# Embryonic Stem Cells in Bone Tissue Engineering

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# EMBRYONIC STEM CELLS IN BONE TISSUE ENGINEERING

# DISSERTATION

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by

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Born on July 11th 1976
In Gouda, The Netherlands

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# List of Publications

## Publications related to this thesis:


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**Both, S.K.**,van den Muizenberg, A., de Boer, J., van Blitterswijk, C. and de Bruin, J.D. (2007) Improved culture conditions for bone tissue engineering using human mesenchymal stem cells. Tissue Eng. 2007 Jan;13(1):3-9

Jukes J.M., **Both S.K.**, Post J.N., van Blitterswijk C.A., Karperien M., de Boer J. Chapter 1, Stem Cells, Tissue engineering textbook, to be published in April 2008, Elsevier

**Both, S.K**., van Apeldoorn A.A., Jukes, J.M., Englund M.C.O., Hyllner, H., van Blitterswijk, C.A. and de Boer, J. Differential bone forming capacity of osteogenic cells from either embryonic stem cells or bone-marrow derived mesenchymal stem cells. Submitted.

**Both, S.K**., Jukes, J.M., Leusink A., Sterk L.M.Th., van Blitterswijk, C.A. and de Boer, J. Effective tissue engineering of bone from embryonic stem cells through a process of endochondral bone formation. Submitted.

**Both, S.K**., Huipin, Y., van Blitterswijk, C.A. and de Boer, J. Osteo-inductive scaffolds stimulate osteogenic differentiation of embryonic stem cells in vitro. Manuscript in preparation.

**Both, S.K**. Fernandes, F.A.M. Huipin, Y, van Blitterswijk, C.A. and de Boer, J. Bone tissue engineering with embryonic stem cells in a rat cranial critical size defect. Manuscript in preparation.

**Both, S.K**., Sterk L.M.Th., Jolink C., van Asbroek-Kamphuis E.B.M., van Blitterswijk, C.A. and de Boer, J. Survival of a tumorigenic population of embryonic stem cells despite an intensive differentiation protocol. Manuscript in preparation.

## Selected abstracts

**Both, S.K**., Huipin, Y., Habibovic, P., van Blitterswijk, C.A. and de Boer, J. Calcium phosphate scaffolds influence the osteogenic differentiation of mouse embryonic stem cells. European Society for Biomaterials (ESB), September 11-15, 2005, Sorrento, Italy, poster presentation.

**Both, S.K**., Huipin, Y., van Blitterswijk, C.A. and de Boer, J.Calcium phosphate scaffolds stimulate the osteogenic differentiation of mouse embryonic stem cells. International Society for Stem Cell Research (ISSCR), July 29-30 August 1, 2006, Toronto, Canada, poster presentation.

**Both, S.K**., Jukes, J.M., van Blitterswijk, C.A. and de Boer, J. Human embryonic stem cells as a source for bone tissue engineering. Tissue engineering international & regenerative Medicine Society (TERMIS). October 8-11, 2006, Rotterdam,The Netherlands, oral presentation.

**Both, S.K**. Fernandes, F.A.M. Huipin, Y, van Blitterswijk, C.A. and de Boer, J. Bone tissue engineering in a rat cranial critical size defect with embryonic stem cells. Dutch society for Biomaterials and Tissue Engineering (NBTE), December 13-13, 2006. Lunteren, The Netherlands, oral presentation

**Both, S.K**., Jukes, J.M., van Blitterswijk, C.A. and de Boer, J. Endochondral bone formation by embryonic stem cells. Orthopaedic Research society (ORS), February 11-14, 2007, San Diego, USA, oral presentation.

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# Chapter 1

# General Introduction and aims

## Bone homeostasis

Bones form the support system which gives stability to vertebrates and is made of a highly specialized form of connective tissue that is constantly being remodeled. The human body contains 206 bones which can be divided into five categories: long bones (limbs), small bones (ankle and wrist), flat bones (skull), sesamoid bones (patella) and irregular bones (spine). Besides physical support, the bone is also important for blood production, calcium and phosphorus homeostasis, attachment of muscle and tendons, sound transduction, as a balance for excessive pH changes in the blood and as a temporary storage of heavy metals [1] [2]. Morphologically, two types of bone are identified; compact (cortical) bone, accounting for 80% of the bone mass and trabecular (cancellous) bone (Figure 1). Compact bone is extremely hard and is mainly located in the shaft of long bones and in the peripheral lining of flat bones. Trabecular bone has a low density and strength but has a very high surface area, and fills the inner cavity of long bones where metabolic responses occur. If we examine bone on the molecular level we can distinguish two types; lamellar and woven bone. Lamellar bone is the strongest and is formed by stacking numerous layers of collagen fibers. The fibers run in opposite directions in alternating layers, assisting in the bone's ability to resist torsion forces. The weak woven bone is abundantly present in newborns and contains randomly orientated

collagen. It is deposited in locations where fast bone formation takes place such as growth plates and during periods of repair.

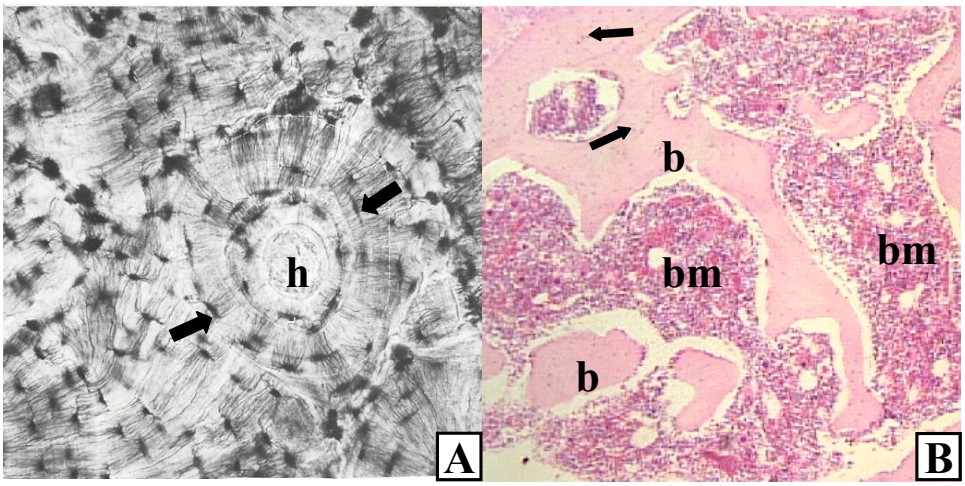


Figure 1. Compact and trabecular bone. *Cross section of compact bone with embedded osteocytes within the bone matrix stained with Indian Ink (A). The Haversian channel (h) that contains the blood vessels and nerves of the bone can be found in a round structure called an osteon. Osteocytes (arrows) can be found within this structure. Cross section of cancellous bone stained with haematoxilin eosin (B). The little specks within the bone (b) are embedded osteocytes (arrows). The spaces between are filled with bone marrow (bm).*

The main building blocks of bone are minerals (calcium phosphate and carbonate), an organic collagenous matrix and water. The minerals constitute for approximately 60% of the weight of bone and provide stiffness and strength. The collagen matrix, including cells, contributes for 30% of the bone weight. It provides flexibility and tensile strength and is composed of a network of proteins from which collagen type I is the most abundant [1, 3]. Other proteins in the bone extracellular matrix include osteocalcin, osteonectin and bone sialoprotein. In the organic matrix four cell types can be found; the tissue resorbing osteoclasts, the matrix producing osteoblasts, which in turn can differentiate into either matrix-embedded osteocytes or bone lining cells which rest along the surface of the bone (Figure 2).

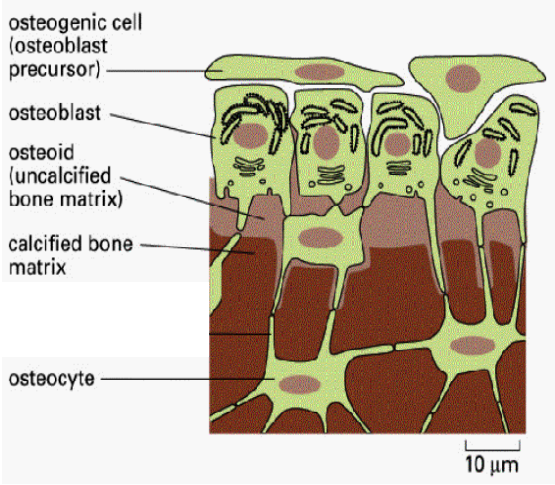


Figure 2. Deposition of bone matrix by osteoblasts *Osteoblasts lining the surface of bone secrete the organic matrix and are converted into osteocytes as they become embedded in this matrix. The matrix calcifies soon after it has been deposited* [77].

From these four bone cell types osteocytes are the most abundant type of cell found in the adult skeleton. Originated from differentiated osteoblasts they are smaller in size and contain less cell organelles [4]. It is believed that osteoclasts function as mechanosensory cells, thereby orchestrating the recruitment of the cells that form and resorb the bone [5, 6]. Osteoblasts are derived from pluripotent mesenchymal stem cells and they are the cells within bone that synthesize the extracellular matrix and regulate its mineralization [7, 8]. These cells have a cuboidal appearance and are located at the bone surface together with their precursors where they form a tight layer of cells. In the end osteoblasts may become “trapped” in their own calcified matrix, changing their phenotype and develop into osteocytes. The osteoclasts are from the haematopoietic lineage, whose precursors are located in the bone marrow (Figure 3). They have the ability to resorb fully mineralized bone and are located at sites called Howship’s lacunae or resorption pits. Macrophages also originate from the haematopoietic lineage and similar to macrophages, osteoclasts are extremely migratory, multinucleated cells, which harbour lysosomal enzymes, used for bone resorption [8]. The last type of bone cells are the lining cells, which originate from osteoblasts. They reside along the bone surface, thus regulating the

passage of calcium into and out of the bone. They respond to hormones by making special proteins that activate the osteoclasts.

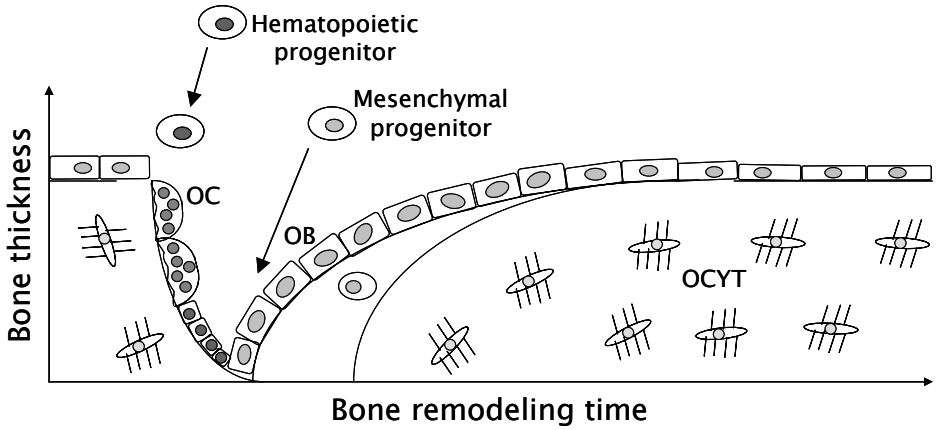


Figure 3. Remodeling of the bone in time. *Bone forming osteoblasts are incorporated into bone, become resting lining cells, or die by apoptosis. Upon mineralization of osteoid, cells are incorporated cells, now called osteocytes. OB, osteoblast; OC, osteoclast; OCYT, osteocytes. Revised with permission of the author R.L.van Bezooyen.*

## *Bone development and repair*

During embryonic development the skeleton is formed from three different cell lineages. Cells from the lateral plate mesoderm will give rise to the limb bud and long bones, mesoderm cells will give rise to the axial skeleton and cells from the neural crest will ultimately develop into the craniofacial skeleton [7]. There are two mechanisms by which the cells form the different types of bone. During intramembranous bone formation the mesenchymal stem cells differentiate into osteoblasts which in turn form the bone tissue. This process occurs for instance during the development of the skull. The other mechanism is referred to as endochondral ossification and involves the differentiation of mesenchymal stem cells into chondrocytes which form a cartilaginous matrix, which will calcify prior to chondrocyte apoptosis (Figure 4). Blood vessels will then penetrate the calcified matrix and osteoblasts follow. The calcified matrix is used as a template for osteoblast differentiation and ossification will occur [9]. Both mechanisms of bone formation may

occur when an injury or fracture occurs in the adult body. Upon bone trauma, healing will start with an inflammatory response, thus cleaning the fracture area. This is followed by the production of an extracellular matrix and the ingrowth of new blood vessels (angiogenesis). Ultimately bone formation is brought about via endochondral ossification or intramembranous ossification. This depends on the oxygen supply at the fracture site and possible changes in the anatomy of the bone. With a stable fracture and unchanged anatomy, intramembranous ossification occurs, whereas unstable fractures typically result in bone deposition through an endochondral process. At first, woven bone is deposited by the osteoblasts but when fracture healing has been completed, the woven bone will be remodeled into lamellar bone [10, 11].

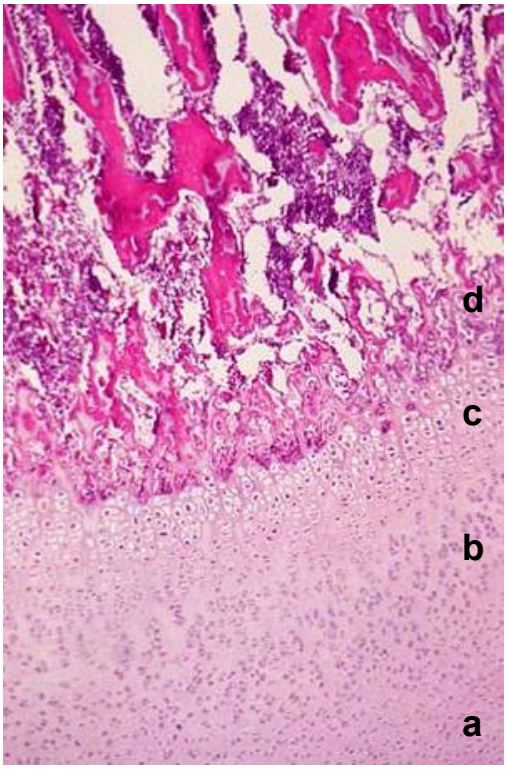


*Figure 4. Process of endochondral bone formation.*
*During the endochondral process the resting cartilage (a) will start proliferating (b), mature and will undergo hypertrophy (c). The chondrocytes which form a cartilaginous matrix, will calcify prior to chondrocyte apoptosis. Blood vessels will then penetrate the calcified matrix and osteoblasts follow (d). The calcified matrix is used as a template for osteoblast differentiation and ossification will occur.*

### *Remodeling of bone*

Bone does not only have self-healing capacity but it can also redefine its mass and morphology to respond to changes in mechanical loading. This adaptation of bone in response to loading was first described in 1892 and is referred to as Wolff's law which dictates that "form follows function" [12]. This adaptation is accomplished by a process called bone remodeling. During osteoclastic resorption pockets are left in the bone which are filled with new bone by osteoblasts. Thus, remodeling of bone results in the maintenance of bone mass. The process of remodeling is affected by several hormones. Via direct stimulation of osteoclast activity, parathyroid hormone (PTH) is able to release calcium and phosphate into the bloodstream while at the same time inducing bone resorption [13]. An estrogen hormone known as 17β-estradiol can protect bone by increasing the level of osteoprotegerin hormone, thereby inhibiting osteoclast differentiation and activation while promoting osteoclast apoptosis [14].

Although the process of remodeling continues throughout the entire lifespan of a human being, the bone mass will decline after the age of 35. Bone resorption then outpaces bone deposition and there will be a net loss of bone. This partial reduction of bone mass is referred to as osteopenia. However there are several bone diseases in which bone formation is affected (either through inhibition of osteoclast activity or induction of osteoblast activity) or in which resorption is affected (induction of osteoclast activity or inhibition of osteoblast activity). When a severe reduction of bone mass occurs it is referred to as osteoporosis, which substantially increases the risk of bone fracture.

### *Necessity of grating material*

Although bone has a tremendous self-healing capacity, there is a large group of patients that need surgical interventions, in which additional bone is required for optimal recovery. This is the case with patients suffering from extensive trauma, i.e. through car-accidents or after removal of a bone tumor. These patients have a so-called critical size defect, which is defined as the smallest size intra-osseous wound that

will not heal spontaneously during a life time [15]. In other words, the bone is not able to bridge the existing lesion. When this bridging does not occur the defect will be filled with fibrous tissue which undermines the structural stability of natural bone. Moreover, in spinal fusion or joint replacement surgery, additional bone tissue is required for optimal healing. Another frequently occurring clinical situation where extra bone tissue is needed is upon loosening of hip implants, which occurs due to bone resorption (osteolysis) at the interface between implant and the surrounding bone tissue (Figure 5). Hip motion is extremely painful for the patients, who therefore require a hip revision surgery. Ideally, the lost bone should be replaced with new bone. The most frequently used bone replacement is autologous or allogeneic bone, but both methods have their drawbacks. Autologous bone harvesting is accompanied by donor site morbidity and post-operative pain and autologous bone has limited availability [16–18]. Allogeneic bone carries the risk of immune reaction and disease transmission [19], although with current techniques such as freezing at very low temperatures and lyophilization these risks are minimized [20, 21]. Unfortunately these techniques do have some negative effect on the treated bone, like a higher resorption rate and slower formation of new bone tissue [18, 22].

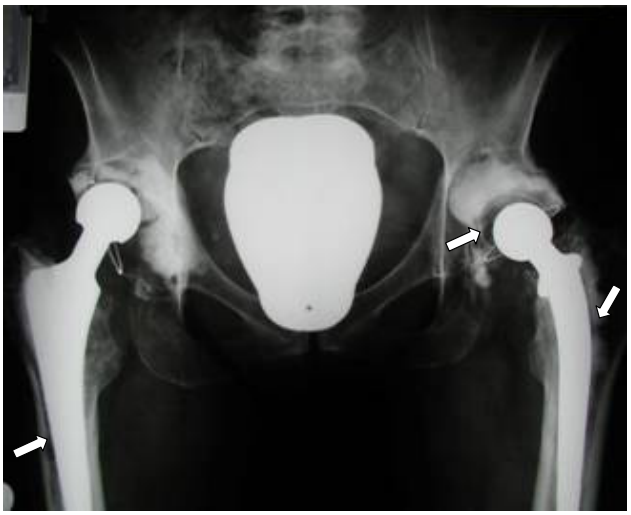

Figure 5. X-ray of a patient with loose implant. *Patients with loose cups and stems. Dark lines around the interface between the cement and bone indicate resorption and osteolysis (arrows).*

*Bone grafts substitutes*

When small bone defects occur, the bone tissue will heal itself, but larger defects can not be repaired and as described earlier natural bone grafts do have their drawbacks. Several bone graft substitutes have been developed in the past decades which provide an option for replacement of bone. A large range of different materials is available, both from organic and in-organic source, which are typically referred to as biomaterials. For instance, metal implants such as titanium and its alloys are frequently used in orthopedic and dental surgery for load bearing applications. When a surgeon performs a total hip replacement the new hip is often made of titanium or one of its alloys. This procedure is commonly used to treat joint failure caused by osteoarthritis. Although metal implants are biocompatible they present a low bonding strength with bone, which in time can result in osteolysis around the implant [23, 24]. If this occurs, a revision replacement may be performed. This is a more complicated surgery, and with each revision surgery, the life-span of the implant decreases. Over the last years several techniques have been applied to introduce microporosity in the implants, thereby providing a better surface for extra cellular matrix (ECM) deposition which has a positive influence on the osteoconductive properties [25, 26]. Because the metals are not biodegradable, they represent prostheses. A lot of research has been devoted to generate biodegradable materials which should aid in the full restoration of the bone defect. For instance, ceramics such as calcium phosphate, calcium carbonate and glass ceramics, are widely studied and used as bone graft substitutes. Particularly, the natural and synthetic forms of calcium phosphate (CaP) are of great interest as bone graft substitutes. CaP materials closely resemble the mineral composition, properties, and micro-architecture of human cancellous bone and they also have a high affinity for proteins [27]. CaP ceramics are often used to fill small bone defects, for example in dental surgery. Both the natural and synthetic form are classified by their chemical composition and include, β-tricalcium phosphate (β-TCP), biphasic calcium phosphate (combination of TCP and hydroxyapatite), hydroxyapatite (HA), and calcium-deficient apatite forms [28].

Interestingly, a number of the CaP materials have osteo-inductive properties [29-31], although the mechanism behind osteoinduction is still unclear. A second family of biomaterials is represented by several polymers which can be easily processed into porous three-dimensional customized structures and are therefore interesting materials for bone grafting. Another advantage of polymers is that growth factors, e.g. bone morphogenic proteins (BMPs), can be incorporated during fabrication and released after implantation in a controlled fashion [32]. Currently this type of biomaterial is not often used as a bone graft substitute, due to limited mechanical properties.

In contrast, demineralized bone matrix (DBM) is widely used in the clinic to heal small bone defects. DBM is made of cortical bone from which the calcium and phosphate as well as the cellular components, are extracted (demineralized). In a seminal paper, Urist *et al.* described how DBM from several species, when implanted in the muscle tissue, induces ectopic bone formation. Although the main component of DBM is collagen (93%), which provides a template for osteoconduction, the most important proteins in DBM are the BMPs [33]. Since DBM is readily available from human tissue banks and is cost-effective it is an attractive bone graft substitute. It is often applied in combination with other biomaterials and is mostly used to treat dental defects and in spinal fusions. In addition, DBM is less immunogenic than mineralized allograft since the demineralization process destroys the antigenic materials present in the bone. However also DBM has its drawbacks, the osteogenic activity is highly dependent upon the method of preparation and the osteoinductive capacity can be affected by the carrier material mixed with the DBM. Also a variation between batches of DBM can hardly be avoided since it is isolated from different donors. DBM is an attractive bone substitute but despite its bone forming capacity, it does not offer any structural or mechanical stability independently of its carrier [34].

An alternative to the strategies described above is the tissue engineering approach, in which osteogenic cells are combined with a biomaterial as a graft to restore the damaged tissue.

## Tissue engineering

The concept of “tissue engineering” was defined by Langer and Vacanti as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" [35]. In practice this term is used for materials or cells replacing or repairing whole or portions of tissues, but the term is also used for substitutes in which cells and materials are combined to create artificial support systems. To date the most successful and commercialized example of tissue engineering is the skin [36-38]. The first skin replacement products became available in 1997 and were composed of fibroblasts, extracellular matrix and a scaffold [37]. Another success story is that of the autologous chondrocyte implantation for patients with deep cartilage knee defects [39, 40]. For this procedure, healthy chondrocytes are obtained from a non-load bearing area of the injured knee, isolated and expanded in the laboratory. The cultured chondrocytes are then injected into the area of the defect. The defect is covered with a sutured periosteal flap to retain the implanted cells at the site of implantation. One of the latest breakthroughs is the tissue engineered bladder, by Dr. Atala and co-workers [41]. Using a bladder biopsy, urothelial and muscle cells were obtained and grown in culture. The cells were seeded onto a biodegradable bladder-shaped scaffold made of collagen. The autologous engineered bladder constructs were then used for reconstruction and implanted in patients. In the approaches mentioned above, tissue-specific cells are used, e.g. chondrocytes, keratinoctyes and urothelial cells but this approach is not feasible for all tissue types, among which bone tissue. Although it is possible to isolate osteoblasts directly from bone it would imply an extra surgery for the patient. With the advent of adult stem cells, a lot of effort has been devoted to the isolation and characterisation of adult sources of stem cells for bone tissue engineering.

*The discovery of stem cells and what defines them*

When a human body is fully grown it is estimated to contain a hundred trillion cells. However it all starts with a single cell. That single cell is the beginning of the formation of all 210 different cell types present in the body. Although this first cell has the potential to differentiate in all cell types, most cells present in a full-grown human body loose their multi-potency and acquire a specific function, like a heart or brain cell. Certain tissues such as blood, skin and bone are continuously rejuvenated. To support tissue maintenance and repair, these tissues contain a population of so-called stem cells, which are unspecialised cells capable of proliferation and self-renewal. Furthermore, stem cell progeny can give rise to specialized cell types, a property which could be useful for tissue engineering strategies. For instance, a heart attack (acute myocardial infarction) is caused by the interruption of blood supply to a part of the heart. This restriction of blood (ischemia) creates an oxygen and nutrient shortage resulting in damage and death of heart tissue. The damage is usually permanent since heart cells are not able to repair it appropriately, resulting in a higher risk of future heart attacks or even death. What if there was a stem cell able to repair this damage? This is one of the reasons scientists became interested in tissue-specific stem cells and their potential application in cell-based therapies.

