ex vivo expansion of the MSCs is actually critical as the expansion phase provides an opportunity to use molecular engineering strategies to target gene defects in the cells. Further, another drawback of the direct seeding approach was that in spite of providing a method to streamline the generation of osteogenic grafts for clinical use, the amount of bone formed was not optimal when compared to the gold standard "autologous bone".

Research by multiple independent researchers have indicated that culturing cells as aggregates leads to a better cross talk between the cells, thereby affecting the signal pathways dictating cell fate determination. In fact, PCR data have indicated the beneficial effects of cell aggregation on osteogenic and chondrogenic differentiation. Keeping these findings in mind, in chapter 4, we investigated the effect of aggregating cells after an initial period of expanding them on monolayer culture in vitro, to enhance their in vivo bone forming capacities. Our results indicated that cell aggregation was indeed beneficial in improving the amounts of bone obtained in vivo. In keeping with the general focus of making the system clinically applicable, pre-formed templates were used to generate the cell aggregates and the culturing of the cell aggregates were done in a serum free medium. Further, platelet lysate obtained from human donors was used as a gel source to bind the cell aggregates on the micro ceramic particles. In the past we had improved the performance of the MSCs by combining them with a compound, cyclic AMP which impinged on the signaling pathways affecting the osteogenic differentiation of the MSCs. Currently, our group is investigating libraries of compounds to identify more such compounds. It would be interesting to conduct future studies employing such compounds with the aggregated cells to further enhance the performance of the MSCs for clinical applications.

In Chapter 6 we tested another approach with a potential to guarantee sufficient amounts of bone using tissue engineered approaches. We investigated the possibility of replacing the autologous MSCs with allogeneic MSCs tested previously to have good in vivo bone forming capacity, in order to provide an "off the shelf" alternative to patients and clinicians in search of tissue engineered substitutes. The motivating factors for this approach were the donor variability of the MSCs to generate bone, lack of markers capable of predicting a priori the bone forming potential of the MSCs,

capability to expand MSCs extensively in culture without significantly affecting the osteogenic performance of the cells, the evidence pointing to the immunoprevileged status of MSCs and the existing use of MSCs as immune modulators in human clinical trials for conditions such as graft versus host disease. We chose the rat ectopic model for testing the effect of allogeneic MSCs as in addition to providing the obvious benefits of working with a small animal model, antibodies to characterize the immune responses in rats are easily available in the market. The main conclusion of this chapter was that the allogeneic MSCs are not intrinsically immunoprevileged. However, use of immunosuppressants offers the possibility of employing allogeneic MSC for bone tissue engineering purposes. In our study, we did not assess the duration for which the MSCs need to be administered or the effects that the MSCs may have in sensitizing the immune system to future allograft transplantations. These may be a few of the clinically interesting questions that may aid in deciding the feasibility of allogeneic implantation for bone tissue engineering in the future. Further, in chapter 4 we described the in vivo enhancement of bone formation achieved by culturing the MSCs as aggregates. There are studies which indicate that the surface marker profile of MSCs cultured as aggregates may differ from those expanded in adherent monolayer cultures. More specifically, these cells may have higher levels of expression of anti-inflammatory proteins TSG-6 as well as STC-1. Studying the immunogenic properties of aggregated cells may offer an alternative to immunosuppressants in the use of allogeneic MSCs for bone tissue engineering.

Chapter 5 addressed the issue of employing MSCs in the clinics without the need for invasive open surgeries for placement of grafts. This chapter was an adaptation of the model used in chapter 4 for generation of in situ forming constructs with enhanced osteogenic capabilities. Similar to the model used in chapter 4, aggregated cells were combined with micro ceramic particles and fibrin and thrombin. However, unlike in chapter 4, fibrin and thrombin components, obtained from human platelet lysate were added to the cell scaffold mixture just prior to introducing into the defect. There was no in vitro culturing of the cell aggregates with the scaffold. The fibrin and thrombin which was liquid at room temperature cross-linked at body temperature enmeshing the cells and scaffolds. The micro particles ensured that the construct could adapt easily to the shape of a defect and could be introduced via a small opening while the liquid state of fibrin and thrombin acted as a delivery vehicle, thus making this construct suitable for introduction into the defect via arthroscopic means. Similar to our findings in chapter 4, the aggregation of cells was found to be a key factor in improving the osteogenicity of the MSCs. This chapter provided evidence that cell based tissue engineered constructs can be introduced into a defect via a minimally invasive route for bone regeneration based applications.