It seems obvious that stem cells are present in tissues which constantly renew such as blood, skin and gut. But stem cells are also detected in brain and heart tissues that in the past where thought to lack renewal capacities. Evidence for this was that heart muscle cells (myocytes) of patients with cardiac failure were dividing (although not to such an extend that repair of damaged tissue was possible) and brain cells of adult mice were identified which were able to form neurons and glia cells [42, 43]. Although the role of adult stem cells is still not fully understood, these studies show us that adult stem cells have the ability to divide and replenish mature cells and it is likely that they thereby maintain and repair the tissue in which they are found [44]. The first adult stem cells were discovered in the 1960's by Till and McCulloch [45] [46]. They

demonstrated that in normal mouse haematopoietic tissue there is a class of cells which, on being transplanted into the spleen of heavily irradiated mice, can proliferate and form macroscopic colonies. The colonies formed in this manner were discrete and easy to count microscopically. Each colony appeared as a cluster of haematopoietic cells, many of which were dividing. Often, within a given colony, cells were observed from three lineages. In some cases, cells from these colonies could be transplanted to secondary hosts, where they were able to contribute to all blood cell lineages [47], which proved the existence of haematopoietic stem cells (HSC) in the bone marrow of adult animals. Further investigation uncovered some of the principles of stem cell biology as we know it today. Stem cells reside in 'niches', which are specific anatomic locations that regulate their participation in tissue generation, maintenance and repair. The niche ensures that stem cells are not depleted, while protecting the host from over-exuberant stem cell proliferation. It integrates signals that mediate the balanced response of stem cells to the needs of organisms. The interaction between stem cells and their niche creates the dynamic system necessary for sustaining the tissue [48]. The criterion of a stem cell is that it possesses the capacity for self-renewal, indefinite growth and the ability to differentiate into a specialized cell type. To identify the stem cell capacity of a given population of cells, mostly *in vivo* testing was performed in the past, whereby cells were transplanted from one animal into another and evaluated for tissue contribution. These days stem cell characterization is also performed *in vitro*, in particular by using stem cell specific cell surface markers which can be identified by antibodies, known as CD (cluster of differentiation) markers. Many CD markers have been identified over the years for certain populations of stem cells, or in some cases, the lack of certain markers. For instance: the mesenchymal stem cell population, as discussed below, is positive for STRO-1, ALP, CD29, CD44, CD90, CD105, CD106, CD146, CD166 and negative for CD34, CD45 and CD150. Over the years stem cells were identified in various tissue such as skin [49], fat, [50, 51] brain [52], muscle [53 54], pancreas [55] and dental pulp [56].

*Mesenchymal stem cells in bone tissue engineering*

A second type of stem cell was discovered in bone marrow, commonly referred to as mesenchymal stem cells but also known as marrow stromal cells or skeletal stem cells. MSCs were discovered a few years after the HSCs and were first described by Friedenstein *et al.* [57, 58] by virtue of their potential to adhere to tissue culture plastic. Due to their fibroblastic morphology, the cells were referred to as colony forming unit-fibroblast (CFU-F). Friedenstein further discovered that MSCs display multilineage differentiation, which eventually led to the name mesenchymal stem cell. With their research Friedenstein and co-workers created a basis for further investigation into the bone forming capacity of MSCs and their possible clinical application [57-61]. Although bone marrow is the main location from which MSCs are isolated for research, they can also be obtained from the femur, spine, sternum, tibia and adipose tissue. Bone is a complex structure that can only be formed *in vivo* and a suitable biomaterial is necessary to provide initial stability for the implanted cells. Some researchers combined autologous MSCs with an appropriate biomaterial and implanted these constructs in the patient, without additional *in vitro* osteogenic stimulation [62, 63]. Other researchers applied a different strategy in which the cells were differentiated towards the osteogenic lineage *in vitro* prior to implantation [64, 65]. In both ectopic and orthotopic implantation sites it has been demonstrated that this procedure leads to new bone formation.

Such a hybrid construct of a biomaterial combined with MSCs has the potential to become the alternative for autologous bone graft, if several problems are solved. To start with MSCs are obtained via a bone marrow aspirate. The skills of the surgeon taking the aspirate and the volume are of significant influence on the quality of the aspirate [66]. Then, in order to obtain sufficient amounts of cells to fill a critical size defect, extensive *in vitro* culture is required, which is associated with a loss in multipotency [67, 68]. For stimulation towards the osteogenic lineage compounds like dexamethason and BMP-2 can be added to the culture

medium [69–71]. After exposing the cells *in vitro* to dexamethasone and BMP-2, an increase in alkaline phosphates (ALP) expression can be measured by flow cytometry, indicating osteogenic differentiation. Literature mentions great variation in the level of ALP expression between donors. In addition, the *in vivo* bone forming potential is highly donor-dependent [68]. Unfortunately, so far it has not possible to correlate *in vitro* data to the *in vivo* bone forming capacities [72], which hampers clinical application of bone tissue engineering using hMSCs.

Before human clinical trials were initiated, large animals such as goats, dogs and sheep were used as models to proof the concept of bone tissue engineering. Autologous cells were isolated and combined with a biomaterial and used for spinal fusion or healing of a critical size defect [73, 74]. Although results indicate that only in the first weeks after implantation the hybrid constructs perform better than pure biomaterials, the studies did prove that bone tissue engineering is feasible. A number of phase I clinical trials have been conducted to demonstrate the safety of a bone tissue engineering approach. The good news from these studies is that the strategy is safe, but the first modest conclusion about efficacy was not very promising. For instance, our lab was involved in a clinical trial in which hMSCs were combined with CaP scaffolds and implanted in intra-oral defects of ten patients [75]. In only one patient there were strong indications that bone formation occurred by the implanted cells. In a parallel control study, control grafts were implanted in immune-deficient mice, and in only seven out of ten mice ectopic bone formation was observed. To solve the underperformance of human MSCs, we need a better understanding of the mechanism by which these cells produce bone. Currently the *in vivo* model is a “black box”. We do not know via which process bone is formed and which cells actually deposit the bone matrix. Bone could be formed by the implanted human cells or perhaps also by the host mouse MSCs. Staining of ectopically implanted hMSCs demonstrated that the implanted MSCs do contribute to ectopic bone formation, but which cells of the starting population contribute to bone formation is still unknown [76].

It is likely that there are components which have more impact on the osteogenic potential of MSC than the ones currently used. Investigating the signaling processes involved in MSC differentiation could provide some new insights. Steps in this direction have already been taken by researchers in our and other groups. If MSCs are to be used as replacements of the current grafting materials several problems need to be solved. Currently MSCs lack indefinite proliferation capacities and are not able to maintain their multipotency. Furthermore they can not be homogenously differentiated into the osteogenic lineage to form a sufficient amount of functional bone able to provide stability in an orthotopic site. The use of another cell source for bone tissue engineering is optional if such a cell source can overcome the current problems faced by MSCs.

### *Embryonic stem cells as a potential source of stem cells in tissue engineering*

Embryonic stem cells (ESCs) are a special group of stem cells since they can only be found during embryonic development. The formation of embryonic stem cells starts with the fertilization of an egg. The egg will then undergo mitotic division, in which exact replicas of the original cell are formed; this period is referred to as the cleavage stage. During the cleavage process there is neither cellular differentiation nor significant growth. The asymmetrical cell divisions, during cleavage, lead to the production of polar bodies. The side were the polar bodies reside is called the animal pole. When the embryo reaches an 8-16 cell stage, the cluster of cells is called a morula. The cleavage will continue and a cavity filled with fluid will form. The embryo then contains two distinct tissues: the trophectoderm and the inner cell mass and is known as a blastocyst [7, 77]. The undifferentiated inner cell mass (ICM) from the blastocyst stage can be maintained in culture as what we call embryonic stem cells (Figure 6).

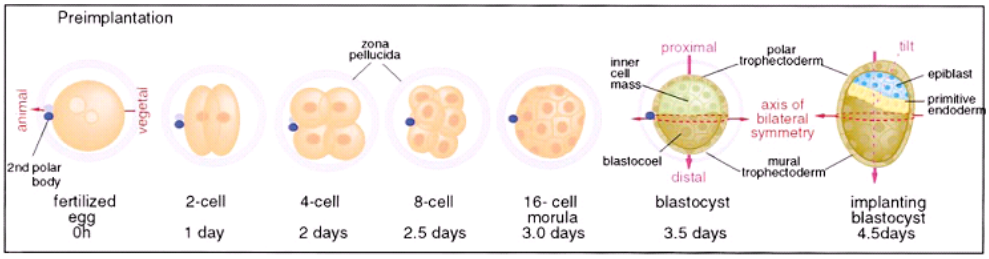


Figure 6. Embryonic development of a blastocyst. *Diagram of the first 4.5 days of mouse development from fertilization until the development of the blastocyst (not drawn to scale)* [7].

## *Embryonic stem cell culture*

From the inner cell mass (ICM) in the blastocyst it is possible to isolate ESCs which was done with mammalian cells for the first time in 1981 by two independent groups [78, 79]. By flushing the ovary ducts of a mouse 3.5 days after fertilization they obtained blastocysts. From these blastocysts the cells from the ICM were isolated and thereby they were able to maintain embryonic stem cells *in vitro*. To reach this ICM it is necessary to remove the outer layer (the trophectoderm) of the blastocyst. The ICM can then be separated from the rest and transferred to a culture dish. If the ICM adheres it can be examined for an undifferentiated morphology. The colony that grows out of these cells can be dissociated by the addition of trypsin and the cells can be replated (Figure 7). In 1998, Thomson *et al.* established the first human embryonic stem cell line [80] thereby providing the prospect of possible clinical application. Human blastocysts are obtained from surplus embryos after *in vitro* fertilization (IVF) and the ICM is isolated using the same methods used for the mouse ESCs. Unlike mouse ESCs, human ESCs do not respond well to dissociation by trypsin. The colonies of human ESCs are therefore mechanically dissected by cutting them in pieces with a knife made of a glass capillary. The pieces are then transferred to a new dish. Although dissociation procedures for human ESC lines are under development [81] the most used culture procedure is still mechanical dissection, thus making human ESC culture a very time consuming business.

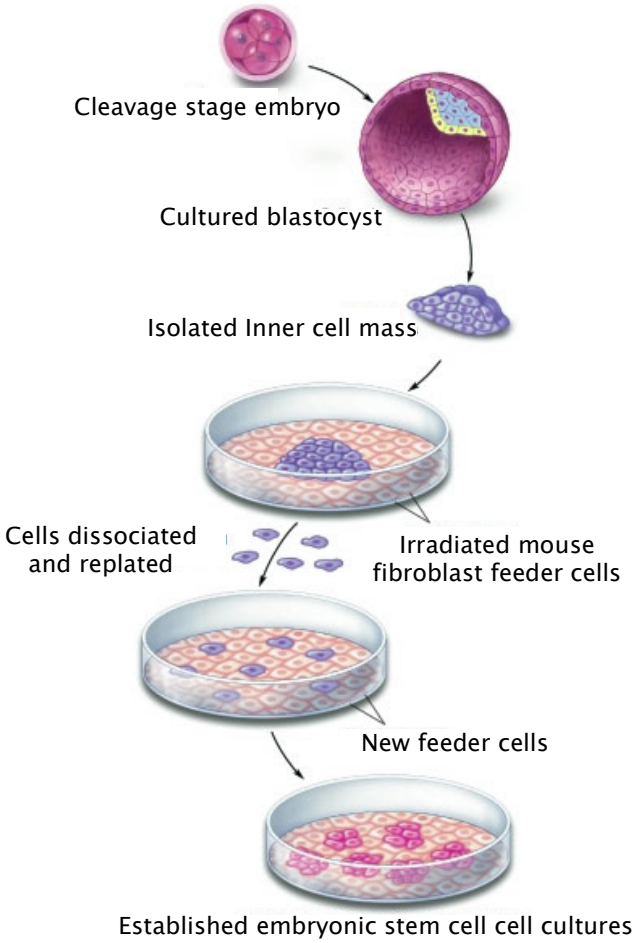


Figure 7. Embryonic stem cell culture. *The inner cell mass (ICM) from a blastocyst is isolated and placed in a culture dish. The colony of ESCs that grows out of these cells can be dissociated by the addition of trypsin and the cells can be replated.*

Maintenance of their undifferentiated state during *in vitro* expansion is important for all ESCs and there are various genetic factors involved. Oct3/4 is a transcription factor, which plays an important role in the processes of cell differentiation and embryonic development. Oct3/4 expression is found exclusively in early embryos, the germline, and

ESCs. In the embryo, Oct3/4 is essential to maintain pluripotency [82]. Without Oct3/4, embryos develop to the blastocyst stage, but the ICM cells are not able to differentiate into multiple lineages. Furthermore, continuous expression of Oct3/4 is required to sustain pluripotency [83]. Another important transcription factor is Nanog whose expression is first detected at the morula stage of a fertilized egg and declines after the blastocyst stage. Over expression of Nanog in mouse ESCs enhances their self-renewal. In the absence of Nanog, mouse embryonic stem cells differentiate into endoderm [84, 85]. Initially, mouse ESCs cultures retained their pluripotent state by growth on a layer of mouse embryonic fibroblasts (MEF). In 1988, researchers discovered that the addition of Leukemia Inhibiting Factor (LIF) to the medium was sufficient to maintain mouse ESCs in a feeder free culture [86] (Figure 8a). For the hESCs however, LIF does not provide the correct stimulus and culture on a MEF layer is still required (Figure 8b). In addition, the serum batch in which ESCs cells are grown is of great importance for maintaining their undifferentiated state. Serum contains bone morphogenetic proteins (BMPs) and by replacing the serum with BMPs pluripotency of mouse ES cells was maintained [87]. The BMP function is dependent on co-stimulation with LIF; in the presence of BMP alone cells will differentiate, whereas in medium with LIF but without serum or BMP, there is limited self-renewal. In light of possible future clinical application, maintenance of human ESCs in a xeno- and possible feeder free environment is essential. So far researchers have been able to replace the MEFs with human neonatal foreskin fibroblasts [88], adult skin fibroblast [89], matrigel and laminin [90]. However the optimal culture method which is both low in cost and feeder free has not yet been found.

Examination of the undifferentiated state of ESCs cells is accomplished via various tests. Because they can grow indefinitely and give rise to cell types from all three germ layers, implantation of ESCs in either the kidney capsule or testicular lumen of a mouse results in a teratoma. This is a tumor with tissue or organ components resembling normal derivatives of all three germ layers. Secondly, similar to the adult stem cells, there are stem cell markers such as Oct-4 and stage-specific embryonic antigens (SSEA3, SSEA4) to identify the embryonic stem cells.

If embryonic stem cells are truly in the similar undifferentiated state as when isolated from the blastocyst they should be able to produce chimeric mice. A chimera is formed when isolated ESCs of one animal are placed in the blastocoel of another animal. If the ESCs are undifferentiated they will take part in the embryonic development process and the resulting animal is a mix of both animals. Of course for ethical reasons such a test can not be performed with human ESCs.

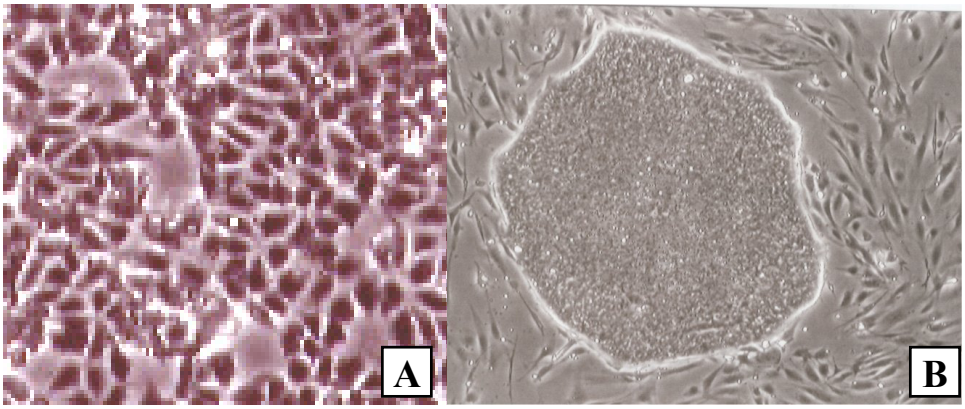


Figure 8. Embryonic stem cells in culture. *MESCs grown on a tissue culture flask in the presence of LIF (A). HESCS grown on a feeder layer of MEF (B).*

### *Differentiation of embryonic stem cells*

Differentiation of ESCs is usually triggered through the formation of so-called embryoid bodies (EBs), which can be formed by several techniques. The hanging drop method uses small drops of cell suspensions placed on a non-tissue culture plate, which is then turned upside down. Another method to produce embryoid bodies is by suspension culture in non-tissue culture treated plates or in spinner flasks. Either method prevents cells from adhering to a surface resulting in the formation of EBs [91] (Figure 9). Differentiation is initiated upon aggregation and the cells partly begin to imitate embryonic development. For this reason EB formation has been widely used as a trigger for *in vitro* differentiation of human and mouse ESCs. After 2-4 days in suspension culture, mouse ESCs form spherical EBs with a morula like structure. After 4-5 days, a central cavity within the EB is

formed, and at this stage the EB is called a cystic EB. Cystic EBs resemble the blastula stage, consisting of a double layered structure with an inner ectodermal layer and an outer endoderm closing the cavity. After 8-10 days in suspension culture, cystic EBs expand to larger cystic structures homologous to the yolk sac of post-implantation embryos [92-94].

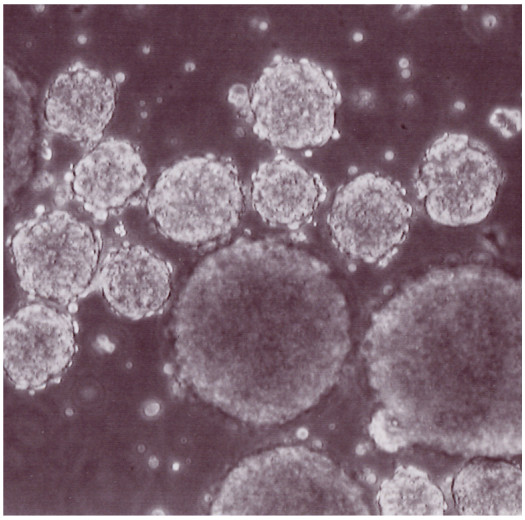

Figure 9. Embryoid bodies. *Embryoid bodies formed by mESCs in a suspension culture.*

Both mouse and human ESC have been induced *in vitro* to form cells from various tissue types such as heart, muscle, liver, brain, cartilage and bone. Although both mouse and human EB formation has thus far failed to induce a uniformly differentiated cell population, differentiation toward a specific lineage can be directed through several means, such as activation of endogenous transcription factors by exposure to reagents in the medium, through transfection of ESCs with transcription factors, exposure of ESCs to selected growth factors or co-culture of ESCs with cell types capable of lineage induction [95]. Noggin, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and retinoic acid are some of the known factors that induce ectoderm development [96, 97]. Co-culture with END-2 cells, BMP 4 and vascular endothelial growth factor (VEGF) induces ESCs into the mesoderm [98-100] while sodium butyrate and dimethylsulfoxide (DMSO) will trigger endoderm differentiation [101].

Although ESCs can be induced to differentiate into various lineages, it is likely that not all cells will follow the intended differentiation pathway. A growth factor like bFGF can influence ectodermal differentiation but also endodermal [102]. After initiating differentiation towards a specific cell lineage the desired cell type can be labeled with a specific antibody. Via cell sorting techniques, either fluorescent-activated cell sorting (FACS) or magnetic affinity cell sorting (MACS), the desired cells can be isolated from the rest. Thus a population of lineage-specific differentiated ESCs can be obtained for further use. Levenberg and co-workers demonstrated the feasibility of such an approach. They used FACS to isolate human embryonic stem cell-derived endothelial cells by using platelet endothelial cell-adhesion molecule-1 (PECAM1) antibodies [103]. They showed that the isolated embryonic PECAM1$^+$ cells, grown in culture, displayed characteristics similar to vessel endothelium. In addition, the cells were able to differentiate and form tube-like structures when cultured on matrigel. When transplanted in immune-deficient mice, the cells formed micro-vessels containing mouse blood cells.

### *Application areas for embryonic stem cells*

As discussed before, there is a broad range of future applications for embryonic stem cells in cell therapeutic approaches. Currently, there is no cure for diabetes but it was reported that human ESCs can be differentiated into insulin-producing islet-like clusters (ILCs) *in vitro* when grown under feeder-free conditions [104]. The temporal pattern of pancreas-specific gene expression in the hESC-derived ILCs showed considerable similarity to *in vivo* pancreas development, and the final population contained representatives of the ductal, exocrine, and endocrine pancreas. Another example is the differentiation of human ESCs into endothelial cells [105]. After transplantation into immune-deficient mice, the differentiated cells contributed to blood vessels that integrated into the host circulatory system and served as blood conduits for 150 days. HESCs could also contribute to cardiac repair. HESCs were differentiated into cardiomyocytes which integrated structurally and functionally with healthy host cardiac tissue, both *in vitro* in co-culture

studies and *in vivo* by pacing the heart in a pig model of slow heart rate [106-108]. All these studies indicate that embryonic stem cells have the potential to be used as regenerative "medicine". But if we truly want to use embryonic stem cells for future clinical therapies there are still several problems to be solved.

Firstly, an optimal differentiation protocol is needed which ensures that the cells are all differentiated into a specific lineage. There can be no remaining undifferentiated ESCs since they have the potential to form teratomas upon implantation. Furthermore, there is evidence that ES cells are genetically unstable in long term culture, and are especially prone to chromosomal abnormalities [109, 110]. Relative to early-passage lines, late-passage human ESC lines had more genomic alterations. Other challenges to be overcome are the allogenic origin, which may cause an immune rejection upon implantation and ethical problems since human ES cells are derived from aborted human embryos. The problem of immune rejection can be solved by a technique called somatic cell nuclear transfer. With this technique, the nucleus from an egg is replaced by the nucleus of a somatic cell [111]. A second option is to fuse an ES cells with a somatic cell [112, 113]. In this way the chromosomes of the somatic cell are reprogrammed via certain factors to an embryonic state. However this does create a cell with tetraploid DNA content. Furthermore both techniques are complicated and do not solve the ethical issue. In a recent breakthrough, several groups of investigators [114-116] demonstrated that a relatively small number of genetic factors define the ES cell identity and can even induce the somatic cell into an embryonic state. These factors are Oct-4 and Sox2, which have a function in maintaining pluripotency in ES cells as well as early embryos, and c-Myc and Klf-4, which contribute to the maintenance ES cell phenotype and their rapid proliferation. These four transcription factors were introduced in mouse fibroblast by retroviral introduction, thereby reprogramming these somatic cells into a pluripotent state. The cells are referred to as induced pluripotent stem (iPS) cells. The iPS cells displayed a similar growth pattern and morphology as ESCs, as well as expressing ES cell markers. Upon subcutaneous implantation in immune-deficient mice, teratomas were formed containing tissues originating from of all

three germ-layers. Although iPS cells were not fully identical to ES cells, they could by-pass the problems that ESCs face in future clinical applications. The latest discovery in this field is the formation of iPS from human somatic cells by Yu and co-workers [117]. The human iPS cells exhibit all the characteristics found in the iPS obtained from mouse cells. Such human induced pluripotent cell lines should be useful in the production of new disease models and in drug development as well as application in transplantation medicine once technical limitations are eliminated. Although clinical applications with human ESCs are not likely to be realized in the near future, they can serve as models in drug development and discovery. The ability to grow pure populations of specific cell types offers a proving ground for chemical compounds that may have medical importance. Treating specific cell types with chemicals and measuring their response offers a short-cut to sort out possible medicines. By rapid screening, hundreds of thousands of chemicals can be tested relatively easily which could reduce the amount of animal tests. Human development studies could also benefits from ESCs research. The earliest stages of human development are difficult or impossible to study. Human ESCs offer insights into early embryonic development which is difficult to study directly in humans. A better understanding of the events that occur at the first stages of development could have potential clinical significance for the prevention or treatment of infertility, birth defects, and pregnancy loss. A thorough knowledge of normal development could ultimately allow the prevention or treatment of abnormal human development.

### *Embryonic development of bone*

The route to bone tissue engineering with embryonic stem cells might differ from the one used for MSC. Therefore a better understanding of how embryonic stem cells develop into bone might hold a clue. After the formation of a blastocyst, as described earlier, a third tissue, a primitive form of endoderm, differentiates on the surface of the ICM (Figure 10). This primitive endoderm will only generate two layers known as parietal and visceral endoderm. The remainder of the ICM is called the epiblast, which will give rise to the entire foetus. A cavity will form in the centre of

the epiblast and it becomes a cup-shaped epithelial tissue. Embryonic patterning starts at 6.5 days after fertilization when the gastrulation process begins. Epiblast cells at one point transform to generate mesoderm and form the primitive streak. This point defines the posterior pole of the future embryo and therefore the opposite site is known as the anterior. In the next 12-24 hours the streak elongates from the rim of the cup to its distal tip. At this anterior end of the streak a specialized structure known as the node forms. The node gives rise to axial mesoderm, which will populate the midline of the embryo between the ectoderm and the definite gut endoderm [7]. Depending on their location, areas of the mesoderm will start differentiating and form the various mesodermal lineages: extra-embryonic mesoderm, lateral plate mesoderm, paraxial mesoderm and dorsal mesoderm.

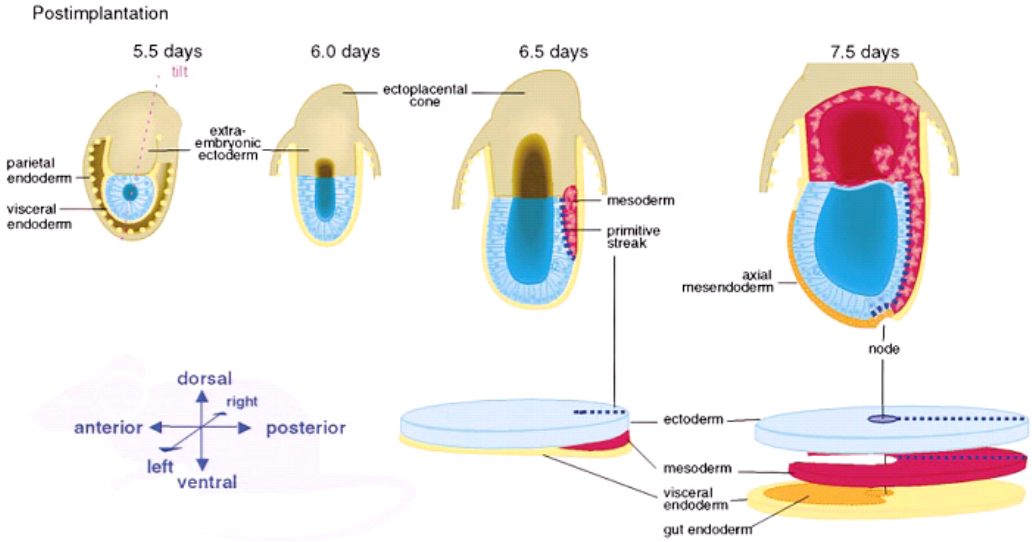


Figure 10. Embryonic development. *Embryonic development of a mouse from the blastocyst stage until the development of the three germ layers. The major axes of the embryo are shown superimposed on the profile of an adult mouse [7].*

Each of the three germ layers will develop in various tissue types, but here we will focus on the processes involved in bone development. The lateral plate mesoderm is split horizontally into the somatic mesoderm and the splanchnic (or visceral) mesoderm. When the somatic layer of the lateral plate mesoderm releases mesenchymal cells, limb development begins. The cells migrate and accumulate under the epidermal tissue of the neurula. On the surface of the embryo a circular bulge will appear which is called a limb bud. Then the mesoderm induces ectodermal cells to elongate and form a special structure the apical ectodermal ridge (AER). Once induced, this AER becomes essential to limb growth and interacts with the mesenchyme. In the limb bud the bone will be formed via endochondral ossification (described in bone development) [9]. Bone of the skull is formed via a different route. Part of the dorsal ectoderm will become neural ectoderm, and its cells become distinguishable by their columnar appearance, this region is called the neural plate. Via a process called neurulation the tissue of the neural plate forms a tube. The dorsalmost region of the neural tube will give rise to neural crest cells. The neural crest cells migrate extensively to generate an exceptional number of differentiated cell types among which many of the skeletal and connective tissue components of the head. Neural crest-derived mesenchymal cells proliferate and condense into compact nodules which will form bone via intramembranous ossification (describe in bone development) [9].

### *Embryonic stem cells in bone tissue engineering*

In contrast to MSCs, embryonic stem cells can be proliferated indefinitely. They differentiate into derivatives of all three germ layers and are easily genetically manipulated. These aspects make them a potential interesting cell source for bone tissue engineering, but moreover an ideal candidate as a model system since donor variation is excluded. The first data on *in vitro* differentiation of mouse ESCs towards bone forming cells were gathered by Buttery and co-workers [118]. Cells derived from embryoid bodies were treated with osteogenic factors such as dexamethasone and retinoic acid to stimulate differentiation.

Osteogenic differentiation was characterized by the presence of mineralized bone nodules that consisted of 50–100 cells within an extracellular matrix of collagen-1 and osteocalcin. Before long, other investigators showed similar results with mouse ESCs, including gene expression of a variety of osteogenic markers, like osteocalcin, osteopontin and Cbfa-1 [119–121]. So far, no *in vivo* research is performed on mouse ESCs to verify that the *in vitro* differentiated ESCs are capable of *in vivo* bone formation. The first results with human ESCs soon followed with similar differentiation protocols as used for the mouse ESCs [122]. They showed comparable results to mESCs. Bielby and colleagues were the first to implant human osteogenic ESCs in an immune-deficient mouse model and to evaluate the cells both *in vitro* and *in vivo* [123]. *In vitro,* they observed mineralized nodules that immunostained positively for osteocalcin and had an increase in expression of an essential bone transcription factor, Runx2. *In vivo,* only mineralized tissue was observed. Evidently, bone tissue engineering using ESCs is a largely unexplored area of research.

## Objectives of this thesis.

For more than two decades, MSCs have been used by many researchers hoping to create a substitute for autologous bone grafts. However there is still no substitute that is comparable to autologous bone. To reach this goal many problems need to be solved and the method by which these substitutes are created should be further optimized. In this thesis the emphasis will be on the osteogenic differentiation of embryonic stem cells. With respect to bone tissue engineering the ESCs are "the new kid on the block". Researchers have explored the osteogenic potential of ESC, but combining the cells with biomaterials and examining their *in vitro* and *in vivo* bone forming potential is a subject that is hardly touched upon. For that reason we will compare the performances of the ESCs to those of MSC and explore the opportunities for ESCs in bone tissue engineering.

The objectives of this thesis are:

- To define the optimum culture method of MSCs for use in clinical settings.
- Osteogenic differentiation of ESCs on calcium phosphate ceramics and the influences of the ceramic materials on the cells.
- Comparing the osteogenic potential of MSCs to ESCs *in vitro* and *in vivo*.
- Optimizing the bone forming capacities of ESCs at both ectopic and orthotopic sites.
- Exploring and reducing the risk of teratoma formation by ESCs.

To reach these objectives we have studied the influences of different culture media and plating densities on human MSCs in chapter 2. The cells were therefore characterized *in vitro* as well as *in vivo*. *In vitro* osteogenic differentiation of ESCs was examined in chapter 3. Furthermore, expression of osteogenic markers was evaluated after combining the ESCs with osteo-inductive and non-osteoinductive calcium phosphate ceramics. Chapter 4 contains a comparative study on

*in vitro* and *in vivo* osteogenic differentiation of human MSCs and ESCs of mouse and human origin. Since bone formation *in vivo* by ESCs is difficult and complex we studied in chapter 5 bone formation via an alternative process. This will be evaluated orthotopically in chapter 6 in a rat critical size cranial defect. As ESCs possess the potential to form teratomas we assessed the possibility to reduce them and examine if this potential is affected by the differentiation process in chapter 7. The final chapter contains a general discussion and conclusion on the performed studies.

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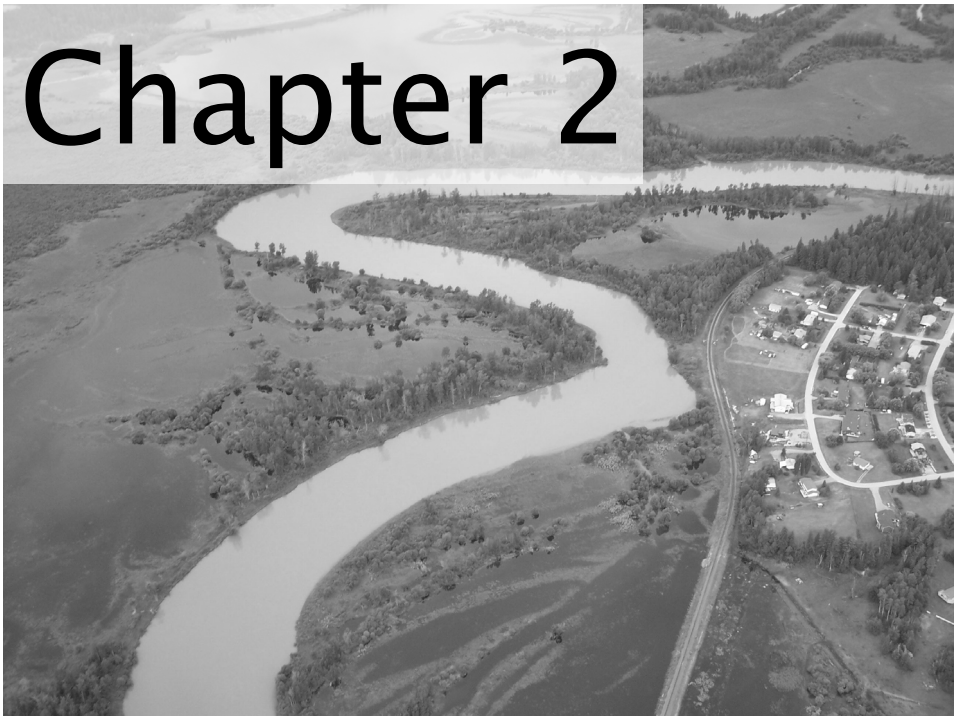
# Chapter 2



# Chapter 2

# A rapid and efficient method for expansion of human mesenchymal stem cells.

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## Abstract

During the past decade, there has been much interest in the use of human mesenchymal stem cells (hMSCs) in bone tissue engineering. HMSCs can be obtained relatively easily and expanded rapidly in culture, but for clinical purposes large numbers are often needed and the cost should be kept to a minimum. A rapid and efficient culturing protocol would therefore be beneficial. In this study, we examined the effect of different medium compositions on the expansion and osteogenic differentiation of bone marrow derived hMSCs from 19 donors. Furthermore, we investigated the effect of low seeding density and dexamethasone on both hMSCs expansion and their in vitro an in vivo osteogenic differentiation capacity. HMSCs seeded at a density of 100 cells/cm$^2$ had a significantly higher growth rate than at 5,000 cell/cm$^2$, which was further improved by the addition of dexamethasone. Expanded hMSCs were characterised in vitro based on a positive staining for CD29, CD44, CD105 and CD166. The in vitro osteogenic potential of expanded hMSCs was assessed by flow cytometric staining for alkaline phosphatase. In vivo bone forming potential of the hMSCs was assessed by seeding the cells in ceramic scaffolds followed by subcutaneous implantation in nude mice and histopathological assessment of de novo bone formation after 6 week implantation. Expanded hMSCs from all donors displayed similar osteogenic potential independent of the culture conditions. Based on these results we have developed an efficient method to culture hMSCs by seeding the cells at 100 cells/cm$^2$ in an αMEM-based medium containing dexamethasone.

## Introduction

The current and most frequently used approach for the treatment of large bone defects is autologous or allogeneic bone grafting. Both methods have distinct drawbacks, including donor site pain and limited availability for autograft, and the risk of immune reaction and disease transmission for allograft. The creation of a tissue engineered implant, which consists of a suitable biomaterial scaffold covered with the patient's own osteogenic cells, could provide an alternative approach.

Human bone marrow contains a multipotent cell population, which has the ability to differentiate into several mesenchymal lineages [1], including bone, cartilage, fat and muscle [2]. These cells were initially isolated by their adherence to tissue culture surfaces [3, 4] and are referred to as colony forming units fibroblasts (CFU-F), marrow stromal cells or mesenchymal stem cells (MSC). Due to their multipotent nature, these cells have attracted much interest for use in cell and gene therapy, as well as for treating large bone defects [5, 6]. When grown under osteogenic differentiation conditions, it has been shown that human mesenchymal stem cells (hMSCs) do not only possess in vitro osteogenic potential [7, 8], but can also form bone tissue *in vivo* as demonstrated by subcutaneous implantation in immune-deficient mice [9-11].

Several markers have been suggested for the identification and osteogenic differentiation of hMSCs [10-14]. HMSCs are positive for the surface expressed antigens endoglin receptors CD105 and CD44, the activated leukocyte cell adhesion molecule CD166 and β-integrin CD29 [12]. Developmental markers such as Alkaline Phosphatase (ALP) and STRO-1 can be used to characterize osteogenic and undifferentiated hMSCs, respectively [13-15].

A number of culture medium supplements have been reported that affect the expansion and differentiation of hMSCs. For instance, basic fibroblastic growth factor (bFGF) and lithium can enhance the proliferation of hMSCs [15, 16]. The addition of the synthetic glucocorticoid dexamethason (dex) to the medium, will induce hMSCs into the osteogenic lineage, thereby changing the shape of the cells [17] and

increasing the expression of osteoblast markers such as alkaline phosphatase [7, 18, 19].

Although in vitro osteogenic differentiation of hMSCs and proof of principle for the creation of an autologous tissue engineered implant has been shown [9, 20, 21], we still lack a well defined protocol for the isolation and expansion of these cells to make this technique available for clinical purposes. Surgical skills, volume of the aspirated bone marrow cells [22] as well as the donor age and variation in growth rate between different donors, significantly influence the creation of a successful living implant [9, 20, 21]. Besides commitment of hMSCs to the osteogenic lineage, a large number of cells are needed for creating a tissue engineered implant that can fill large bone defects. Since expansion of hMSCs is labour intensive and therefore expensive, the use of an optimal culture system is desirable. For example, based on using 8 million cells/cc scaffold [23], 20 cc of tissue engineered implant used for spinal fusion surgery would require about 160 million cells. Using previously established protocols [9, 19, 24], it would take 15-29 days and approximately 24 man-hours to reach a sufficient amount of cells. Although this protocol was previously used to create tissue engineered living bone equivalents, which were also used in a clinical trial [25], it was time consuming and labour intensive due to the amount of subculturing needed. There is therefore a need for an optimal protocol for expansion and osteogenic differentiation of hMSCs [20]. In this report we describe a method for the expansion of osteogenic hMSCs under optimized conditions to allow treatment of bone defects.

## Materials and methods

### *Harvest and culture of human mesenchymal stem cells*

Bone marrow aspirates were obtained from 19 donors, undergoing a total hip replacement surgery, who had given an informed consent (donor information in Table 1). Nucleated cells were counted in the aspirate and plated at a density of 500,000 cells/cm$^2$ in aMEM proliferation medium (see below; donors 1-15) or aMEM osteogenic

medium (see below; donors 11-15). The hMSCs from donors 16-19 were harvested and proliferated as described above and then cryopreserved. After defrosting, they were plated at 5,000 cells/cm$^2$ and grown in the different culture media as described below (n=5, for each donor).
Alpha MEM proliferation medium contained minimal essential medium (αMEM, Gibco), 10% fetal bovine serum of a selected batch (FBS, BioWhittaker, lot: 0S025F); 0.2 mM L-ascorbic acid-2-phosphate (Sigma), penicillin G (100 u/ml, Invitrogen); streptomycin (100 μg/ml, Invitrogen); 2 mM L-glutamine (Sigma) and 1ng/ml basic fibroblast growth factor (Instruchemie). Alpha MEM osteogenic medium was comprised of the proliferation medium supplemented with 10nM dexamethason (Sigma). DMEM proliferation medium comprised 10% fetal bovine serum (FBS; BioWhittaker), 1% non-essential amino acids 100x (Sigma), penicillin G (100 u/ml; Invitrogen), streptomycin (100 μg/ml; Invitrogen), 2mM L-glutamine (Sigma), 1 mM sodium pyruvate (Gibco), 1mM Hepes (Gibco) and 1ng/ml basic fibroblast growth factor (Instruchemie). DMEM osteogenic medium was comprised of DMEM proliferation medium supplemented with 10nM dexamethason (Sigma). The batch of FBS selected for the HMSCs in this study was tested *in vitro* for cell growth, expression of alkaline phosphatase (ALP) by FACS and the enzymatic expression of ALP and *in vivo* for bone formation of the hMSCs. The cells were grown at 37°C in a humidified atmosphere of 5% $CO_2$. Cultures were refreshed twice a week. When the cells reached near confluence (80-90%), they were washed with phosphate buffered saline (PBS, Sigma), detached with 0.05 % trypsin (Gibco), containing 1mM EDTA and subcultured.

### *Flow cytometry*

For flow cytometry, hMSCs from donors 1-15 were detached and centrifuged at 100 g for 10 minutes at 4°C. The cell pellets were resuspended in PBS containing 5 % bovine serum albumin (Sigma) and 0.05 % $NaN_3$ (Sigma) and stored on ice for 15 minutes. Next, the cells were incubated in block buffer containing control mouse anti-human IgG2a (Dako); anti-ALP antibody (hybridoma B4-78), anti-CD29

monoclonal antibody (Dako), anti-CD44 (Pharming) antibody, anti-CD105 (Dako) antibody or anti-CD166 (RDI) monoclonal antibody. The cells were incubated on ice for 45 minutes and washed three times with PBS containing 1% BSA and 0.05% $NaN_3$ (wash buffer). Incubation with the secondary antibody, goat anti-mouse-IgG-FITC (Dako), was performed for 45 minutes on ice. After washing the hMSCs three times with wash buffer, the cells were incubated for 10 minutes with 10 µl Viaprobe (Becton Dickinson) for live-dead staining. The cell suspension was analysed using a flow cytometer (Becton Dickinson). From each sample ten thousand events were collected in duplicate and the data was analysed via Cell Quest software (Becton Dickinson). For statistical analysis of the percentages of positive cells of the donors a student t-test was performed, significance was determined at $p < 0.05$.

### *Alkaline Phosphatase activity*

The enzyme activity of Alkaline Phosphatase was measured in triplicate for donors 16-19 by washing the cells with PBS and incubating them for 10-15 minutes in a 20mM paranitrophenol solution (Sigma) in 1M diethanolamine (Sigma), 1mM $MgCl_2$ (Sigma). The activity of the Alkaline Phosphatase enzyme was measured with a micro plate reader (BIOTEC instruments) at 310nm and normalised for cell number.

### *In Vivo implantation*

To assess the *in vivo* bone forming capacity of various passages of hMSCs from donors 1 to 15, they were seeded onto porous biphasic calcium phosphate (BCP) scaffolds (2-4mm) and implanted in nude mice. The hMSCs were seeded by adding a 100 µl drop cell suspension, containing 200,000 cells, on each biphasic calcium scaffold scaffold (n=6 per experiment). After 2 hours of adherence, the medium was added and scaffolds were cultured for 7 days in αMEM osteogenic medium. Prior to implantation, the particles were placed in serum free medium for approximately 1-2 hours and washed 3 times with PBS. Immunodeficient mice (HsdCpb:NMRI-nu, Harlan) were anaesthetised by an intramuscular injection of 0.05 ml Ketamine, Xylazine and Atropine

mixture. Before surgery, the skin on both lateral sites of the spine was cleaned with 70% alcohol and 6 subcutaneous pockets were created in each mouse. In each pocket, three tissue engineered samples were inserted and each culture condition was divided over three mice. Six weeks post-operatively, the animals were euthanized by $CO_2$. The explanted samples were fixated in 1.5% glutaraldehyde (Sigma) in 0.14M cacodylic acid buffer, dehydrated by increasing ethanol steps and embedded in methylmethacrylate (Sigma). Sections were made using a diamond saw (Leica SP1600) and stained with 1 % methylene blue (Sigma) and 0.3 % basic fuchsin solution (Sigma) [26].

| Donor | Aspiration site | Sex | Age | αMEM versus DMEM | 100 cells/cm² versus 5000 cells/cm² | +/- dexamethason | In vivo | Days needed to reach 200x10⁶ cells | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | 100 cells | 5000 cells |
| 1 | Crista | F | 72 | | √ | | √ | 15 | 18 |
| 2 | Crista | F | 87 | | √ | | √ | 19 | 24 |
| 3 | Crista | F | 61 | | √ | | √ | 21 | 29 |
| 4 | Crista | F | 63 | | √ | | √ | 16 | 17 |
| 5 | Crista | F | 69 | | √ | | √ | 19 | 23 |
| 6 | Acetabulum | M | 29 | | √ | | √ | 12 | 15 |
| 7 | Acetabulum | F | 63 | | √ | | √ | 15 | 19 |
| 8 | Acetabulum | M | 56 | | √ | | √ | 12 | 19 |
| 9 | Iliac Crest | F | 28 | | √ | | √ | 14 | 20 |
| 10 | Acetabulum | F | 25 | | √ | | √ | 13 | 16 |
| 11 | Crista | F | 77 | | √ | √ | √ | 14 | 15 |
| 12 | Acetabulum | F | 44 | | √ | √ | √ | 16 | 18 |
| 13 | Iliac Crest | F | 26 | | √ | √ | √ | 14 | 16 |
| 14 | Acetabulum | M | 48 | | √ | √ | √ | 16 | 18 |
| 15 | Iliac Crest | M | 56 | | √ | √ | √ | 16 | 19 |
| 16 | Acetabulum | F | 28 | √ | | | | | |
| 17 | Acetabulum | M | 49 | √ | | | | | |
| 18 | Iliac Crest | M | 42 | √ | | | | | |
| 19 | Iliac crest | F | 36 | √ | | | | | |

Tabel 1: Donor and experiment information (M=male, F=female).

## Results

### *Effect of culture medium on hMSC growth*

The cells of donors 16-19 were cultured for two passages in the two different basal media. When hMSCs were proliferated in αMEM for 4 days the amount of cells obtained was significantly higher for al 4 donors compared to hMSC proliferated in a DMEM proliferation medium, regardless the passage number (n=4 for each donor, $p<0.05$) (Fig 1a).

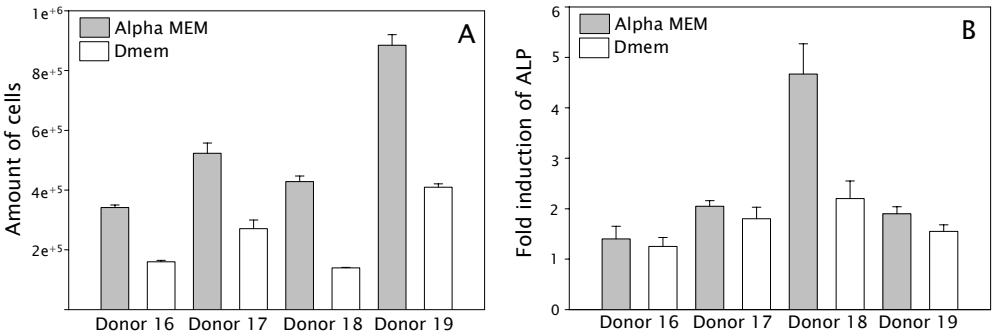


Figure 1. A. Proliferation of hMSCs in αMEM versus DMEM media. *When hMSCs were proliferated in αMEM for 4 days, the amount of cells obtained was significantly higher for al 4 donors compared to hMSCs proliferated in a DMEM proliferation medium regardless the passage number (n=4, p<0.05).* B. Effect of αMEM or DMEM medium on alkaline phosphates enzyme activity. *HMSCs were proliferated in αMEM and DMEM proliferation and differentiation medium the ALP enzyme activity was measured as activity per cell. The fold increase in ALP enzyme activity of the proliferation versus osteogenic medium is displayed in the graph.*

To investigate the effect of different media on the osteogenic potential of hMSCs, the cells were cultured for 7 days in osteogenic αMEM- and DMEM-based medium. When grown in αMEM-based medium, all four donors (16-19) displayed increased ALP enzyme activity when dexamethasone (dex) had been added (differentiation medium). In DMEM-based medium, this difference was less apparent (fig 1b). In

addition, the overall ALP enzyme activity was higher when the cells were cultured in αMEM medium as compared to DMEM.

### *Effect of seeding density and dexamethasone on hMSCs proliferation.*

With all 15 donors tested, the cultures seeded at a density of 100 cells/cm$^2$ reached 200 million cells within 12-21 days of passage one (figure 1). In contrast, hMSCs seeded at 5,000 cells/cm$^2$, as described in our previous culturing protocol [9, 19], had to be subcultured for at least two passages (most donors three passages) and cultured for 15-29 days, before 200 million cells were reached (fig 2, table 1).

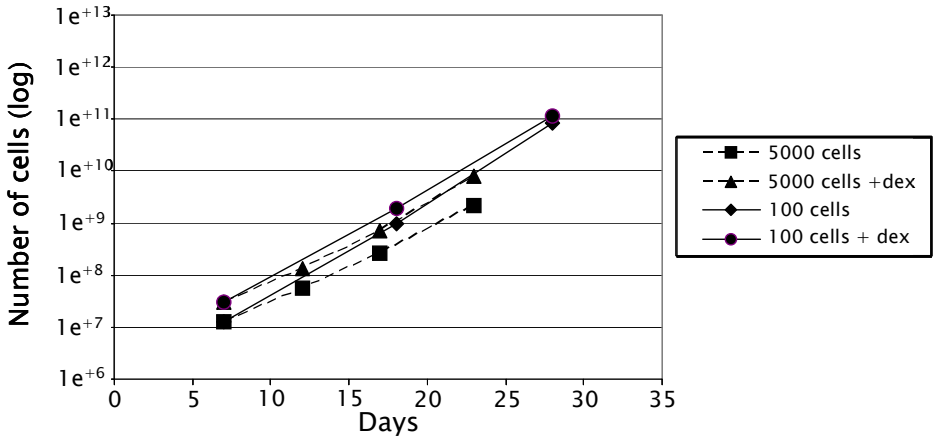


Figure 2. The effect of seeding density and culture medium on hMSC proliferation. *Progressive cell numbers are expressed against culture time. The cells were seeded at 5,000 or 100 cells/cm$^2$ in proliferation and osteogenic medium (n=5 for each donor). Error bars for each passage were too small to be visualised in the graph. Because of variants in proliferation rate between donors a representative graph of the growth curve of donor 2 is given.*

By using the lower seeding density, the cultures therefore reached 200 million cells approximately 4.1 days earlier compared to cells seeded at 5,000 cells/cm$^2$. Dexamethasone stimulates differentiation of hMSCs and some hMSCs culture protocols use it throughout the entire culture period [27]. Therefore we investigated the effect of dexamethasone on hMSCs proliferation by culturing them in either proliferation medium or osteogenic medium throughout the entire proliferation period. The amount of cells obtained from the primary culture was higher for all five donors when grown in osteogenic medium compared to proliferation medium, also at later passages independent of the seeding density (fig 3). Furthermore dexamethason appeared to stimulate hMSCs proliferation at later passages as well.