All the above studies were performed in an ectopic location, either in the nude mouse or in immunocompetent rats. These are well-accepted models in bone tissue engineering to provide in vivo proof of concept prior to proceeding to the more technically challenging critically sized bone defect model or the orthotopic model. However, the latter provides a better simulation of the biochemical and mechanical environment that the tissue engineered constructs are likely to face in an actual clinical set up. It has been well documented that the osteogenic cells in the vicinity, the surrounding hematoma, the mechanical stresses experienced by the implanted graft within the defect can all contribute to the final outcome of the tissue engineered graft. Thus in chapter 7, we performed studies to evaluate the healing of a critical sized femoral defect in a rat with two commonly used ceramics, β-TCP and HA. HA and β-TCP fall at the two extreme ends of the spectrum with regard to their osteoinductivity and biodegradability. The aim of this chapter was to study the relation between superior osteoinductive performances at ectopic sites with bone healing capacity at the orthotopic locations as well as to determine the influence of increased biodegradability on inflammation and bone formation

8.3. Future perspectives

The challenge of engineering a tissue that can function in the in vivo environment as efficiently and safely as the original tissue is enormous. Within this thesis, we have focused mainly on the osteogenic capacities of the MSC, which is just one aspect of making a clinically viable graft. Providing a scaffolding material which can support the ingrowth of the newly forming bone without adversely affecting the viability of the implanted cells, the local milieu or the mechanical stability of the defect is equally crucial. Another crucial factor is the vascularization of the grafts which is essential to provide nourishment to the cells and thus guarantee the survival of the cells. While it is the combination of all the factors that will ultimately determine the success of the tissue engineered graft with respect to an autologous construct, the complexity makes it necessary to address individual problems and optimize them before attempting to address the problem as a whole. Below, we list a few areas relevant to the work presented in this thesis which we believe can potentially improve the osteogenicity of the graft while keeping with the focus of developing a clinically relevant product.

• Choosing the right source of cells

Though in this thesis, we have mostly worked with MSCs from the bone marrow, more and more research is being performed to isolate MSCs from "waste" sources such as amniotic fluid, umbilical cord, placenta and adipose tissue [13]. Each of these different sources offers distinct advantages. While adipose tissue contains a

significantly greater population of MSCs (according to some reports, almost 500 time more as compared to an equivalent amount of bone marrow) [14], several studies have reported superior cell biological properties such as improved proliferative capacity, life span and differentiation potential of MSC from birth-associated tissues such as placenta[15, 16] , umbilical cord [17] and amniotic fluid over bone marrow derived MSCs. More research efforts to better characterize the properties of the MSCs from these sources can result in obtaining banked autologous cells for future applications from tissue sources that have no or only limited use in the post-embryonic life of an individual. Further, there are reports which suggest that MSCs from placenta and umbilical cord have greater hypo-immunogenic potential that those from the bone marrow, though these results are mainly from in vitro studies [18, 19]. We have demonstrated that there can be discrepancies between the in vitro and in vivo results, thus necessitating further studies in animal models to verify the in vitro findings. In addition, studies are also needed to determine the possibility of employing induced pluripotent stem cells iPSCs, which are adult cells that have been genetically reprogrammed to an embryonic stem cell–like state by being forced to express genes and factors important for maintaining the defining properties of embryonic stem cells [20]. However, currently there are major issues relating to the method of reprogramming (viral vs. nonviral), completeness of reprogramming, epigenetic changes, and genomic instability which need to be addressed [21].

• Optimizing culture conditions of the MSCs in vitro

The treatment of MSCs in vitro can affect their differentiation potential in vivo [22, 23]. More and more studies demonstrate the effect of the 3D culturing environment on the differentiation potential of the cells. For example, Brannvall et al. found that neuroprogenitor cells in 3D hyaluronan/collagen I scaffolds generated up to 70% neurons compared to 15% in 2D cultures [24]. Daley et al. noted that stem cell renewal and differentiation are regulated by the 3D environment within the stem cell niche [25]. Hwang et al. noted that a 3D PEG scaffold environment together with an appropriate growth factor microenvironment promoted differentiation of embryonic stem cells in chondrocytes [26]. Taking cues from research in other tissue engineering fields, the field of bone tissue engineering also needs to focus greater attention on the importance of osteogenically differentiating MSCs in a controlled 3D microenvironment. We observed the distinct advantage offered by culturing cells as aggregates (chapter 4) as well as by seeding whole bone marrow directly on 3D scaffolds, thus bypassing the 2D expansion phase (chapter 3). However, it must be admitted that lack of feedback in our current methods makes it difficult to provide a rigorously controlled microenvironment to the MSCs, which may in turn influence the ultimate outcome. This is of special significance when researchers attempt to create