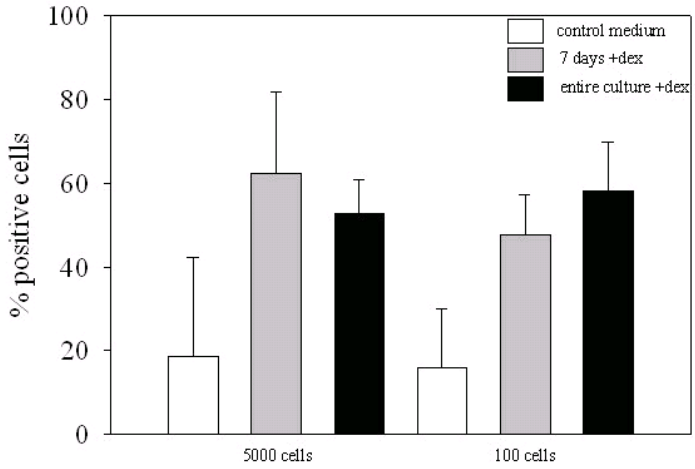


Figure 3. The effect of seeding density and culture medium on the expression of ALP of hMSCs. *The percentage positive cells expressing ALP of donors 6-11 were measured after proliferating the cells at 5,000 and 100 cells/cm2. Cells proliferated without dexamethasone were cultured without (A) and with (B) dexamethason for 7 days, or they were continuously cultured in the presence of dexamethasone (C). A significant increase of ALP activity is seen for all 6 donors when dexamethasone is present in the media,, regardless the proliferation medium previous used ($p<0.05$). Proliferating hMSCs with or without dexamethasone has no significant influence on the ALP expression and also seeding densities have no significant effect.*

*Osteogenic differentiation*

Cells grown at 100 cells/cm² in the presence of dexamethasone, resulted in the fastest and most labour efficient method to expand hMSCs. To confirm that hMSCs expanded using this protocol retained their osteogenic potential, we first analysed the effect of seeding density and dexamethasone on the hMSC phenotype with a panel of hMSCs markers. The hMSC markers CD29, CD44, CD105 and CD166 were expressed in hMSCs of all donors (1-15) in all culture conditions and the expression varied between 95-100% (Fig. 4).

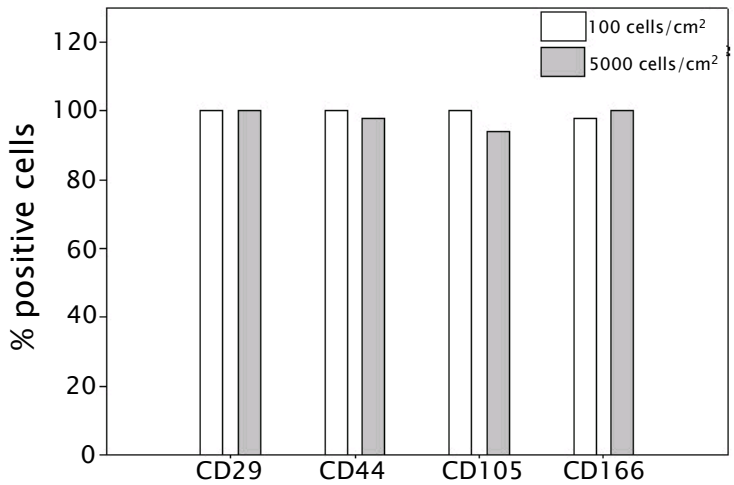


Figure 4. The effect of seeding density on the expression of the hMSCs related markers CD29, CD44, CD105 and CD166. *Cells of donor 7 were cultured at a seeding density of 100 cells/cm² and 5,000 cells/cm² in proliferation medium until the amount of 200 million was reached.*

To investigate whether the culture conditions had an influence on the osteogenic potential of the hMSCs we measured expression of the osteogenic marker ALP. From previous experiments (data not shown) we determined that after approximately 7 days of culture in the presence of dexamethasone, ALP expression of the cells reaches its optimum. To assess if the continuous presence of dexamethasone had an influence on the osteogenic potential of the hMSCs, cells previously cultured without

dexamethason were also cultured with dexamethason for 7 days. At both seeding densities, there was a significant increase in the ALP expression when the cells were cultured in the presence of dexamethasone compared to cells cultured in proliferation medium (Fig. 3). ALP induction was similar between cells seeded at different densities and with comparable population doublings. There was no significant difference on the induction of ALP by dexamethason, irrespective of whether hMSCs had been cultured in previous passages in the presence of dexamethason or only for 7 days.

### *In vivo bone forming potential of hMSCs*

After six weeks of implantation the samples of all donors exhibited direct deposition of bone tissue, irrespective of seeding density or presence of dexamethason in the culture medium. The newly formed bone comprised osteocytes embedded within the bone matrix and layers of osteoblasts (fig 5). Besides fibrous tissue and newly formed bone no other connective tissue formation was observed with any of the donors. Microscopic observation was performed blinded by three independent observers, who each examined 5-8 slides of each implanted condition. Between the different donors the amount of newly formed bone varied but not within the different culture conditions from one single donor.

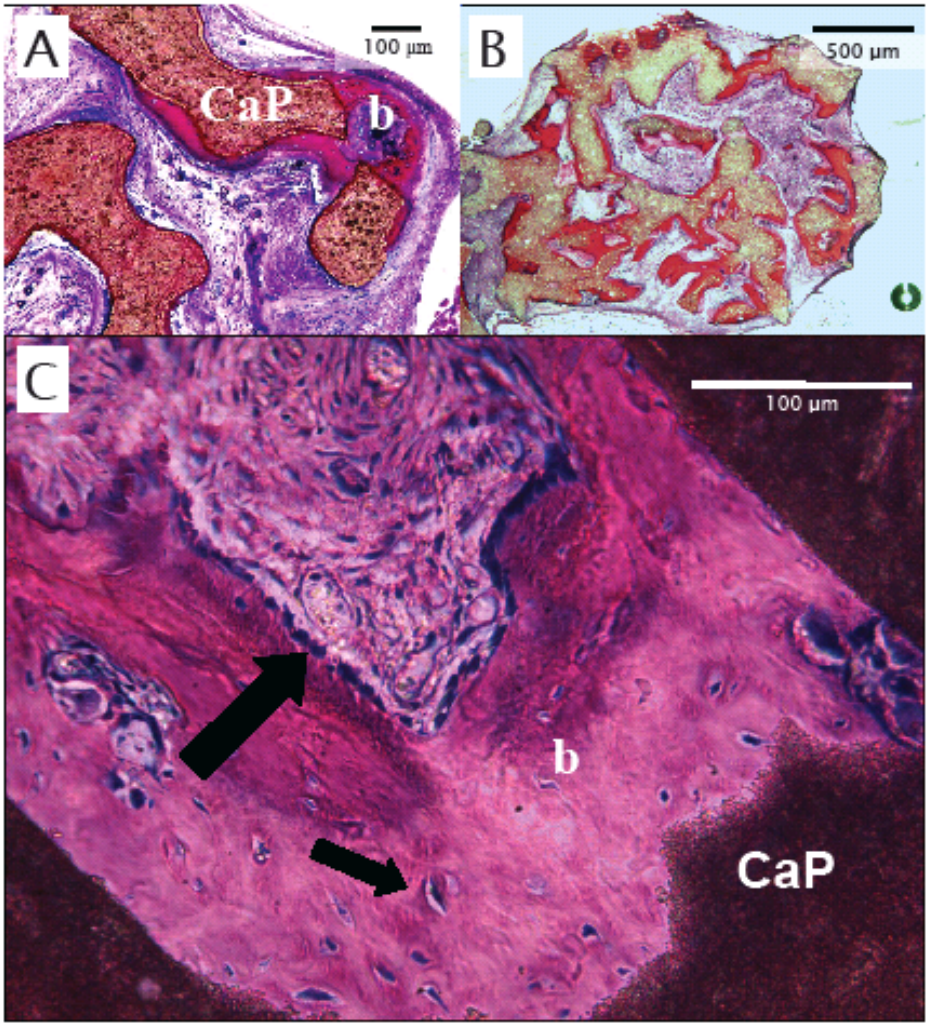


Figure 5. In vivo bone formation by hMSCs. *Bone was formed on porous BCP particles after 6 weeks of subcutaneous implantation in nude mice. The newly formed bone (b) was deposited against the material (CaP) surfaces (a,b,c,) in some samples there was an abundant amount of newly formed bone present (b). Embedded osteocytes (small arrow) and a layer of osteoblasts (big arrow) were observed within the newly formed bone. Irrespective of culture medium or culture method; hMSCs from all donors (1-15) exhibited in vivo bone formation.*

## Discussion

In this study, we demonstrate that the use of an αMEM-based medium is superior for both expansion as well as the osteogenic differentiation of hMSCs, as compared to a DMEM based medium. The expansion of hMSCs at low density results in a higher proliferation rate compared to cells proliferated at a higher density, which is further stimulated by the addition of dexamethason. Both seeding densities and the addition of dexamethasone do not interfere with the osteogenic *in vitro* and *in vivo* potential of the hMSCs.

The use of αMEM or DMEM medium for the expansion of hMSCs, has been investigated before by Jaiswal et al. [7]. Interestingly, they found that fold increase of ALP enzyme activity was much higher when cultured in DMEM, which is in contrast with our results. Moreover, they also state that mineralization was sparsely detected in hMSCs cultured in αMEM osteogenic medium, which is again in contrast with previous found results [28]. In this paper we describe that hMSCs proliferate faster at lower seeding densities. To investigate the optimal density, we tested the growth rate of the hMSCs also at densities lower than 100 cells/cm$^2$ and concluded that the growth rate at those lower densities was even higher then when seeded at 100 cells/cm$^2$ (data not shown). Unfortunately, at these low densities the cells form a few isolated cell colonies, which do not fill up the entire culture flask. Therefore, we concluded that seeding 100 cells/cm$^2$ represents the most efficient expansion. To create an even better expansion environment for the hMSCs, it might be beneficial to refresh not the entire but part of the culture media. With refreshing the entire culture, media components secreted by the cells will be removed and the cells will have to adept to the new environment. When cells are seeded at low density, they display a lag phase of 3-5 days before going into a rapid exceptional growth phase. At the end of the lag phase it has been reported that the cells secrete high levels of dickkopf-1(DKK-1), inhibitor of the Wnt-signaling pathway [29]. High levels of DKK-1 allow the hMSCs to re-enter the cell cycle and therefore being responsible for the rapid growth. However,

this data is in contradiction with the results of de Boer et al [16], which demonstrate that the addition of Lithium, a stimulant of the Wnt-signaling pathway, increases the expansion rate of hMSC. At this moment no real conclusion can be made to what causes the cells to grow at a higher rate when seeded at low densities, very likely several different factors contribute to this phenomenon.

The effect of increased expansion of hMSCs cultured at lower densities, has also been reported by Colter et all [30]. They reported that there might be a minor population of small agranular cells, also referred to as recycling stem cells, that give rise to new population of small densely granular cells and are thereby responsible for the rapid expansion when hMSCs are seeded at lower densities. They also reported that their hMSCs in replated low density passages, retained their ability to generate single cell derived colonies and thereby retaining their multipotentially for differentiation. This is in contrast with an observation we made. We observed that the osteogenic potential of the cells *in vitro* but also *in vivo,* seemed to be decreasing with repeated passages of the hMSCs, indicating that the cells might lose their multipotentially for differentiation. This change can be observed in vitro by flow cytometry. It has been reported by Mendes et al. [18], that an increase of ALP induction by dexamethasone is related to the occurrence of bone formation in vivo. The addition of dexamethasone to the medium is not a new concept. Rat MSCs cultures are always expanded in the presence of dexamethason [27, 31], but for hMSCs it is almost only used for differentiation of the cells and not for expansion. We clearly show herein that dexamethason does not only influence on the osteogenic potential of hMSCs but also enhances their proliferation rate, without interfering with the *in vivo* bone forming capacities of the hMSCs. In conclusion, based on these results the optimal way to rapidly expand hMSCs is by seeding the cells at the density of 100 cells/cm$^2$ in an α MEM based medium containing dexamethasone.

## Acknowledgement

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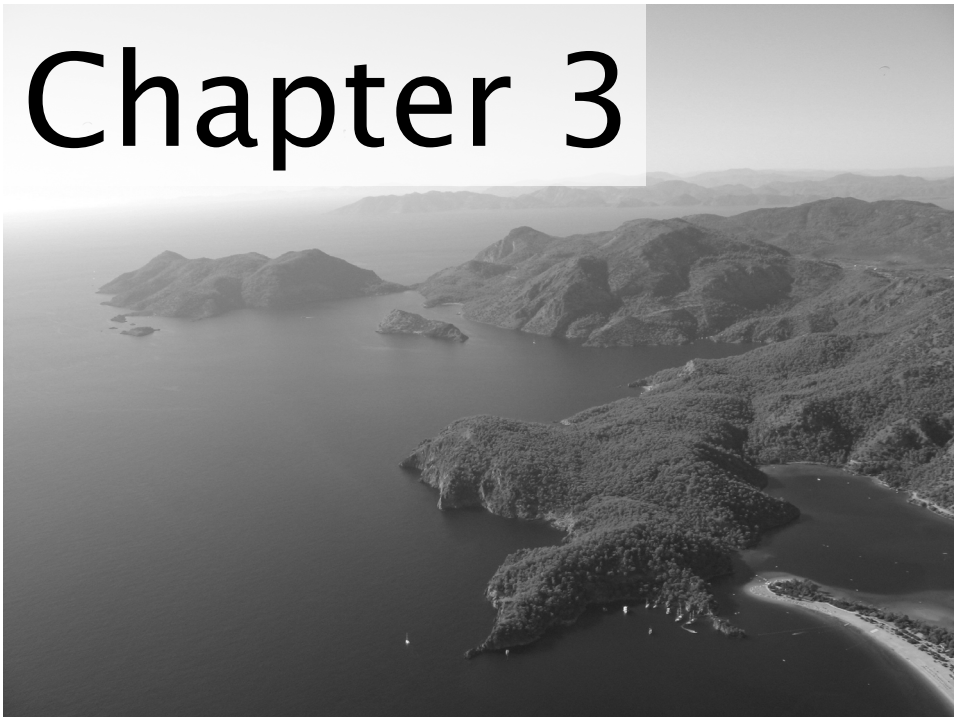
# Chapter 3



# Chapter 3

# Calcium phosphate ceramics stimulate osteogenic differentiation of embryonic stem cells by influencing the culture medium composition.

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*Twente University, Institute for Biomedical Technology, Drienerlolaan 5, 7522 NB Enschede, The Netherlands*

## Abstract

Calcium phosphate ceramics used in bone replacement surgery display different osteogenic properties when implanted in osseous and non-osseous sites in goats. Tri-calcium phosphate (TCP) and bi-phasic calcium phosphate ceramics sintered at 1150°C (BCP1150) induce bone whereas bi-phasic calcium phosphate sintered at 1300°C (BCP1300) does not. Currently, no *in vitro* osteogenic model system has been able to replicate these results. We therefore assessed whether mouse embryonic stem cells (mESCs), which can be induced into the osteogenic lineage, could be used as a model.

First, qPCR of osteogenic markers revealed that differentiation of mESCs on calcium phosphate scaffolds was enhanced compared to mESCs differentiating on tissue culture plastic. Interestingly, differentiation was more robust on BCP1150 and TCP than on BCP1300, reflecting the *in vivo* osteogenic behaviour. We investigated whether the ceramics influence differentiation by affecting medium composition and detected a strong decrease in calcium and phosphate concentrations in the medium after addition of BCP1150 and TCP but not in BCP1300-treated medium. In agreement with this, we observed higher protein absorption to BCP1150 than to BCP1300, but we found no qualitative differences by SDS-PAGE. In conclusion, osteogenic differentiation of mESCs is clearly enhanced by calcium phosphate ceramics at least in part by influencing medium composition. mESCs can be used as a cell source for further investigating the osteogenic potential of different calcium phosphate ceramics and the underlying molecular mechanism

## Introduction

The golden standard for bone replacement surgery is autologous or allogeneic bone grafting. However, this method has its drawbacks, such as donor site complications [1], limited availability and the use of allogeneic bone furthermore carries the risk of disease transmission. A promising alternative is tissue engineering autologous bone. Creation of a tissue-engineered implant, which consists of a suitable biomaterial scaffold covered with the patient's own osteogenic stem cells, provides an alternative approach. Mesenchymal stem cells isolated from the bone marrow are multipotent and have the ability to differentiate into several mesenchymal lineages [2] including bone, cartilage, fat and muscle [3]. By combining biomaterials with human mesenchymal stem cells, we and others have demonstrated *in vivo* bone formation [4] [5] [6, 7], providing prove of principle for clinical use of a tissue engineered construct.
Calcium phosphate (CaP) ceramics have a chemical composition similar to bone and are therefore used as synthetic bone graft substitutes[8]. However, there is a large variety of CaP ceramics available with various compositions and porosities, several of which have osteo-inductive properties [9], [10-13]. We and others have demonstrated that osteo-induction of biphasic calcium phosphate (BCP) ceramics in goat muscle correlates to the microporosity of the material, which in itself may correlate with the sintering temperature of the ceramics[14]. Further improvement of osteo-inductive properties of ceramics is hampered by the lack of knowledge about the molecular mechanism, partly because there is no *in vitro* model that faithfully reproduces the osteo-inductive activity of ceramics in vitro. In this study, we explore the potential use of embryonic stem cells as a cell source. Embryonic stem cells (ESCs) are pluripotent cells derived from early stage embryos and are capable of indefinite proliferation while maintaining their pluripotent status. They can be induced to differentiate into cells of all three germ layers of the human body (endoderm, mesoderm and ectoderm) *in vitro* as well as *in vivo* [15-17]. Differentiation of mouse ESCs is initiated upon the withdrawal of leukemia inhibitory factor and suspension growth. They

spontaneously aggregate and form so-called embryoid bodies (EB) thereby undergoing differentiation [18]. By providing the right cocktail of stimuli, it is possible to direct the differentiation into a certain lineage. It has been demonstrated that mouse embryonic stem cells (mESCs) can differentiate into the osteogenic lineage through stimulation by various factors [19, 20 21, 22]. The aim of this study was to explore the possibility of using mouse embryonic stem cells for further in vitro studies to the molecular mechanism underlying osteoinductive ceramics.

## Materials and methods

### *Culture of mouse embryonic stem cells.*

The mouse embryonic stem cell (mES) line, IB10 [23] was grown on tissue culture polystyrene, coated with 0.1% gelatin (Sigma) and maintained in DMEM (Cambrex, Biowhitakker) medium supplemented with 10% FBS (Greiner, selected batch for mES), 1% non-essential amino acids 100x (Sigma), 4mM L-glutamine (Sigma), penicillin G (100 u/ml, Invitrogen); streptomycin (100 μg/ml, Invitrogen) and 100 μM 2-mercaptoethanol (Invitrogen) (basic medium). To maintain the undifferentiated state, cells were cultured in the presence of Buffalo rat liver cell-conditioned (BRL) medium and 1000 units/ml of Leukemia inhibiting factor (Esgro, Chemicon international). The cells were grown in a 37°C humid atmosphere at 5% $CO_2$ and upon reaching near confluence (80-90%), they were washed with phosphate buffered saline (PBS, Sigma) and detached with 0.05 % trypsin (Gibco), containing 1 mM ethylenediaminetetraacetic acid (EDTA).

### *Embryoid body formation and differentiation of mouse embryonic stem cells.*

For formation of so-called embryoid bodies (EBs), 175,000 cells were cultured in suspension for 4 days, in 10 $cm^2$ non-adherent plates in basic medium containing 100 μM 2-mercapto-ethanol. Next, cells were dispersed with trypsin and grown in either basic medium or differentiation medium consisting of control medium supplemented with

$10^{-7}$M retinoic acid (Sigma). After three days, differentiation medium was changed by mineralisation medium, consisting of basic medium supplemented by 2.5 µM Compactin (Sigma) and 0.01 M β-glycerol phosphate (Sigma) and basic medium was supplemented with 0.01M β-glycerol phosphate.

### *MC3T3 and C2C12 cell culture*

C2C12 cells [24] were seeded at a density of 2000 cells/cm2 in control medium containing DMEM (Invitrogen),10% fetal bovine serum (FBS, Gibco), penicillin G (100 u/ml) and streptomycin (100 µg/ml). MC3T3 cells [25] were plated at a density of 5000 cells/cm2 in control medium containing αMEM (Invitrogen), 10% FBS, penicillin G (100 u/ml), streptomycin (100 µg/ml), 2mM L-glutamine and 1 mM sodium pyruvate (Gibco) The cells were grown in a 37ºC humid atmosphere at 5% CO2 and culture medium was refreshed twice a week. When the C2C12 cells reached 60-70% confluence and the MC3T3 cells reached 80-90% confluence, they were washed with phosphate buffered saline (PBS, Sigma) and detached with 0.05 % trypsin, containing 1 mM EDTA.

### *Differentiation on tissue culture plates and calcium phosphate scaffolds.*

For differentiation of mES, the cells were either seeded on tissue culture plates treated with 0.1% gelatin or calcium phosphate scaffolds. When seeded on TC plates, 3 day old EBs were transferred to a 10 $cm^2$ tissue culture polystyrene plate for attachment and cultured in control and differentiation medium for 3 days followed by control and mineralization medium. For differentiation on calcium phosphate scaffolds, three day old EBs were washed with PBS, made into a single cell suspension using 0.25% trypsin (Gibco) containing 1 mM EDTA and passed over a 100 µm cell strainer (BD Falcon). mES, C2C12 and MC3T3 cells were seeded onto porous granules of bi-phasic calcium phosphate sintered at 1150°C (BCP1150) or 1300°C (BCP1300) and tri-calcium phosphate sintered at 1100°C with an average diameter of 2-4 mm[11]. The cells were seeded at a density of 250,000 cells/scaffold in a non-adherent plate (n=3,

consisting of 8 cell-seeded particles each) and cultured for 3 days in control and differentiation medium.

### *RNA isolation and quantitative PCR*

To analyze the expression of osteogenic markers by mES we isolated total RNA using the TriZol method according to the manufacturer's protocol (Invitrogen). The quality and quantity of RNA was analyzed by gel electrophoresis and spectrophotometry. One µg of RNA was used for first strand cDNA synthesis using Superscript II (Invitrogen) according to the manufacturer's protocol. One µl of 100x diluted cDNA was used for GAPDH (5'-AACGACCCCTTCATTGAC-3' and 5'-TCCACGACATACTCAGC AC-3') amplification and 1 µl of undiluted cDNA was used for osteocalcin (OC) (5'-CAGACCTAGCAGACACCATGAG-3' and 5'-AGGTCAGAGAGACAGAGCGCA-3') and bone saline protein (BSP) (5'-CAGAGGAGGCAAGCGTCACT-3' and 5'-CTGTCTGGGTGCCAACACTG-3') amplification. PCR was performed on a Light Cycler real time PCR machine (Roche) using SYBR green I master mix (Invitrogen). Data was analyzed using Light Cycler software version 3.5.3, using fit point method by setting the noise band to the exponential phase of the reaction to exclude background fluorescence. Expression of osteogenic marker genes are calculated relative to GAPDH levels by the comparative $\Delta$CT method and the statistical significance was found using Student's t test at $P<0.05$.

### *Mineralisation and von Kossa staining.*

To study mineralisation, cells were grown in control or mineralisation medium for 21 days. When abundant mineralization was observed in the positive control by phase contract microscopy, the cells were washed with PBS and fixated overnight in 4 % paraformaldehyde (Merck). After washing and 15 minutes incubation with demineralised water, the cells were incubated with 5% silver nitrate (Sigma) under a UV-lamp, until black staining was observed in the positive control.

### *Protein analysis*

For analyzing differences in protein absorption to ceramics, 150 mg of TCP, 150 mg of BCP1150 and 190 mg of BCP130 particles (n=2) were incubated in FBS for 2 hour or 3 days and the absorbed proteins were retrieved from the particles by incubation in Laemli sample buffer (BioRad). Protein concentrations were measured using using a micro BCA$^{tm}$ protein assay kit (Perbio) and equal amounts of protein were loaded onto a 7% acryl/bis-acrylamide gel. The proteins were stained with Coomassie blue

### *Ion analysis*

Two milli liters of Dmem were incubaded with 1 particle for 3 days with Either BCP1300, BCP1150 or TCP particles. The concentration of $Ca^{2+}$, $Mg^{2+}$ and $PO_4^{3-}$ in the medium were measured via Inductively Coupled Plasma - Atomic Emission Spectrometer (ICP-AES).

## Results

### *Osteogenic differentiation of mouse embryonic stem cells*

As previously described [19, 21, 22], mESCs can undergo osteogenic differentiation, and to confirm this we exposed IB10 mESCs to an osteogenic differentiation protocol. Quantitative PCR was used to monitor the up-regulation of mature osteoblast markers osteocalcin (OC) and bone sialoprotein (BSP). After three days, differentiation medium enhances the expression of OC and BSP in differentiation versus control medium at 2- and 2.5-fold respectively (figure 1C,D). Although the up-regulation of osteogenic markers is relatively mild, osteogenic medium had an all or nothing effect on mineralisation. As shown in figures 1 A and B, mESCs display extensive mineralization, when cultured in mineralization medium whereas mESCs cultured in control medium do not. Positive staining was mainly confined to multi-layered nodules. These data show that IB10 mESCs can be induced into the osteogenic lineage.