special conditions such as hypoxia to stimulate angiogenic pathways or improve oxygen levels within the core of a construct to improve the survival and thus the bone formation by the implanted MSC [27]. Bioreactors which are intended as a means to generate and maintain a controlled physicochemical culture environment are a definite need, both for generating constructs with more uniform performance as well as for scaling up the tissue engineered constructs. However, the problems associated with operating most of the currently available bioreactor systems, has impeded the routine use of this technology in the tissue engineering field [28]. More research efforts should be focused on developing automated, commercially viable, safe and regulatory-compliant manufacture of bioreactors that can be applied within hospitals.

• Identifying markers to predict the in vivo bone potential of MSCs from different donors

Large donor-donor variation in the in vivo bone forming capacity of the MSCs is a major bottleneck in ensuring a reproducible clinical outcome using autologous cells. To overcome this drawback, chapter 6 focused on the use of allogeneic MSCs with the aim of identifying good MSC donors and using the MSCs from these selected donors as a ready source of progenitor cells for bone tissue engineering. However, our results demonstrated that allogeneic MSCs mount an immune response thus necessitating the use of concomitant immunosuppressant therapies. Another option would then be to identify genes using microarray techniques to predict the performance of the MSCs to form bone in an in vivo situation. Though such a strategy was described for the field of cartilage regeneration by multiple researchers [29, 30], there are currently no such strategies for the field of bone tissue engineering. Identification of such markers can then be used for development of specific antibodies against the marker. This in turn can offer opportunities to enrich cell populations with good in vivo bone forming capacity from a particular donor using cell sorting techniques, thus ensuring more reproducible clinical outcomes

• Investigating the fate of MSCs in vivo

To develop and optimize cell based tissue engineered constructs for implantation in the in vivo environment, detailed understanding of the behaviour and homing of MSC in vivo is required. Though in vitro studies can provide some information on the behaviour of the cells, a recent study indicates that more than 6500 genes are differentially regulated during bone healing [31]. This suggests a molecularly complex environment which is difficult to replicate ex vivo. Further, for introduction of cell based products into the market, the new European Union (EU) Directive 2009/120/EC, requests data on bio distribution, persistence and long-term

engraftment of the somatic cellular components [32]. Thus research into technologies such as quantum dot labeling or iron oxide based nanoparticle incorporation which can enable non-invasive tracking of the fate of the implanted cells can prove useful for both basic science research as well as for product development and business related purposes [33].

In conclusion, the field of bone tissue engineering has the potential to revolutionize treatment of orthopedic, maxillofacial, reconstructive and plastic surgery. Though a lot of work has been done in the past, newer approaches to consolidate and refine this work to develop products for clinical use are currently needed. The combined efforts of physicians, researchers and industry will eventually guide the tissue engineered products from the laboratory to the bedside.

8.4. References

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Selected abstracts for oral presentation

Chatterjea A, Alves H.A., Renard AJ, van Blitterswijk CA, de Boer J, *Generation of an osteogenic graft by direct seeding of bone marrow onto ceramic scaffolds*, Netherlands society for Biomaterials and Tissue Engineering (NBTE), Lunteren, The Netherlands, December 2007.

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Curriculum Vitae

Anindita Chatterjea was born on the 10th of March 1978 in Kolkata, India. She obtained her medical degree from Mumbai University in 2001. She specialized in Paediatrics and worked as a Paediatrician at various hospitals in India. She also experienced working in a neonatal intensive care unit within a tertiary hospital in Townsville, Australia. She moved to the Netherlands in 2005 and started working as a trainee researcher in the Biophysical Engineering Group, University of Twente. In September 2007, she started her PhD at the Tissue Regeneration group, University of Twente, under the supervision of Prof. Clemens van Blitterswijk and Prof. Jan de Boer. Her research primarily focused on streamlining and optimizing bone tissue engineering strategies in order to increase their routine use in the clinics. The results of her research are described in this thesis. During the course of her PhD, she obtained a research grant from the Anna Fonds foundation and did an internship at the Department of Biomedicine, University Hospital Basel in Switzerland. Apart from her research activities, she also served as the President of the Indian Students' Association, University of Twente and was a regular contributor to the English page section of the university newspaper, UT Nieuws.