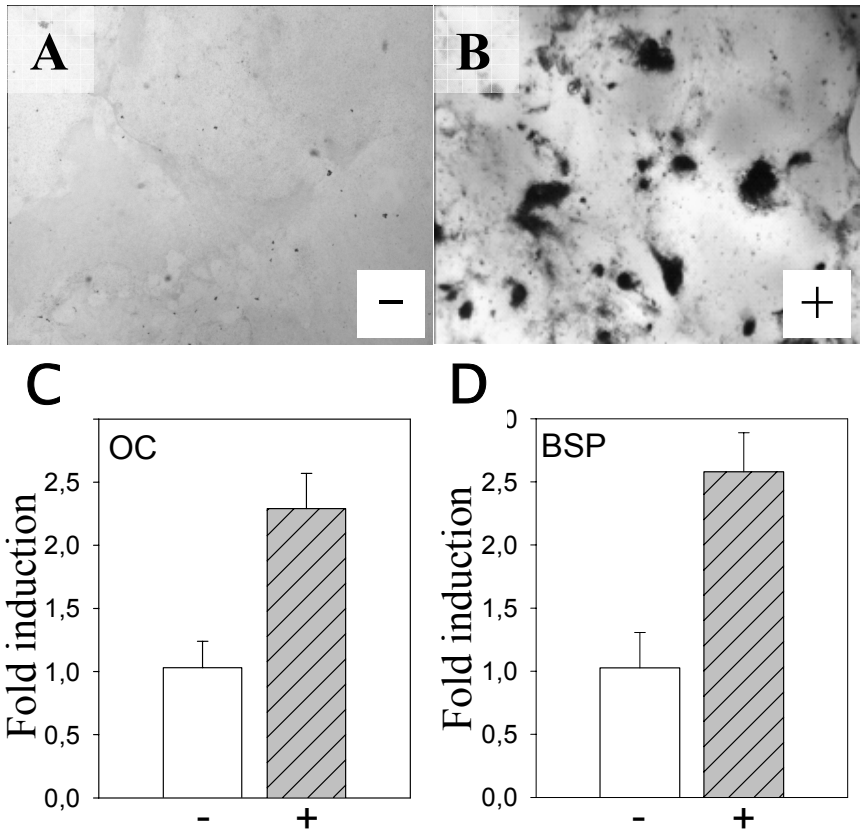


Figure 1. Osteogenesis of mESCs in 2D cultures. *(A,B) Cells were cultured in either control medium (-) or differentiation medium (+) and analysed for mineral deposition by Von Kossa staining (black colour). (C,D) Expression of the osteocalcin (OC) gene and bone sialoprotein (BSP) grown on tissue culture plastic in control (C) and differentiation (D) medium. Expression was normalized to GAPDH levels. Standard deviation is represented by error bars.*

### *In vitro differentiation on calcium phosphate ceramics*

Our approach to bone tissue engineering comprises of seeding osteogenic cell on porous calcium phosphate ceramics. Ceramics with different biological properties have been described and we investigated whether osteogenic differentiation of mESCs could be promoted by seeding on so-called osteoinductive calcium phosphate ceramics [10, 11, 13].

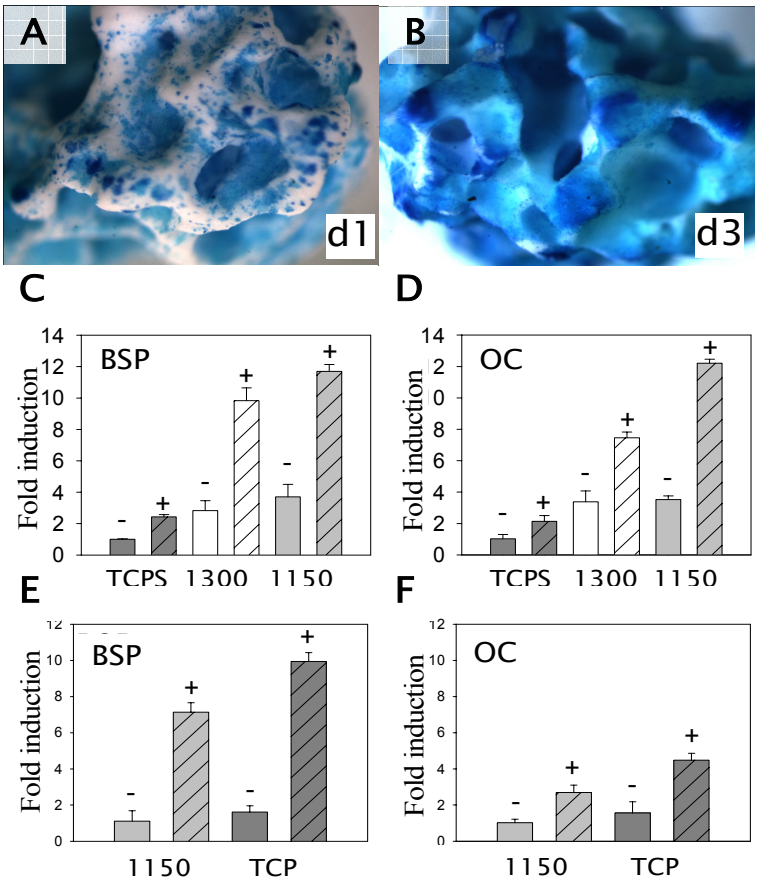


Figure 2. Differentiation of mouse embryonic stem cells on calcium phosphate ceramics *(A,B) The increase in cell growth on calcium phosphate scaffolds from day 1 to 3 days is visualised by methylene blue staining. (C,D) Osteocalcin (OC) and bone sialoprotein (BSP) expression of mESCs in control (-) and differentiation medium (+) on tissue culture plastic (TCPS), BCP1300 (1300) and BCP1150 (1150). MESC seeded on calcium phosphate scaffold have a higher expression of osteogenic markers then when cultured on TCPS. A significant increase of OC can be observed when mESC are cultured on BCP1150 compared to BCP1300 ($p<0.05$) (E,F) Osteocalcin (OC) and bone sialoprotein (BSP) expression of mouse embryonic stem cells on BCP1150 (1150) and TCP particles. The enhancement of OC is significantly higher for cells seeded on TCP compared to cells seeded on BCP1150 ($p<0.05$). Expression was normalized to GAPDH levels. Standard deviation is represented by error bars.*

First, we demonstrated that mESCs can proliferate on calcium phosphate ceramics by culturing the cells for 1 to 3 days on BCP1300 particles. Methylene blue staining visualised the increase in cell number (figure 2A,B). We proceeded by testing three materials; biphasic calcium phosphate sintered at 1150°C (BCP1150) and 1300°C (BCP1300) and tri-calcium phosphate (TCP). BCP1150 and TCP are osteoinductive materials *in vivo* whereas BCP1300 is not. MESCs were either seeded onto tissue culture plastic, onto BCP1300 or BCP1150 in control and differentiation medium for three days. In all cases, differentiation medium enhanced expression of OC and BSP (figure 2C,D). Importantly, mESCs seeded on calcium phosphate ceramics display a more robust up-regulation of BSP and OC than cells cultured on tissue culture plastic, a three times higher induction in control medium and six times higher in differentiation medium. Moreover, cells grown in control medium on BCP1150 and TCP displayed a higher level of OC and BSP, than cells grown in differentiation medium on TCPS, suggesting that the ceramics have an intrinsic osteogenic property. In line with this, we observed that osteogenic marker gene expression was higher on BCP1150 than on BCP1300, reflecting the osteogenic behaviour *in vivo*. mESCs on TCP displayed an even higher expression of BSP and OC (fig 2E,F), compared to cells grown on BCP1150. We demonstrate that mESCs osteogenic differentiation is influenced by the material properties, which seems to reflect the osteo-inductive properties of the materials *in vitro*.

### *Differentiation of lineage-committed cell lines on calcium phosphate ceramics.*

We next investigated whether the osteo-inductive ceramics could also induce differentiation of more committed osteogenic cells. We seeded the pre-osteoblast cell line MC3T3 and the pre-myogenic cell line C2C12 onto the different ceramics in control and differentiation medium for three days and investigated the expression of OC. Although both MC3TC and C2C12 respond to the differentiation medium by up regulation of OC expression (figure 3A B), we did not observe a

difference in osteogenic differentiation on different ceramics. In MC3T3 cells, a reverse trend is even apparent.

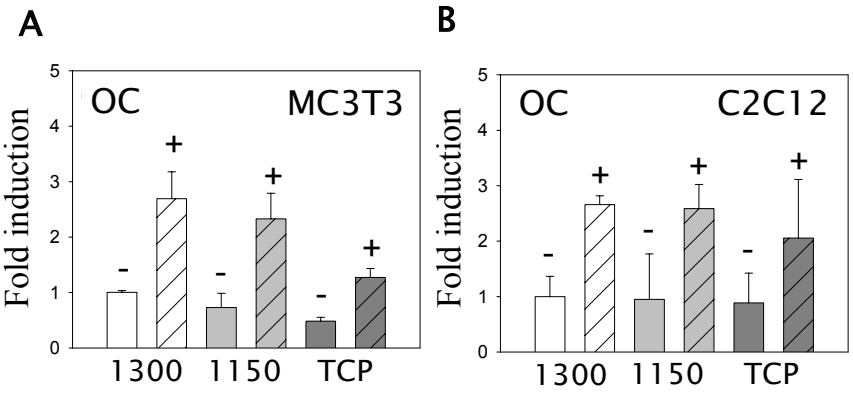


Figure 3. Differentiation of lineage-committed cell lines on calcium phosphate ceramics. *Osteocalcin (OC) and bone sialoprotein (BSP) expression of C2C12 mesenchymal stem cells and MC3T3 pre-osteoblast cells seeded on BCP1300 (1300) BCP1150 (1150) and TCP1100 (TCP). in control (-) and differentiation medium (+). Both cells lines have increased expression of osteogenic markers when cultured in differentiation medium but do not respond to difference in materials. Expression was normalized to GAPDH levels. Standard deviation is represented by error bars.*

### *Differential protein binding and cell attachment.*

Protein absorption to materials is of great importance for the cellular response to biomaterials. To investigate whether differential protein binding underlies the osteogenic behaviour of the ceramics,, we analysed protein binding from FBS to TCP, BCP1150 and BCP1300 particles after two hours and three days. As shown in figure 4A, more proteins adhere per gram of material to TCP than to BCP1150 and more to BCP1150 than to BCP1300. As discussed previously, BCP1150 is a microporous material, which could explain the difference in protein absorption. To study whether BCP1150, BCP1300 and TCP have a qualitative difference in protein absorption, we loaded equal amounts of protein onto an SDS-PAGE gel and stained the proteins. There was no obvious difference in signal intensity for any protein visible on the gel

(figure 4B). Because serum protein such as fibronectin and vitronectin play an important role in cell adhesion, we investigated whether ES attachment differed between the different ceramics. Two hours after seeding, we observed more adhered and spread cells on TCP1150 than on BCP1150 (figure 5A,B), but no apparent difference was observed between BCP1150 and BCP1300.

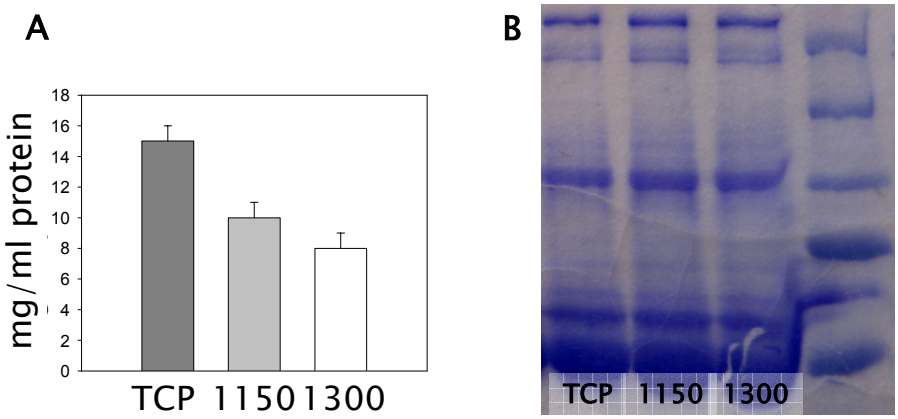


Figure 4. Protein adhesion on calcium phosphate ceramics. *(A). Protein adhesion to BCP1150 and BCP1300 quantified by a micro protein assay. (B) Adhesion of FBS proteins to BCP1150 and BCP1300 was visualised by SDS-PAGE. Equal amounts of proteins were loaded in both lanes. Note that no apparent differences can be observed.*

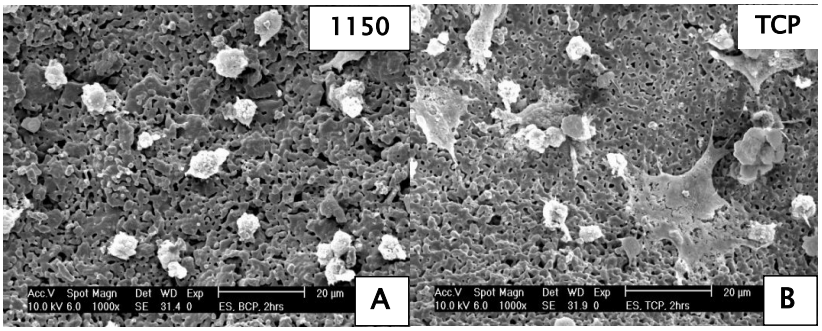


Figure 5. Cell adhesion on BCP1150 and TCP. *After two hours mESC seeded on BCP1150 and TCP were visualised by ESEM. A trend was observed that cells adhere faster to TCP compared to BCP1150.*

*Calcium phosphate ceramics control osteogenesis through changing medium composition.*

Ceramics may influence osteogenic differentiation of mESCs through direct cell/material interaction (e.g. differential protein adhesion can influence adhesion and differentiation) or can be indirect through the effect of the material on proteins or ions in the culture medium. To investigate the latter possibility, we seeded cells onto BCP1300 in control and differentiation medium and added an empty scaffold of BCP1150 in the same well. Interestingly, the extra scaffold resulted in an increase in expression of BSP and OC (Figure 6A,B), suggesting that a change in medium composition by BCP1150 enhances osteogenic differentiation of the cells on BCP1300 scaffolds. We studied whether BCP1300, BCP1150 and TCP could modify the medium composition by incubation of the medium with BCP1300, BCP1150 or TCP particles and by measuring calcium, phosphate and magnesium ion concentrations. We found a small decrease in calcium and phosphate concentrations in the medium after the addition of BCP1300 but a strong decrease after addition of BCP1150 and TCP (Table 1). In contrast, the magnesium concentration was hardly affected.

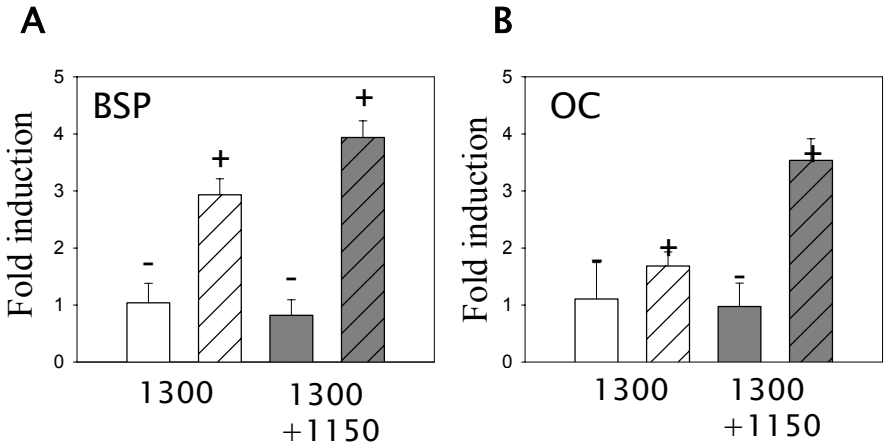


Figure 6. Trans-effect of BCP1150 on mESCs differentiation. *(A,B) Osteocalcin (OC) and bone sialoprotein (BSP) expression of mouse embryonic stem cells in control (-) and differentiation medium (+) seeded onto BCP1300 in the presence (+1150) or absence of an empty BCP1150 particle in the same well.*

*The addition of an extra scaffold to the medium induces an increase in expression in differentiation medium of BSP and a significant increase for OC ($p<0.05$) Expression was normalized to GAPDH levels. Standard deviation is represented by error bars.*

| | $Ca^{2+}$ (mg/L) | $Mg^{2+}$ (mg/L) | $PO_4^{3-}$ (mg/L) |
|---|---|---|---|
| Dmem | 75 | 20 | 25 |
| Dmem BCP 1300 | 70 | 20 | 24 |
| Dmem BCP 1150 | 55 | 22 | 18 |
| TCP | 55 | 20 | 18 |
| Dmem BCP 1300 +1150 | 55 | 22 | 18 |

Table 1. *Concentrations of $Ca^{2+}$, $Mg^{2+}$ and $PO_4^{3-}$ in medium treated with and without BCP1150, BCP1300 and TCP scaffolds*

## Discussion

In this study we demonstrate that in vitro osteogenic differentiation of mESCs can be influenced by the properties of ceramic materials and seems to reflect the osteo-inductive properties of the materials *in vitro*. Thus mESCs can be used as a cell source for investigation into the osteogenic potential of different calcium phosphate ceramics. We surprisingly found that the osteogenic potential of ceramics can be mediated via its effect on medium composition.

Most studies investigating differences in osteoinduction by ceramics are performed in vivo in goat and dogs [10, 11, 14, 26]. With these studies we are able to examine the biological performance and safety of the ceramics and the differences in osteoinduction between ceramics. Though there studies are not suited to investigate multiple parameters as only limited numbers of samples can be investigated and these studies do not reveal insight in the process causing osteoinduction. However *in vivo* studies are a black-box in which so many biological factors vary. Therefore it is very difficult to investigate the osteoinduction phenomenon *in vivo*. One of the few known hypothesis on osteoinduction by calcium phosphate

ceramics is related to the geometry of the implant and the absorption of bone morphogenic proteins (BMPs) onto the surface [27-30]. Ripamonti *et al* descibed that the *intrinsic osteoinductivity* appears to be dependent on the surface characteristics, geometric configuration, and correlates to adsorption of endogenous, circulating, or locally produced BMPs/OPs onto the hydroxyapatite [31, 32]. He demonstrated that BMP-3 and OP-1 localize at the interface of sintered hydroxyapatites, thereby suggesting that BMP's play a role in osteoinduction. The causal relationship has sofar not been demonstrated.

Ceramics with cultured mesenchymal stem cells have also been implanted subcutaneously in immune-deficient mice to compare between ceramics by Arinzeh *et al* [5] but no thorough investigation into the mechanism was performed either.

A number of studies have been published on *in vitro* studies on osteoinduction by ceramics. Besides C2C12 and MC3T3 cells used in our studies, differentiation of the human osteoblast like cell-line SaOS-2, was also not affected by ceramic composition. In two different studies Wang *et al* [33] used hydroxyapatite (HA), TCP, combination of both and also HA sintered at different temperatures without finding any differences in the gene expression of the SaOS-2 cells cultured on these ceramics. As such, our studies with mESCs provide the first example where osteogenic differentiation in vitro correlates to osteo-induction in vivo.

Osteoinduction is related to the porosity and thus surface area of the material. A porous material has better osteoinductive performances compared to a non porous material and increased surface area stimulates bone formation [14, 34]. When comparing this hypothesis with our data we see a relation. MESCs are far more susceptible to osteogenic differentiation when cultured on a three dimensional calcium phosphate ceramic compared to a flat surface such as tissue culture plastic. This is in accordance with the finding that when mESCs mineralise on tissue culture plastic the mineralisation areas are concentrated in nodules, suggesting that osteogenic differentiation is stimulated in a three dimensional environment. To test this, we could study the osteogenic

differentiation of mESCs seeded in a three dimensional environment of for instance a porous polymer compared to a sheet of the same polymer. However, our results demonstrate that in our model system geometry can not be the only factor contributing to enhanced osteoinduction. We demonstrated this by the addition of an empty particle of BCP1150 into the medium of cells seeded on BCP1300, which caused an additional increase in the osteogenic differentiation of the seeded cells. In this experiment, the geometry of substrate remained the same but differentiation was altered.

We also investigated the hypothesis that changes in the ion concentrations in the medium influence the mESCs differentiation. Upon implantation, highly resorbable ceramics such as $\alpha$- and $\beta$-tricalcium phosphate will initiate a dynamic process of calcium- and phosphate release, followed by the formation of a biological apatite layer [35]. This dissolution-reprecipitation process is suggested to be at the origin of the calcium-phosphates bioactivity [36, 37]. The ceramics used in the present study consisted for about 80% of the largely non-resorbable hydroxyapatite phase and for about 20% of $\beta$-tricalcium phosphate. Due to their chemical composition, biphasic BCP ceramics do not show significant resorption in vivo, as is indicated by various studies in which these ceramics were implanted ectopically and orthotopically for up to 6 months. However, in a study by Habibovic et al. [14] in which BCP1150 and BCP1300 were implanted subcutaneously in goats inside diffusion chambers, presence of carbonate was observed already 1 week after implantation, which suggested the formation of a carbonated apatite layer. Although no considerable degradation of the ceramic was observed, it is plausible that at micro level, inside the pores of the material, the above described dissolution-reprecipitation process takes place, followed by the formation of the biological apatite layer. Another possibility is that the surface of the ceramics acts as a collection of nucleation sites for the formation of carbonated apatite layer from calcium- and phosphate-saturated body fluids. Due to a higher specific surface area, this process is more pronounced on the surface of the highly microporous BCP1150 as compared to BCP1300. This hypothesis

is fully supported by our data on the calcium and phosphate levels in medium incubated with BCP1150 and BCP1300. The former, more microporous, ceramic caused a drastic decrease in calcium and phosphate levels.

From earlier *in vitro* experiments it is known that when calcium phosphate ceramics are submerged in medium, calcium and phosphate ions from the medium are removed by deposition on the immersed ceramics, which is suggested to have negative influences on cell adhesion [38]. In contrast with these findings Knabe *et al.* in similar studies observe an increase in phosphorus ions in the medium from the ceramics, which is suggested to have an inhibitory effect on cellular growth [39]. When we compare these findings with our data we find a decrease in calcium and phosphate which has no negative effect on our cells, in contrast TCP and BCP1150 both have a higher decrease in ions compared to BCP1300 but cells cultured on these materials are more susceptible to osteogenic differentiation. Also in contrast with our findings it is reported that increased calcium concentrations increase the number of osteoblasts [40]. Although in our studies it seems there might be a positive influence of the decrease in ions, this will need to be examined by osteogenic differentiation of mESCs cultured in calcium or phosphate depleted medium or in medium which was priory conditioned by the ceramics. Data from a preliminary study (data not shown) indicated that osteogenic differentiation of mESCs is not effected by ion concentrations.

Another hypothesis could be that osteogenic differentiation is affected because differential absorption of proteins to the ceramics causes a difference in protein abundance in the medium. Foetal bovine serum is a complex cocktail of proteins, some of which might be involved in osteogenesis like vitronectin [41]. Indeed, we observed a higher amount of proteins on the osteo-inductive BCP1150 and TCP, compared to the non-inductive BCP1300. If large differences in the amount of protein adhesion had occurred it could have affected cell adhesion, but no qualitative difference in adhesion of proteins on the different ceramics were found. However the outcome of the experiment with the addition of

the extra particle to BCP1300 indicates that protein adhesion might play an important role.

Based on the observation that differential protein binding occurs on osteo-inductive versus non-inductive materials and on the observation that osteo-inductive ceramics can influence mES differentiation *in trans,* we hypothesize that protein binding to the material stimulates osteogenic differentiation of mESCs (Figure 7). The BMP binding hypothesis of Ripamonti could be in line: we indeed find that osteo-inductive materials stimulate differentiation through binding of compounds from the medium surrounding them. Because it is not known whether improved sequestration of BMP to a ceramic particle would have a net positive (local sink of BMP affecting differentiation) or net negative effect (BMP immobilised to a ceramic is presumed biologically inactive) is not known. Alternatively, ceramics may bind and inhibit negative regulators of bone formation, e.g. noggin or sclerostin, and thus promote differentiation. We therefore suggest an alternative hypothesis which states that the osteoinductive capacities of ceramics depend on their capacity to bind negative regulators of bone formation. This theory is supported by our findings that adding BCP1150 to the medium of cells seeded on BCP1300 increased their osteogenic differentiation. Thus, the osteo-inductive activity of ceramics could be molecularly similar to that of patients with fibrodusplasia ossificans, who display progressive heterotopic bone formation due to a defect in the feedback pathway between BMP-4 and one of its extra cellular antagonist, thereby enhancing the BMP activity [14 42].

In future studies we will investigate the osteoinductive factor responsible for the osteogenic differentiation of mESCs. A micro-array study might give us a better insight in differences of gene expression by cells seeded on both materials and identify the molecular pathway responsible for the enhanced osteogenic differentiation. The next step would be to investigate the involvement of the pathway in osteo-induction in vivo. Although we still need to prove our protein binding hypothesis it seems likely that the possibility of a combined effect should not to be excluded, especially with the improved adherence of ES cells to TCP in mind.

Whether the in vitro data we found on the osteoinductive capacities of ceramics can be directly correlated to *in vivo* results is not sure. However recent *in vivo* studies suggest that TCP is a more osteoinductive ceramic compared to BCP1150 which in its turn is more osteoinductive then BCP1300 (HY, manuscript in preparation).

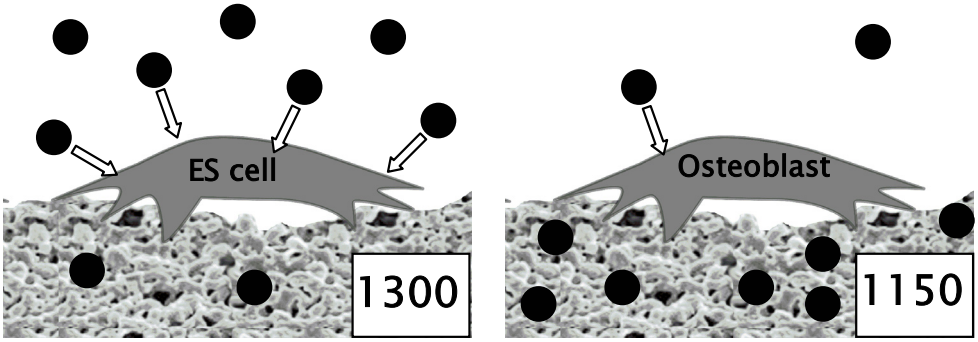


Figure 7. Mechanism of osteoinductivety by calcium phosphate ceramics. *By binding certain factors to the surface of the ceramic BCP1150 stimulates osteogenic differentiation of mESCs more effective then BCP1300.*

## Conclusion

Osteogenic differentiation of mESCs is clearly enhanced by calcium phosphate ceramics, at least in part by influencing medium composition. Our findings confirm that mESCs are a valid in vitro model to predict osteo-inductivity as well as for investigating the underlying molecular mechanism.

## Acknowledgements:

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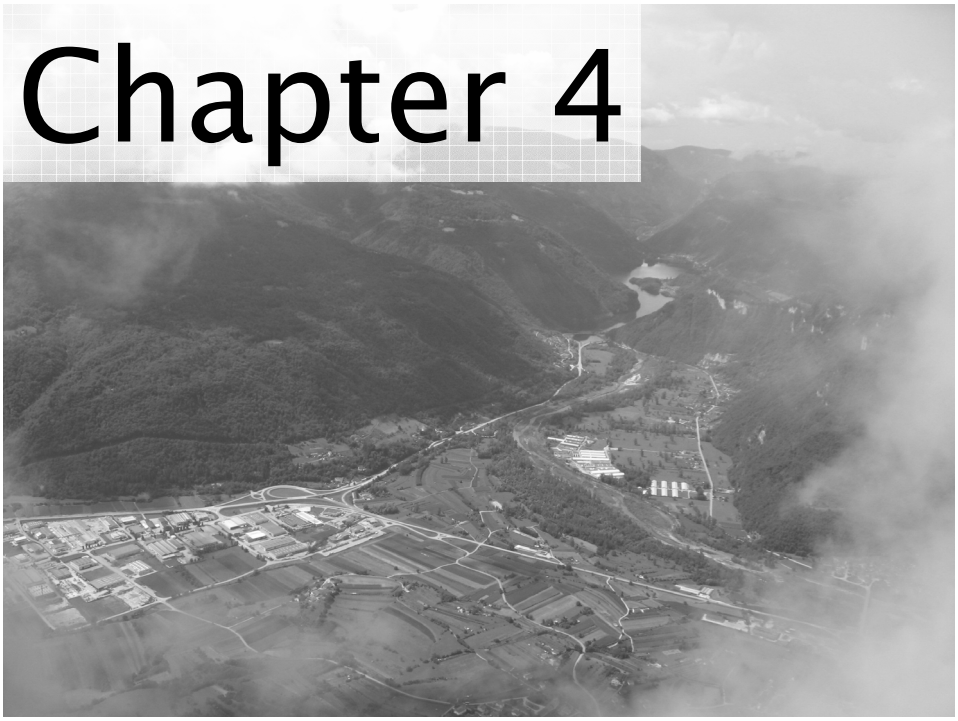
# Chapter 4



# Chapter 4

# Differential bone forming capacity of either embryonic stem cells or bone-marrow derived mesenchymal stem cells.

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## Abstract

For more then a decade, human mesenchymal stem cells (hMSCs) have been used in bone tissue engineering research and more recently some of the focus in this field has shifted towards the use of embryonic stem cells. From hMSCs it is well known that they are able to form bone when implanted subcutaneously in immune-deficient mice, but the osteogenic potential of embryonic stem cells has been assessed only *in vitro*. Therefore, we performed a series of studies in which we compare the *in vitro* and *in vivo* osteogenic capacities of human and mouse embryonic stem cells to hMSCs. Embryonic and mesenchymal stem cells showed all characteristic signs of osteogenic differentiation *in vitro* when cultured in osteogenic medium, including the deposition of a mineralised matrix and expression of genes involved in osteogenic differentiation. As such, osteogenic ES cells could not be discriminated from osteogenic hMSCs. Nevertheless, although osteogenic hMSCs formed bone upon implantation, osteogenic cells derived from both human and mouse embryonic stem cells did not form functional bone, indicated by absence of osteocytes and bone marrow and absence of lamellar bone. Although embryonic stem cells show all signs of osteogenic differentiation *in vitro*, it appears that in contrast to mesenchymal stem cells they do not possess the ability to form bone in vivo when a standard differentiation protocol was applied.

## Introduction

In orthopedic surgery, autologous bone grafting is still the most commonly used approach for the restoration of skeletal function. Besides autologous bone, alternative graft materials are allogeneic bone, demineralized bone matrix and synthetic biomaterials. Although autologous bone outperforms its alternatives, it also has it drawbacks, such as donor side pain when harvested and limited availability. The risk of immune reaction and disease transmission is associated with allograft transplantation so the search for alternatives is ongoing.

Since it was reported that human mesenchymal stem cells (hMSCs) are precursors to osteoblasts [1], a large body of research has focused on the use of these stem cells for skeletal tissue repair. hMSCs are an accessible source of stem cells harvested from the bone marrow, and it contains a multipotent cell population that has the ability to differentiate into several mesenchymal lineages [2], not only bone but also cartilage, fat and muscle [1]. It has been shown that hMSCs do not only possess *in vitro* osteogenic potential when grown under osteogenic differentiation conditions [3, 4], but can also form bone tissue *in vivo* when implanted subcutaneously into immune-deficient mice [5, 6]. One of the strategies of bone tissue engineering is to create a tissue-engineered construct that can be used for bone replacement surgery. To this end, mesenchymal stem cells from the bone marrow are isolated and expanded *in vitro* until a sufficient amount of cells is reached. Then, they are seeded onto a suitable scaffold material, for instance calcium phosphate ceramics, and exposed to osteogenic stimuli prior to implantation into the defect site. Unfortunately, hMSCs have their limitations. During expansion, hMSCs gradually loose their multipotency [7], large donor variation is observed with respect to bone forming capacity and until now the amount of bone formed is insufficient for clinical application [8].

After Thomson and co-workers first described that an embryonic cell line was derived from a human blastocyst, embryonic stem cells have been considered as an alternative cell source for bone tissue engineering [9]. Embryonic stem cells are pluripotent cells derived from the inner cell

mass of blastocyst-stage embryos. They can proliferate indefinitely and differentiate into to all tissues types. Since their discovery, many different human embryonic stem cell lines have been isolated [10-12], which inspired the tissue engineering field to use human embryonic stem cells (hESCs). Different reports describe that using specific differentiation protocols, hESCs can be induced into many different cell types like cardiomyocytes [13], neural precursors [14, 15], insulin-producing cells [16], cartilage and liver cells [17].

The first successful osteogenic differentiation experiment with embryonic stem cells *in vitro* was performed with mouse embryonic stem cells (mESCs) [18-21]. MESCs can form so-called embryoid bodies in hanging drop or suspension culture. Embryoid bodies are then transferred from a three- into a two-dimensional culture and are exposed to a cocktail of supplements such as retinoic acid, ascorbic acid, dexamethason and compactin, which initiates osteogenic differentiation. Osteogenic differentiation of mESCs was characterized by the formation of mineralised nodules and the expression of bone-specific genes such as osteocalcin, cbfa-1 and bone sialoprotein. There are however, to our knowledge, no reports describing *in vivo* bone formation by mESCs in a tissue engineering setting.

When research focused on osteogenic differentiation of hESCs, similar culturing protocols were used as for mESCs [22-24]. Again, *in vitro* these cells display signs of osteogenic differentiation such as formation of mineralised nodules and expression of bone-specific genes. Recently, Bielby et al described that osteogenic hESCs implanted subcutaneously in immune-deficient mice give rise to regions of mineralized tissue [22] but from the report it is not clear whether mature bone tissue was formed or not. Hence, in view of the numerous potential applications of embryonic stem cells we aimed in this study at comparing the ability of mESCs and hESCs versus hMSCs to differentiate into the osteogenic lineage in vitro and to form mature bone tissue *in vivo*.

## Materials and methods

### *Culture of mouse embryonic stem cells.*

The mouse embryonic stem cell line IB10 [25] was grown on tissue culture polystyrene, coated with 0.1% gelatine (Sigma) and maintained in basic medium comprising DMEM (Cambrex, Biowhitakker) medium supplemented with 10% foetal bovine serum (FBS, Greiner, selected batch for mESCs), 1% 100x non-essential amino acids (Sigma), 4 mM L-glutamine (Sigma), penicillin G (100 u/ml, Invitrogen), streptomycin (100 μg/ml, Invitrogen) and 100 μM 2-mercapto-ethanol (Invitrogen) [25]. To maintain the undifferentiated state, cells were cultured in the presence of Buffalo rat liver cell-conditioned (BRL) medium and 1000 units/ml of Leukemia inhibiting factor (Esgro, Chemicon international). The cells were grown in a 37°C humid atmosphere at 5% $CO_2$ and upon reaching near confluence (80-90%), they were washed with phosphate buffered saline (PBS, Sigma) and detached with 0.05 % trypsin (Gibco), containing 1 mM ethylenediaminetetraacetic acid (EDTA).

### *Embryoid body formation and differentiation of mouse embryonic stem cells.*

For formation of embryoid bodies (EBs), 175,000 cells were cultured in suspension for 4 days, in 10 $cm^2$ non-adherent plates in basic medium containing 100 μM 2-mercapto-ethanol. Next, cells were dispersed with trypsin and grown in either control medium or differentiation medium consisting of control medium supplemented with $10^{-7}$M retinoic acid (Sigma). After three days, differentiation medium was changed for osteogenic medium, consisting of basic medium supplemented with 2.5 μM compactin (Sigma) [21] and 0.01 M β-glycerol phosphate (Sigma) and basic medium was supplemented with 0.01M β-glycerol phosphate. In some experiments the compactin was replaced by 10 nM dexamethason (Sigma) or by 10 ng/ml BMP-2 (R&D systems)

### *Culture of human embryonic stem cells.*

The human embryonic stem cell line (hESC) SA002, was established as described by Heins *et al.* [10, 26, 27]. Briefly, the hESCs were cultured using mitomycin-C inactivated mouse embryonic fibroblast (MEF) feeder layers and VitroHES™ medium (Vitrolife AB, Kungsbacka, Sweden) supplemented with 4 ng/ml human recombinant basic fibroblast growth factor (Invitrogen). The cells were grown in a 37°C humid atmosphere at 5% $CO_2$ and medium was refreshed every 2-3 days. The undifferentiated hESCs were passaged every 4-5 days by mechanical dissociation with a Stem Cell Cutting Tool (Swemed by Vitrolife, Vitrolife AB, Kungsbacka, Sweden).

### *Differentiation of human embryonic stem cells*

Cell aggregates from undifferentiated hESCs colonies were removed with the cutting tool and plated onto tissue culture plastic [28] in VitroHES™ medium. After 2 days the colonies were grown in control medium or osteogenic medium. The control medium consisted of αMEM (Invitrogen) containing 10% FBS of a selected batch (BioWhittaker, lot: 0S025F), 0.2 mM L-ascorbic acid-2-phosphate (Sigma), penicillin G (100 u/ml, Invitrogen); streptomycin (100 μg/ml, Invitrogen), 2 mM L-glutamine (Sigma) and 1 ng/ml basic fibroblast growth factor (Instruchemie). In the osteogenic medium, basic fibroblast growth factor was removed from the proliferation medium and supplemented with 10 nM dexamethasone.

### *Culture of human mesenchymal stem cells*

Cryo-preserved human mesenchymal stem cells (hMSCs), previously harvested from bone marrow aspirates of donors undergoing total hip replacement surgery [5]. The hMSCs were obtained form 4 donors form either the Acetabulum or the Iliac Crest. They were plated at 5,000 cells/$cm^2$ and were proliferated in control medium and differentiated in osteogenic medium, as described above for the hESCs culture. The cells were grown in a 37°C humid atmosphere at 5% $CO_2$ and medium was refreshed every 3-4 days. Upon reaching near confluence (80-90%), they

were washed with phosphate-buffered saline and detached with 0.05 % trypsin, containing 1 mM ethylenediaminetetraacetic acid (EDTA).

### *RNA isolation and quantitative PCR of hESCs and mESCs*

To analyze the expression of osteogenic marker genes by mESCs and hESCs we isolated total RNA using the TriZol method according to the manufacturer's protocol (Invitrogen). The quality and quantity of RNA was analyzed by gel electrophoresis and by spectrophotometry. One µg of RNA was used for first strand cDNA synthesis using Superscript II (Invitrogen) according to the manufacturer's protocol. For mESCs one µl of 100x diluted cDNA was used for GAPDH (5'-AACG ACCCCTTCATTGAC-3'and 5'-TCCACGACATACTCAGCAC-3') amplification and 1 µl of undiluted cDNA was used for osteocalcin (OC) (5'-CAGACCTAGCAGACACCATGAG-3' and 5'-AGGTCAGAGAGACAGAGCGC A-3') and for bone sialoprotein (BSP) (5'-CAGAGGAGGCAAGCGTCACT-3' and 5'-CTGTCTGGGTGCCAACACTG-3') amplification. For hESCs one µl of 100x diluted cDNA was used for 18s RNA (5'-CG GCTACCACATCCAAGGAA-3' and 5'-GCTGGAATTACCGCGGCT-3') amplification and 1 µl of undiluted cDNA was used for osteocalcin (OC) (5'-GGCAGCGAGGTAGTGAAGAG-3' and 5'-GATGTGGTCAGCCAACTC GT-3') and for osteopontin (OP) (5'-CCAAGTAAGTCCAACGAAAG-3' and 5'-GGTGATGTCCTCGTCTGTA-3') amplification. PCR was performed on a Light Cycler real time PCR machine (Roche) using a SYBR green I master mix (Invitrogen). Data was analyzed using Light Cycler software version 3.5.3, using fit point method by setting the noise band to the exponential phase of the reaction to exclude background fluorescence. Expression of osteogenic marker genes are calculated relative to GAPDH or 18S levels by the comparative $\Delta$CT method and the statistical significance was found using Student's t test at $P<0.05$.

### *Flow cytometry of hMSCs*

For flow cytometry, hMSCs from donors 1-3 were detached and centrifuged at 100 g for 10 minutes at 4°C. The cell pellets were resuspended in PBS containing 5 % bovine serum albumin (Sigma) and

0.05 % $NaN_3$ (Sigma) and stored on ice for 15 minutes. Next, the cells were incubated in block buffer containing control mouse anti-human IgG2a (Dako) or anti-ALP antibody (DSHB, B4-78). The cells were incubated on ice for 45 minutes and washed three times with PBS containing 1% BSA and 0.05% $NaN_3$ (wash buffer). Incubation with the secondary antibody, goat anti-mouse-IgG-FITC (Dako), was performed for 45 minutes on ice. After washing the hMSCs three times with wash buffer, the cells were incubated for 10 minutes with 10 μl Viaprobe (Becton Dickinson) for live-dead staining. The cell suspension was analysed using a flow cytometer (Becton Dickinson). From each sample ten thousand events were collected in duplicate and the data was analysed using Cell Quest software (Becton Dickinson).

### *Mineralisation and von Kossa staining.*

To study mineralisation, cells were detached and grown for 21 days in control medium or osteogenic mouse or human medium, respectively. All media contained 0.01M β-glycerophosphate (Sigma). For this experiment, hMSCs from donors 2 and 4 were used. When abundant mineralisation was observed in the positive control by phase contract microscopy, the cells were washed with PBS and fixated overnight in 4 % paraformaldehyde (Merck). After washing and 15 minutes incubation with demineralised water, the cells were incubated with 5% silver nitrate (Sigma) under a UV-lamp, until black staining was observed in the positive control

### *Confocal Raman spectroscopy*

To determine the chemical composition of the mineralised matrix produced by the mESCs, they were grown in control and differentiation medium. After culture the cells were washed with PBS and then fixed with 4% paraformaldehyde for 30 minutes at 4°C. After fixation, the samples were washed with PBS to remove excess fixative and then stored in PBS. Raman measurements were performed on an in-house build confocal Raman system as described previously [29]. At least 8 areas within the culture were randomly selected and the Raman signal was

collected of 15 by 15 micrometer raster scanned areas. The laser was focused onto the cell layer and 64 by 64 pixels were scanned during 30 seconds. The total signal of these areas was then depicted into an "average" Raman spectrum. The Raman spectra were baseline corrected and an average spectrum was then calculated using Microcal Origin 7.5 SR 6 software (Originlab corporation, Northampton, MA, USA). The final results were then plotted as Raman spectra of 200 to 2000 wave numbers for comparison of the so-called Raman "fingerprint region" of the different cell cultures.

### *Scanning electron microscopy*

After Raman measurements were taken, the same samples were prepared for scanning electron microscopy. The samples were dehydrated using an increasing ethanol series from 70% to 100%, critical point dried using a Baltzer critical point drier, and gold coated using a Cressington 105 Automated goldsputter coater (Cressington, Watford, UK) for examination by Scanning Electron Microscopy using an ESEM, XL-30 (Philips, Eindhoven, the Netherlands).

### *In vivo bone formation*

To assess the *in vivo* bone forming capacity of the cells, they were seeded onto porous biphasic calcium phosphate (BCP) ceramics (2-4mm) and implanted in nude mice [5]. The mESCs and hMSCs (donors 2 and 4) were both seeded by adding a 100 µl cell suspension, containing 200,000 cells, onto each BCP scaffold (n=6 per experiment). The hESCs were seeded by adding 100 µl of mechanically dissociated cell colonies with approximately 1,000,000 cells onto each BCP scaffold (n=6 per experiment). After 2 hours, medium was added and scaffolds were cultured for 7 days in mouse or human osteogenic medium, respectively. The presence of cells on the BCP scaffolds was visualized by staining with 1% methylene blue (Sigma) in 0.1 M Borax (Sigma). Prior to implantation, the particles were placed in serum-free medium for approximately 1-2 hours and washed 3 times with PBS. Immune-deficient mice (HsdCpb:NMRI-nu, Harlan) received an subcutaneous

injection with Temgesic for pain relief and were anaesthetised by Isoflurane. Before surgery, the skin on both lateral sites of the spine was cleaned with 70% alcohol and 4 subcutaneous pockets were created in each mouse. In each pocket, three tissue engineered samples were inserted and each culture condition was divided over six mice. Six weeks post-operatively, the animals were euthanized by $CO_2$. The explanted samples were fixated in 1.5% glutaraldehyde (Sigma) in 0.14M cacodylic acid buffer, dehydrated by increasing ethanol steps and embedded in methylmethacrylate (Sigma). Sections were made using a diamond saw (Leica SP1600) and stained with 1 % methylene blue (Sigma) and 0.3 % basic fuchsin solution (Sigma) [30]. The animals were housed at the Central Animal Laboratory institute and the experiments were approved by the local animal experiment committee.

### *Teratoma formation by human embryonic stem cells*

In vivo differentiation of hESCs was assessed as previously described [10, 26]. Briefly, 20-40,000 undifferentiated hESCs were surgically placed under the kidney capsule of 5-weeks old severe combined immune-deficient mice (C.B-17/IcrCrl-ScidBR, Charles River Laboratories, Wilmington, MA, USA). After 8 weeks of implantation the mice were sacrificed and the teratomas evaluated (Heins et al., 2004). All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee at Göteborg University in accordance with the policy regarding the use and care of laboratory animals. The obtained tumors were fixated in 4 % paraformaldehyde for 24 hours. Paraffin was infiltrated (Sakura Fine Techinical, Japan) and 6-8 µm section were made from the embedded samples (Microm HM360, Micro GmbH, Germany) and stained for light microscopy evaluation with hematoxylin and eosin (Skura DRS-601 stainer).

## Results

### *In vitro osteogenic differentiation of stem cells*

As previously described, hMSCs [5, 6], hESCs [22, 23, 28]and mESCs [19, 20]can undergo osteogenic differentiation when exposed to an osteogenic differentiation protocol. To investigate the osteogenic potential of our hMSCs we used techniques commonly used by other investigators for examining osteogenic differentiation. For the hMSCs isolated from bone marrow of three donors, we measured expression of the osteogenic marker ALP in response to the osteogenic reagent dexamethasone at day 5 and 7. At both days, ALP expression significantly increased when the cells were cultured in the presence of osteogenic medium compared to cells cultured in control medium (Figure 1).

Although there are differences between the donors, as reported by Sidappa et al [7], osteogenic medium always substantially stimulated the expression of ALP for each donor. When cells were cultured for 28 days in mineralisation medium, we observed widespread mineralisation indicating the in vitro osteogenic potential of hMSCs (Figure 2).

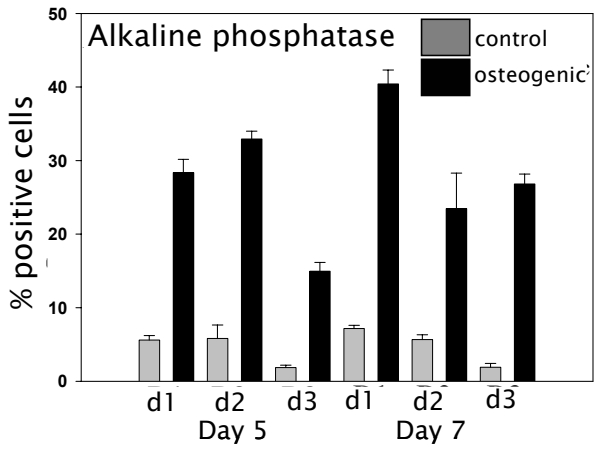


Figure 1. Effect of osteogenic medium on alkaline phosphatise expression of hMSCs. *The percentage of positive cells expressing ALP of donors 1-3 were measured after 5 and 7 days. ALP activity increased significantly for all 3 donors when cultured in osteogenic medium.*

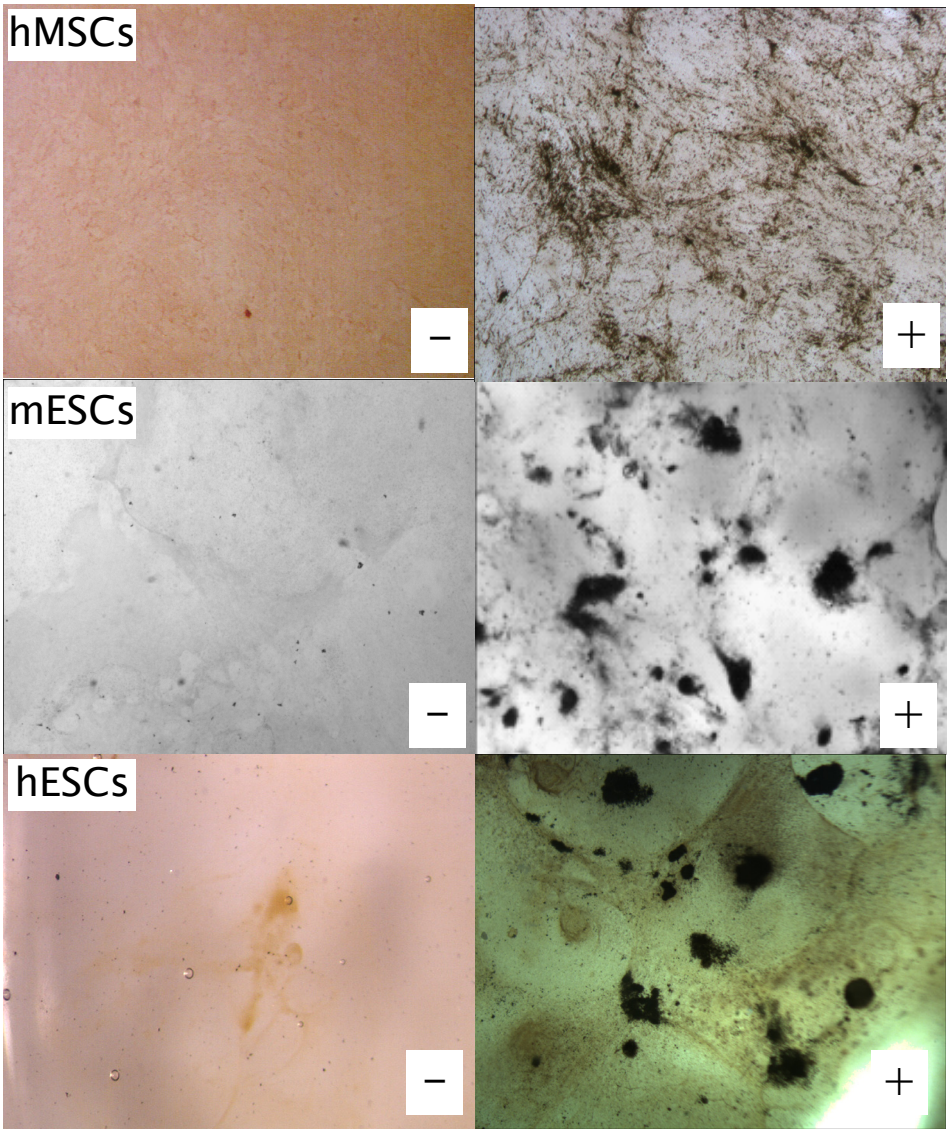


Figure 2. Osteogenesis of hMSCs, mESCs and hESCs in 2D cultures. (A,B,C) Cells were cultured in either control medium (–) or differentiation medium (+) and analysed for mineral deposition by Von Kossa staining (black colour).

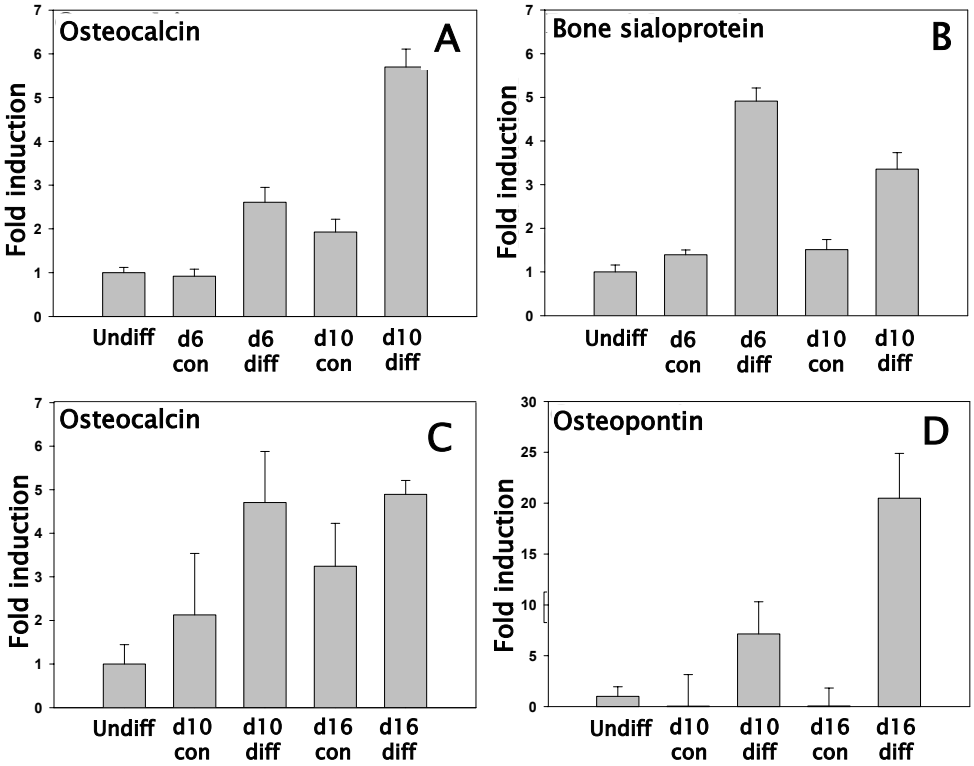


Figure 3. Differentiation of embryonic stem cells. *Expression of the osteocalcin gene and bone sialoprotein in control and differentiation medium by mESCs (A,B). Expression of the osteocalcin gene and osteopontin in control and differentiation medium by hESCs (C,D). Expression was normalized to GAPDH levels for mESCs and 18sRNA for hESCs. Standard deviation is represented by error bars.*

To analyze the *in vitro* osteogenic differentiation of embryonic stem cells we used quantitative PCR to monitor the expression of mature osteoblast markers osteocalcin (OC) and bone sialoprotein (BSP) for mESCs and OC and osteopontin (OP) for hESCs. After embryoid body formation, both mESCs and hESCs were cultured on tissue-culture polystyrene in osteogenic and control medium. As shown in Figure 3A and B, osteogenic medium enhances the expression of both osteocalcin and bone sialoprotein in mESCs at day 6 and 10 compared to control medium. Using von Kossa staining, we observed mineralised nodules in

the mESCs osteogenic culture whereas the control culture did not mineralise (Figure 2). Interestingly, OC expression in control medium of the mESCs is induced at day 10, even though mineralisation is not observed in this culture. A similar phenomenon is seen with the hESCs. At day ten, osteogenic medium enhances the expression of OC and OP 2.5- and 7-fold respectively in comparison to control medium (Figure 3 C, D). Similar to what we observed with the mESCs there is an increase in expression of osteocalcin in time with the control, even though only the cells cultured in osteogenic medium do mineralise (Figure 2). For OP expression there is however an increase (20-fold) in osteogenic medium while there is no increase in the control medium. Although the up-regulation of osteogenic markers is relatively mild, as reported previously [31], osteogenic medium had an all or nothing effect on mineralisation for both mESCs and hESCs. These data show that the embryonic stem cells can be induced *in vitro* into the osteogenic lineage similar to the hMSCs cultures.

### *The composition mESCs-derived mineralised nodules*

To further investigate that the areas displayed by the von Kossa staining are not the result of precipitation of ions, but represent true mineralisation, we analyzed mineralised areas of the mESCs by Raman spectroscopy. Several Raman bands can be observed in the data collected from both control and differentiation medium cell cultures specific for cellular and extra cellular matrix components (Figure 4AB). Several of these bands have been described before by others and can be assigned to protein, phosphate and DNA specific Raman bands. In Table 1, we have summarized the most prominent Raman bands and their respective assignments.

Comparing the spectra obtained from cells grown in either control or mineralization medium demonstrates the appearance of Raman positive bands specific for phosphate and carbonate, indicative of mineralization. In addition, several bands can be observed which can be assigned to the organic part of the extra cellular matrix, mainly collagen type I. We also observed these protein bands in the control samples, which concurs with

the SEM observation of fiber deposition in the control samples (Figure 4CD). Moreover, some of the measurements showed no mineral positive Raman bands, which confirms the observation of heterogeneous distribution of mineralized areas observed by light microscopy after von Kossa staining. Another interesting observation in osteogenic mESCs is the fact that the main phosphate band shows some asymmetry, a small shoulder can be seen around 945 $cm^{-1}$, which might indicate the presence of amorphous calcium phosphate as described by Crane and coworkers [32].

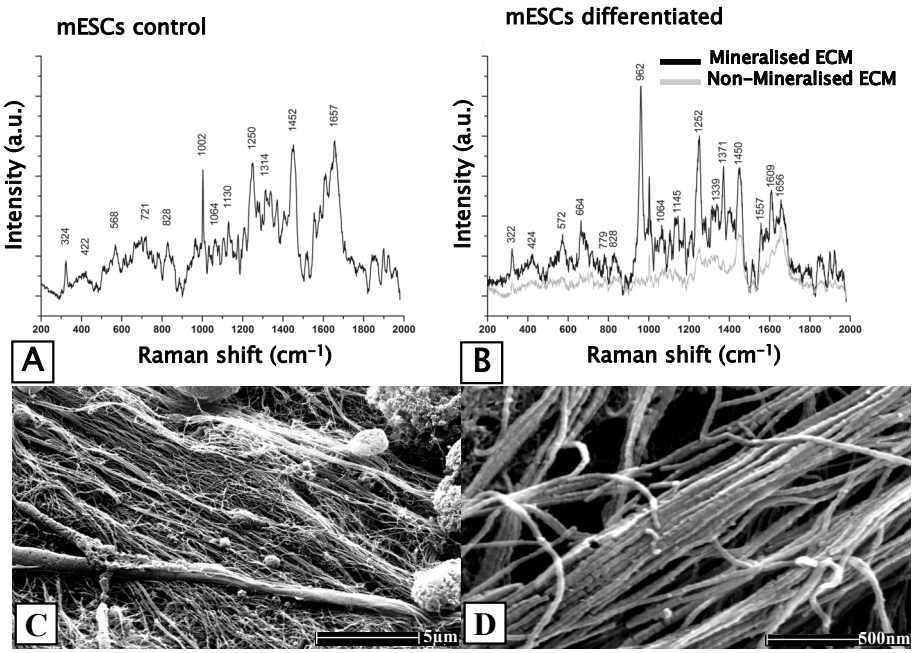


Figure 4. Raman and ESEM imaging. *Spectra obtained from control (A) and osteogenic (B) mESCs cells show the appearance of Raman positive bands specific for phosphate and carbonate, indicative of mineralization. When cultured in osteogenic medium mESCs deposited collagen-like fibers, as visualised by the ESEM (C). Close up of collagen-like fibers with banding pattern (D)*

| Peak position ($cm^{-1}$) | Assignment |
|---|---|
| 422 | $PO_4^{3-}$ $\nu_2$ |
| 550–590 | $PO_4^{3-}$ $\nu_4$ |
| 828 | Nucleotide backbone O–P–O, Tyrosine |
| 880 | Hydroxyproline ring |
| 962 | $PO_4^{3-}$ $\nu_1$ |
| 1002 | Phenylalanine |
| 1031 | $PO_4^{3-}$ $\nu_3$ |
| 1064 | $CO_3^{2-}$ $\nu_1$, P–O symmetric stretch |
| 1130–1150 | C–N, Tyrosine, Phenylalanine |
| 1245–1250 | Amide III, adenine |
| 1339–1375 | Adenine, thymine, Guanine |
| 1450 | C–H, $CH_2$ wag |
| 1650–1660 | Amide I |

Table 1. *Raman band assignments of control and differentiation medium treated mESCs*

### *In vivo bone-forming potential*

HMSCs, hESCs and mESCs were seeded onto calcium phosphate ceramics and cultured for one week in their designated osteogenic culture medium. Prior to implantation, the adherence of the cells was visualized by staining several samples of each cell line with methylene blue (Figure 5). Cells from all three sources adhered to and proliferated on the porous ceramics. The tissue engineered constructs were implanted subcutaneously into immune-deficient mice.

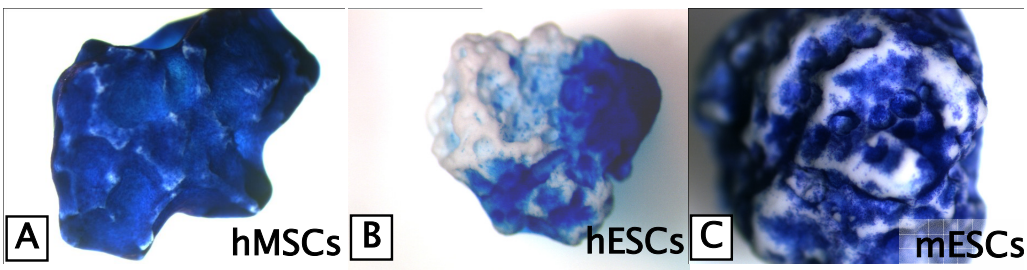


Figure 5. Adherence of stem cells on calcium phosphate ceramics. *The presence of hMSCs (A), mESCs (B) and hESCs (C) on calcium phosphate scaffolds after 7 days of culture is visualised by methylene blue staining.*

After 6 weeks the mice were sacrificed and the samples were explanted, dehydrated and embedded. Sections were made and stained with methylene blue and basic fuchsine and histologically examined. As reported before, the implanted hMSCs exhibited direct deposition of bone tissue [5]. The newly formed bone consisted of osteocytes embedded within the bone matrix, bone marrow and layers of osteoblasts (Fig. 6A,B). Photographs were taken using polarized light, which visualized that lamellar bone was formed (Fig. 6c). Next we evaluated the sections of the implanted mESCs. In none of the examined samples did we observe mature bone tissue as described above (Figure 6D). From our previous research on hMSCs, we were aware of several parameters which influence the performance of hMSCs-induced bone formation such as the *in vitro* culturing time and composition of the differentiation medium [5, 6]. To investigate the effect of the *in vitro* culture time on bone formation by mESCs, we repeated the ectopic bone formation experiment but this time the mESCs were cultured an additional 14 and 21 days prior to implantation. Unfortunately, also this did not result in the formation of mature bone tissue. Next, we varied the osteogenic medium composition of the mESCs by using either dexamethason or BMP-2 as alternative osteogenic compounds to compactin and implanted the constructs. With von Kossa staining we confirmed that also these osteogenic components induced the cells into the osteogenic lineage (data not shown). Unfortunately, again no bone formation was observed (Figure 6 D). Another parameter which may influence the osteogenic process is the composition of the scaffold material. So far, we used bi-phasic calcium phosphate ceramics which do not display osteo-inductivity upon implantation in the muscle of goats [33]. To examine if osteo-inductive ceramics could have a positive effect on *in vivo* bone formation by mESCs we seeded them onto osteo-inductive bi-phasic calcium phosphate ceramics and osteo-inductive tri-calcium phosphate ceramics [33] and cultured them for one week in osteogenic medium and implanted them. From the *in vivo* evaluation, no bone was observed in any of the sections. We conclude that using the most optimal tissue

engineering protocols in use for human MSCs, we were unable to achieve bone formation by mESCs.

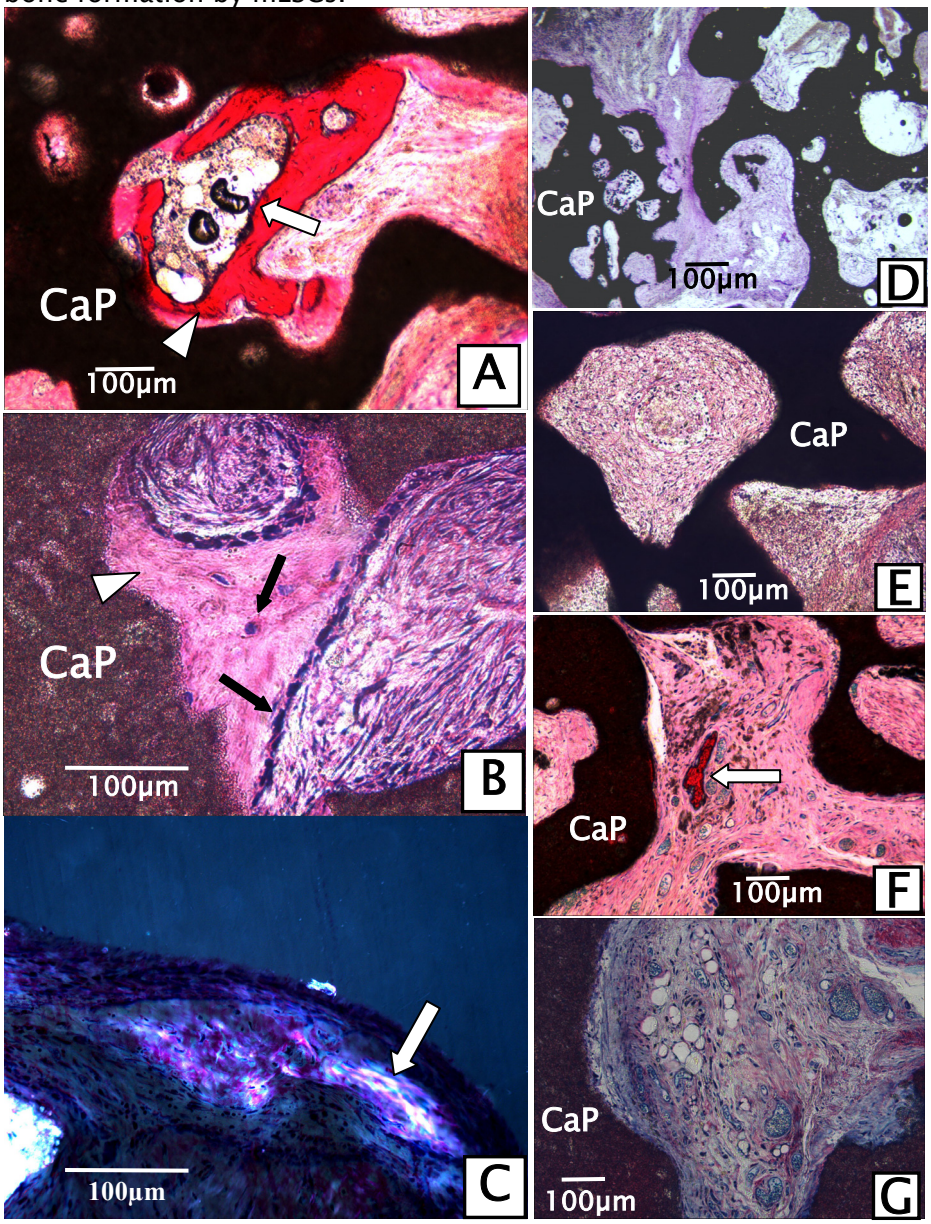


Figure 6. In vivo bone formation by stem cells. *Bone was only formed by hMSCs on porous biphasic calcium phosphate particles after 6 weeks of subcutaneous implantation in immune deficient mice(A,B). The newly formed*

*bone (arrowheads) was deposited against the material (CaP) surfaces. Embedded osteocytes, bone lining cells (small arrows) and bone marrow (big arrow) were observed within the newly formed bone. Bone formed by hMSCs was photographed with polarized light (C), which shows that lamellar bone is formed (arrow). In the sections of both mESCs (D,E) and hESCs (F,G) no bone was observed, also the addition of BMP-2 did not induce the mESCs to form bone bone (E,G), in sections of the hESCs a few areas resembling mineralization (arrow) were observed (F).*

The examination of the hESCs samples, which were cultured for one week in osteogenic medium, also revealed no bone formation but we did find small areas that appeared to be mineralised (Figure 6f). Histologically these areas stain pink which indicates bone formation but no osteoblasts, osteocytes or bone lining cells could be observed. Also with the hESCs we varied the osteogenic medium and replaced dexamethason with BMP-2, however this change did not stimulate the hESCs to *in vivo* bone formation (Figure 6g).

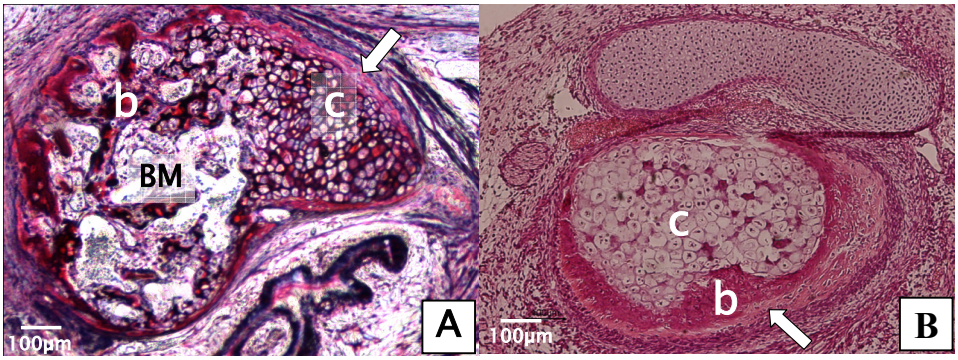


Figure 7. *In vivo* bone formation by stem cells. *Teratomas were formed by both mESCs (A) and hESCs (B). In the teratomas formed by the mESCs the cartilage (c) is partly surrounded by osteoid (arrow) and adjacent calcified bone (b) is formed including bone marrow (BM). The cartilage (c) in the teratoma produced by the hESCs is surrounded by a rim of calcified bone (b) that is sequentially surrounded by osteoid (arrow).*

To demonstrate that our hESCs culture could in principle result in bone formation, we implanted undifferentiated hESCs under the kidney capsule, which favors the formation of teratomas. Histological

examination of the teratomas after 8 weeks demonstrated the formation of bone and osteoid inside the teratomas (Figure 7AB). Interestingly, bone tissue was mostly observed adjacent to areas of cartilage formation, suggesting that the bone tissue is formed through a process of endochondral bone formation rather than through direct, intramembranous, ossification.

## Discussion

In this manuscript, we describe how osteogenic embryonic stem cells fail to produce bone upon implantation in vivo, in contrast to osteogenic hMSCs. Although *in vitro,* all data indicate that at least part of the embryonic stem cell population is differentiating towards mineralising osteoblasts, implantation of these cells *in vivo* does not result in the deposition of mature bone tissue. This in contrast to hMSCs, which form functional bone upon subcutaneous implantation in immune-deficient mice. This observation has important implications for the approach taken in skeletal tissue engineering.

There are a number of potential explanations for the lack of bone formation. First, it may be due to a difference in the number of osteoprogenitors which were implanted. From light microscope observation, it appears that proliferation of ES cells on the ceramics is less efficient then that of hMSCs and we know from our own experience that the amount of implanted cells correlates with the amount of bone formed in vivo. We estimate that the amount of implanted cells is roughly half of that of the hMSCs, which should suffice to result in bone formation. Furthermore, we did observe high cell density on distinct parts of the constructs seeded and with light microscope observation we found tissue formation in and around the ceramics which was a-typical for the fibroblastic cells which normally invade porous biomaterials. This tissue is very likely ES cell-derived. Furthermore, in some cases, the cell density obtained was sufficient to detect mineralising tissue in the hESCs seeded constructs, in which no cells typically present in bone were detected.

Secondly, the efficacy of osteogenic differentiation may be less efficient in our current ES protocols than it is for hMSCs. In the case of ES cells, the cells have to go from a pluripotent state, through a mesenchymal intermediate to eventually form a mineralising osteoblast. In contrast, hMSCs have committed to the mesenchymal lineage and a sub-fraction of the hMSCs in culture even display expression of the typical osteoblast marker ALP. In our experiments, up to about 10% of the undifferentiated hMSCs expressed the osteogenic marker ALP (see Figure 1).

In the osteogenic ES cultures, we observed mineralisation only in nodules, whereas mineralisation in hMSCs was widespread and also observed in single cell layers. Most of the nodules were positive for von Kossa staining and the nodules represented a substantial fraction of all the cells in culture. The implanted cells were allowed to grow up to 21 days *in vitro* on the ceramic scaffolds in osteogenic medium, which presents the ES cells a three-dimensional environment which seems to favour their differentiation. Indeed, we observed that osteogenic differentiation was more effective on the scaffolds than on the culture plate, as assessed by quantitative PCR (data not shown). As such, we have implanted substantial numbers of mineralising osteoblasts derived from mouse and human embryonic stem cells but were not able to observe bone formation from them. This observation raises the question about the nature of the bone-forming cells in bone tissue engineering. It is generally assumed that implantation of committed, mineralising osteoblasts results in bone formation but our ES data and earlier observations with hMSCs argue against this. We and others use ALP as a marker for osteogenic differentiation by hMSCs [5, 34]. Although we frequently observe that up to fifty percent of the hMSCs are ALP positive [7], this does not automatically mean that all ALP-positive cells have the capacity to form bone in vivo. In fact, we were unable to correlate ALP expression to the amount of bone formed after *in vivo* implantation in a panel of 60 donors (unpublished data). Staining of ectopically implanted hMSCs has demonstrated that they do contribute to ectopic bone formation but which cells of the heterogeneous starting population contribute to this bone formation is still unknown [35]. Bone formation is a

highly orchestrated process during development, fracture healing and bone remodeling in which osteo-progenitors are recruited to the desired location and interact with locally present cells and extracellular matrix proteins. *In vitro* differentiation of osteogenic cells can only recapitulate the process to a limited degree, evidenced by the fact that *in vitro* bone formation has not been reported in the literature so far. Apparently, a signal required for the cells to organise themselves into bone-forming units is present at the ectopic site. This signal is able to support bone formation by implanted hMSCs but not by osteogenic hESCs or mESCs.
Another point of concern in the application of ES cells in tissue engineering is the maintenance of their pluripotency during expansion of the cells. When undifferentiated embryonic stem cells are implanted in mice they will form teratomas, which contain tissue from all three germ layers including bone [9, 11]. We did observe bone formation in the teratomas derived from both mouse and human ES cells, thus demonstrating that these ES cells in principle can be differentiated towards the osteogenic lineage. An interesting observation we made was that in both mouse and human ES-derived teratomas we observed bone formation adjacent to cartilage tissue. This organisation is reminiscent of endochondral bone formation, in which a cartilage *anlagen* precedes the formation of bone tissue. We are currently investigating whether bone tissue engineering can be approached through a cartilaginous intermediate.

## Acknowledgements:

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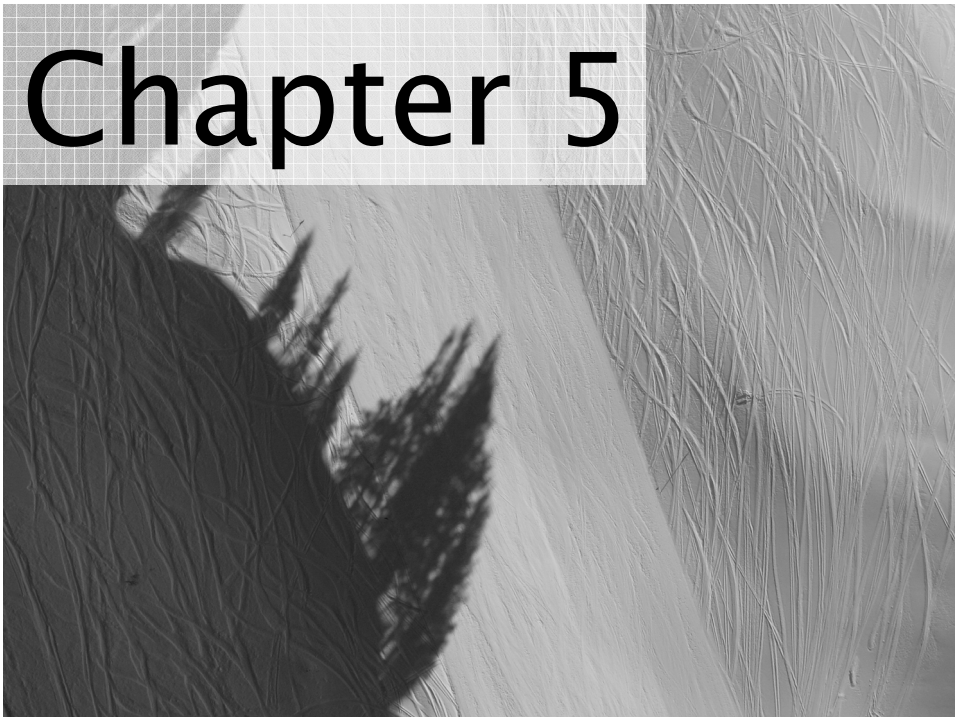
# Chapter 5



# Chapter 5

# Endochondral bone tissue engineering using embryonic stem cells

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## ABSTRACT

Embryonic stem cells can provide an unlimited supply of pluripotent cells for tissue engineering applications. Bone tissue engineering by directly differentiating embryonic stem cells (ESCs) into osteoblasts was unsuccessful so far. Therefore, we investigated an alternative approach, based on the process of endochondral ossification. A cartilage matrix was formed *in vitro* by mouse ESCs seeded on a scaffold. When these cartilage tissue engineered constructs (CTECs) were implanted subcutaneously, the cartilage matured, became hypertrophic, calcified and was ultimately replaced by bone tissue in the course of 21 days. Bone aligning hypertrophic cartilage was observed frequently. Using various chondrogenic differentiation periods *in vitro,* we demonstrated that a cartilage matrix is required for bone formation by ESCs. Chondrogenic differentiation of mesenchymal stem cells and articular chondrocytes showed that a cartilage matrix alone was not sufficient to drive endochondral bone formation. Moreover, when CTECs were implanted orthotopically into critical size cranial defects in rats, efficient bone formation was observed. We report for the first time ESC-derived bone tissue engineering under controlled, reproducible conditions. Furthermore, our data indicate that ESCs can also be used as a model system to study endochondral bone formation.

## INTRODUCTION

Bone tissue engineering is generally approached by combining osteogenic cells with a porous biodegradable ceramic scaffold. Human bone marrow-derived mesenchymal stem cells (MSCs) can be differentiated into the osteogenic lineage by culturing the cells in the presence of the osteogenic differentiation supplements dexamethasone, ascorbic acid and ß-glycerol phosphate [1, 2]. Mineralized bone matrix is deposited *in vitro* as a result of the expression of osteogenic genes [3] and *de novo* bone formation is observed when human MSCs are implanted into an ectopic or orthotopic site. Even though MSCs from most human donors show osteogenic potential, there is large variation in bone forming capacity by human MSCs, and multipotency is gradually lost upon expansion [4]. Most importantly, bone formation by MSCs is currently insufficient for successful tissue engineering [5]. Besides efforts to increase bone formation by MSCs *in vivo*, we also explore embryonic stem cells (ESCs) [6-8] as a potential source for bone tissue engineering. ESCs are capable of indefinite, undifferentiated proliferation *in vitro* and can provide an unlimited supply of cells which can be differentiated into various cell types [9].

Before ESCs can be used in clinical applications, some technical issues have to be addressed, such as the labor-intensive procedure and the use of animal-derived reagents to expand human ESCs, the immunogenicity of allogeneic ESCs and the potential risk of tumorigenicity. Moreover, a differentiation scheme has to be designed to obtain the desired cell or tissue type. Osteogenic differentiation of mouse and human ESCs has been established *in vitro*, by culturing the cells in medium supplemented with ascorbic acid, ß-glycerol phosphate, dexamethasone[10-12], BMP2 [13], compactin [13] or vitamin D3 [14]. Mineralization was observed and qPCR analysis showed up-regulation of osteogenic markers such as Cbfa-1/Runx2, osteopontin, bone sialoprotein and osteocalcin. We observed similar results when mouse and human ESCs were differentiated into the osteogenic lineage *in vitro* (manuscript in preparation). To assess bone tissue engineering using ESCs, we seeded human or mouse ESCs onto

ceramic scaffolds and cultured them in osteogenic media for 7 or 21 days. Six weeks after implantation into immuno-deficient mice, no bone tissue was observed in samples of mouse ESCs (manuscript in preparation). For human ESCs, we observed some *in vivo* mineralized tissue, but no bone tissue, as reported previously [11]. So far, *in vivo* bone formation by ESCs has only been observed in teratomas. Strikingly, it occurred to us that bone tissue in teratomas frequently aligns hypertrophic cartilage, which resembles the process of endochondral ossification. Most bones in the body are formed via endochondral ossification, which involves the formation of cartilage tissue from condensed mesenchymal cells, and the subsequent replacement of the cartilage template by bone. In contrast, direct conversion of mesenchymal tissue into bone is called intramembranous ossification, which occurs primarily in the craniofacial skeleton. In this manuscript, we describe an alternative approach to *in vivo* bone formation by ESCs, based on the process of endochondral ossification.

## Materials and methods

### *Cell culture*

Mouse ESC line IB10 was cultured and embryoid bodies were formed as described previously [15]. ESC-derived embryoid body cells were used for differentiation experiments. Human MSCs were isolated from bone marrow aspirates from donors that had given written informed consent [1]. Goat MSCs and calf articular chondrocytes were isolated as described earlier [16] [17].

### *Differentiation*

Aliquots of 1.5 million cells were seeded onto 3 ceramic particles of 2-3 mm, prepared as described by Yuan *et al.* [18]. For cranial implants, 1 million cells were seeded statically on both sides of the 8 x 1.5 mm disc. Further differentiation of stem cells into the chondrogenic lineage was performed in serum-free chondrogenic medium containing TGFß3 [19, 20]. For differentiation of human MSCs, the chondrogenic medium was

supplemented with 250 ng/ml human BMP6 (Biovision) [21]. For differentiation of ESCs into the osteogenic lineage, cells were cultured for another 3 days in medium supplemented with $10^{-7}$ M retinoic acid (Sigma) and subsequently in medium supplemented with 0.2 mM ascorbic acid, 2.5 μM compactin (Sigma) and 0.01 M β-glycerol phosphate (Sigma) (adapted from [13]). Osteogenic differentiation of human MSC was described by Both *et al.* [1].

### *In vivo studies*

Samples were precultured in chondrogenic or osteogenic medium for 21 days and subsequently implanted into immuno-deficient mice (HsdCpb:NMRI-nu Harlan, n=6) for 21 days, unless indicated otherwise [15]. For cranial implantation, immuno-deficient rats (Crl:NIH-*Foxn*$^{rnu}$, Charles River) were injected subcutaneously with 0.02 mg/kg buprenorphine (Temgesic) for pain relief. The rats were induced with 4-5% isoflurane and during the operation they were maintained with a mixture of isoflurane (1.5-3%), $O_2$ (200-300 ml/min) and $N_2O$ (50-200 ml/min). An incision was made in the skin over the cranium from the middle of the nasal bones to the posterior nuchal line. The periostium was sedated with Lidocaine (2%) and removed. An 8 mm trephine dental bur (ACEuropa, Lda) was used to mark the defect site and a 0.7 mm drill (Synthes) was used to remove the bone to realize the craniotomy. An implant was press fitted into the defect site. Six rats received a sham implant and 7 rats received a CTEC. The overlaying tissue was sutured back in layers. After 4 weeks, implants were removed and processed histologically as described below. Animals were housed at the Central Laboratory Animal Institute (Utrecht University, The Netherlands) and experiments were approved by the local Animal Care and Use committee.

### *Histological staining and light microscopical analysis*

Samples were fixed in 0.25% glutaraldehyde (Merck) in 0.14 M cacodylate buffer and dehydrated using sequential ethanol series. Scaffolds were embedded in methyl methacrylate (LTI, Bilthoven, The

Netherlands) and sections were processed on a histological diamond saw (Leica SP1600; Bensheim, Germany). Sections were etched with an HCl/ethanol mixture and sequentially stained to visualize cartilage and bone. Cartilage formation was visualized by 0.04% thionin (Sigma) in 0.1 M sodium acetate (Merck), which stained cells blue and glycosaminoglycans pink. Bone formation was visualized by 1% methylene blue (Sigma) and 0.03% basic fuchsin (Sigma), which stained cells blue and bone pink. Histological sections were analyzed using a light microscope (E600 Nikon). For mineralization studies, ESC-derived EBs or MSCs were grown on tissue culture plates in osteogenic medium for 21 days, fixed and incubated with 5% silver nitrate (Sigma) under a UV-lamp, until black staining was observed.

### *Histomorphometry*

Histomorphometry was performed on particles which were explanted at different time points and on the inner circle of cranial implants. Low magnification images were made from 2-3 sections per sample. Scaffold, bone and cartilage were pseudo-coloured and image analysis was performed with KS400 software (Carl Zeiss Vision, Oberkochen, Germany). A custom-made program (University of Utrecht, The Netherlands) was used to measure percentage of cartilage or bone compared to scaffold area.

### *Statistical analysis*

Statistical calculations were performed with SPSS 14.0 software (Chicago, IL). Histomorphometric data for particles was not normally distributed. Therefore, we used non-parametric tests to compare the amount of cartilage and bone in time (Kruskal-Wallis), and the amount of cartilage and bone at different time points (Wilcoxon signed rank test). For the cranial implants, the samples of the sham group showed little to no bone in-growth in the inner circle whereas bone was formed in all TE samples. Due to the lack of variation in the sham group, we calculated the mean of the three images for each sample and a Mann-Whitney U test was used to detect a difference between the two groups.

## Results

### *Chondrogenic differentiation of mouse ESCs in vitro and bone formation in vivo*

It is well established that osteogenic human MSC mineralize *in vitro* (Figure 1A) and form bone *in vivo* (Figure 1B) [1]. This process of ossification occurs through intramembranous ossification, without the intermediate production of cartilage (data not shown). Moreover, it is known that mouse ESCs, like MSCs, can be induced into the osteogenic lineage *in vitro* as indicated by mineralization (Figure 1C) and the up-regulation of osteogenic genes. However, in contrast to human MSCs, these osteogenic mouse ESCs did not form bone upon implantation (Figure 1D). Therefore, we assessed an alternative approach: endochondral ossification, in which ESCs first deposit cartilage, which may serve as a template for ossification. In previous studies, we have shown cartilage formation by mouse ESCs in pellets, on polymeric scaffolds and in hydrogels [15]. We now assessed the chondrogenic potential of mouse ESCs on ceramic scaffolds. ESC-derived embryoid body (EB) cells were seeded on ceramic particles and cultured in serum-free chondrogenic differentiation medium containing TGFβ3 for 21 days. Regions with typical cartilage morphology, being round cells with lacunae surrounded by extracellular matrix, were found on the outside of the particles and inside the pores (Figure 1E). Cartilage was formed in each sample cultured in chondrogenic medium and about a tenth to a third of the cells on the particles differentiated into chondrocytes. Collagen type II expression was substantially up-regulated compared to control cultures (data not shown). Particles cultured in control or osteogenic medium did not show formation of cartilage (data not shown).

After creating a cartilage template on ceramic particles by differentiating mouse ESCs into the chondrogenic lineage for 21 days *in vitro* (from now on referred to as cartilage tissue engineered constructs, CTECs), the next step was to demonstrate *in vivo* bone formation. Therefore, CTECs were implanted subcutaneously in the back of immuno-deficient mice for 21

days. Bone-like tissue was formed in all samples which were differentiated into the chondrogenic lineage (Figure 1F), in contrast to the samples which were differentiated into the osteogenic lineage *in vitro* (Figure 1D), in which bone tissue was never observed. The newly formed bone, also known as osteoid, was aligned with osteoblasts, and osteocytes were visible in the mature and mineralized bone tissue (Figure 1G). Bone was formed both on the outside of the particles, as within the pores, and the tissue consisted of lamellar bone as demonstrated by polarized light (Figure 1H). For the first time, we demonstrate directed, reproducible bone formation by mouse ESCs *in vivo*.

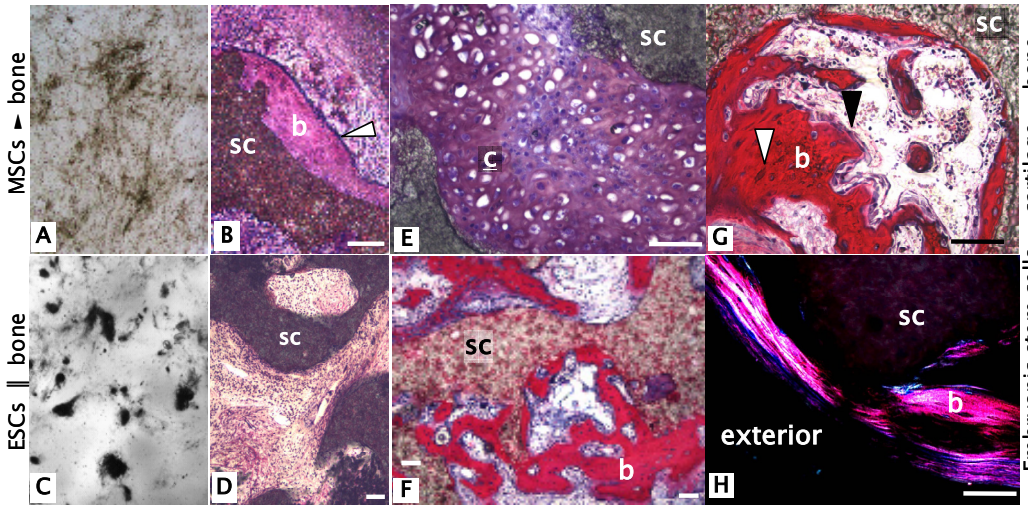


Figure 1. Two approaches for *in vivo* bone formation by MSCs and ESCs.
*(A) In vitro osteogenic differentiation of human MSCs cultured on tissue culture plastic for 21 days, indicated by von Kossa staining, which stains mineralized matrix black. (B) In vivo, bone is formed by human MSCs, as shown by the methylene blue and basic fuchsin stained sections of cells grown on ceramic particles for 7 days and implanted subcutaneously into immuno-deficient mice for 6 weeks. Bone tissue stains pink and bone-lining cells are indicated by a black arrow head. (C) In vitro osteogenic differentiation of mouse ESCs cultured on tissue culture plastic for 21 days, indicated by black von Kossa staining of the mineralized matrix. (D) After 21 days, no bone is formed in vivo by mouse ESCs, which were precultured in vitro for 21 days on ceramic particles in osteogenic*

*medium. (E) In the process of endochondral ossification, bone is formed on a cartilage template. Mouse ESCs were cultured in chondrogenic medium on ceramic particles for 21 days. Cells displayed a chondrocyte phenotype as indicated by round cells in lacunae surrounded by extracellular matrix, which stained positive for glycosaminoglycans (indicated by pink thionin staining). (F) CTECs were implanted subcutaneously for another 21 days to demonstrate bone formation. Bone tissue is stained dark pink by basic fuchsin. (G) Higher magnification of bone tissue observed on implanted CTECs. Bone-lining cells are indicated by a black arrow head and osteocytes by an open arrowhead. (H) The bone tissue that was formed consisted of lamellar bone as indicated by polarized light. Ceramic scaffolds are indicated by sc, bone by b and cartilage by c. Scale bar represents 100 µm.*

### *A time course of endochondral bone formation by mouse ESCs*

Next, we investigated the fate of *in vitro* formed cartilage after implantation and the process of bone formation *in vivo*. Therefore, we analyzed the CTECs 2, 7, 14 or 21 days after implantation (Figure 2A). At the time of implantation, cartilage tissue was present on the CTECs and hardly any cellular stroma was observed. After 2 days *in vivo*, cartilaginous tissue was still present on the implanted CTECs, indicating the survival of the implanted tissue. The CTECs showed different amounts of fibrous immature scar-like tissue that showed resemblance to mesenchymal cells in tissue cultures. After 7 days *in vivo*, the cartilage showed the beginning of maturation indicated by larger lacunae with smaller uniform nuclei. The onset of endochondral calcification was indicated by slight basic fuchsin staining within the mature cartilage. Furthermore, the fibrous stroma became more cellular and dense and contained more vessels. Bone formation was first seen after 14 days *in vivo*. Bone surrounded hypertrophic chondrocytes and the mineralized cartilage matrix (Figure 2B). In some regions, cartilage seemed to be totally replaced by bone and in other regions, mature cartilage was still present. After 21 days *in vivo*, most of the cartilage was replaced by bone. We even observed tissue resembling bone marrow in some bone lacunae (Figure 2A).

The gradual decrease in the amount of cartilage and a gradual increase in the amount of bone tissue in time were confirmed by histomorphometric analysis. After 21 days, there was significantly less cartilage and significantly more bone per scaffold area than at earlier time points. There was also significantly more bone than cartilage after 21 days (Figure 2C). Thus, in the course of 21 days *in vivo*, almost all cartilage matured and was replaced by bone tissue.

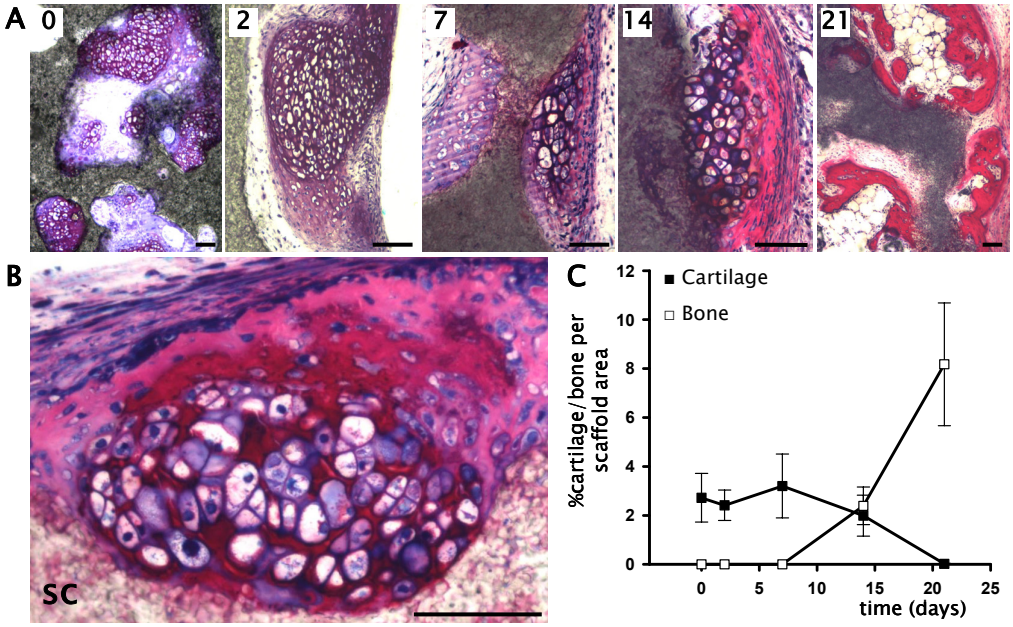


Figure 2. *In vivo* bone formation throughout time. *(A) Representative images of histological sections of CTECs 0, 2, 7, 14 and 21 days after implantation. At 0 and 2 days in vivo, cartilage matrix is visualized by pink thionin staining of glycosaminoglycans. At 7, 14 and 21 days in vivo, bone tissue is stained by methylene blue and basic fuchsin staining, which stains cells blue and bone tissue dark pink. Cartilage can still be recognized by morphology, being an extracellular matrix in which single cells in a lacuna can be distinguished. (B) Higher magnification image of a CTEC after 14 days in vivo, showing the process of endochondral ossification. Hypertrophic chondrocytes in mineralized cartilage were surrounded by bone tissue. Scale bars represent 100 µm. (C) Histomorphometric analysis of the amount of cartilage and bone per available scaffold area in time.*

### *A cartilage template from ESCs is necessary for endochondral bone formation*

We investigated whether either chondrogenic stimulation or cartilaginous tissue was required for *in vivo* bone formation. Therefore, we differentiated cells *in vitro* for 3, 7, 14 and 21 days, and subsequently implanted these samples for another 21 days into immuno-deficient mice. *In vitro* chondrogenic differentiation for 3 and 7 days did not result in tissue with typical cartilage morphology. After 14 days, the first cartilage could be observed and more cartilage tissue was formed after 21 days of *in vitro* culture (Figure 3A).

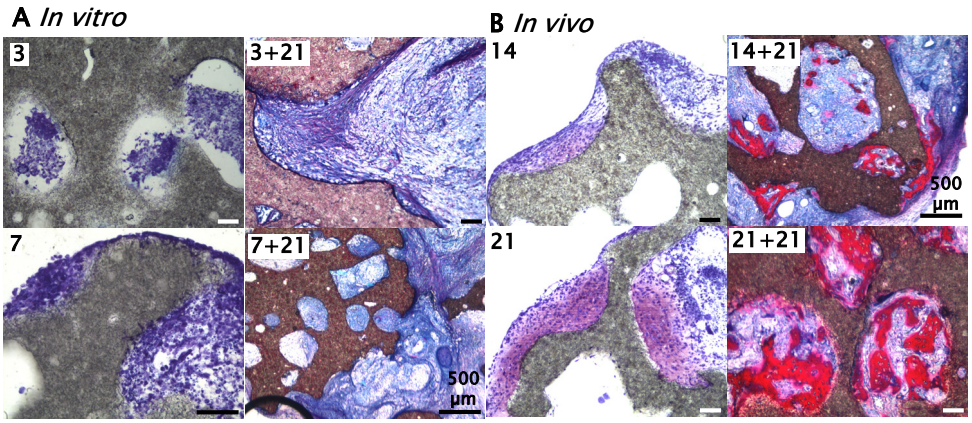


Figure 3. The necessity of a cartilage template for *in vivo* bone formation by mouse ESCs. *(A) Chondrogenic differentiation of mouse ESCs for 3, 7, 14 and 21 days in vitro, as indicated by thionin staining. Cartilage matrix was first observed after 14 days of chondrogenic differentiation and the amount increased in time as seen after 21 days. (B) Subsequent implantation for 21 days after in vitro differentiation for 3, 7, 14 and 21 days. Bone tissue was observed in the 14+21 and 21+21 days samples as indicated by basic fuchsin staining. Scale bars represent 100 µm.*

After subsequent implantation for 21 days, we scored the amount of bone nodules aligning the ceramic particle in all histological sections (5-7 sections per sample, 6 mice per time point). No bone nodules were

observed in the 3 days differentiated samples. For the samples that had been differentiated *in vitro* for 7 days, we observed 1 bone nodule in a few sections, with an average of 0.2 bone nodules/section. For the 14 days-CTECs, we observed 0 to 13 bone nodules in the sections, with an average of 4.6 bone nodule/section. For the 21 day CTECs, we observed 2 to 20 bone nodules, with an average of 9.2 bone nodules/section. Thus, bone was mainly observed in the samples that had been differentiated into the chondrogenic lineage for 14 days and 21 days (Figure 3B). In consistency with the higher amount of cartilage *in vitro*, the highest amount of bone was found in the 21 days samples. We conclude that a cartilage template is required for bone formation.

### *A cartilage template is not sufficient for endochondral bone formation*

To investigate whether any cartilage template will mature, calcify and will be replaced by bone, we implanted cartilage derived from articular chondrocytes and adult stem cells.

Freshly isolated calf chondrocytes were cultured on ceramic particles in chondrocyte proliferation medium. After 21 days *in vitro*, cartilage and some fibrous tissue was formed on the ceramic particles (Figure 4A). Subsequently, these constructs were implanted into immuno-deficient mice. The cartilage phenotype was stable *in vivo* and more cartilage matrix was deposited (Figure 4B). Hypertrophy and calcification of the cartilage matrix was not observed. No signs of endochondral ossification were observed when articular chondrocyte-derived cartilage was implanted.

We also investigated the fate of a cartilage template derived from adult stem cells. Given that even non-stimulated MSCs can form bone *in vivo*, we had to look for signs of endochondral ossification other than sheer bone formation, such as hypertrophic chondrocytes, calcified matrix and regions where bone aligns and replaces cartilage. We seeded goat and human MSCs on ceramic particles and differentiated these cells into the chondrogenic lineage as described for ESCs. Cartilaginous tissue was formed by goat MSCs, which was almost homogeneous in some samples

(Figure 4A). Human MSCs proliferated on the ceramic particles and a small amount of GAG positive tissue was observed (Figure 4A), limited to one region per sample. Next, these CTECs were implanted into immuno-deficient mice for an additional 21 days. Cartilaginous tissue was still observed and bone was formed *in vivo* (Figure 4B). Although the cartilage and bone areas were not quantified, from light microscopical analysis of histological slides, it was obvious that goat MSCs formed substantially more cartilage *in vitro* and more bone *in vivo* than human MSC.

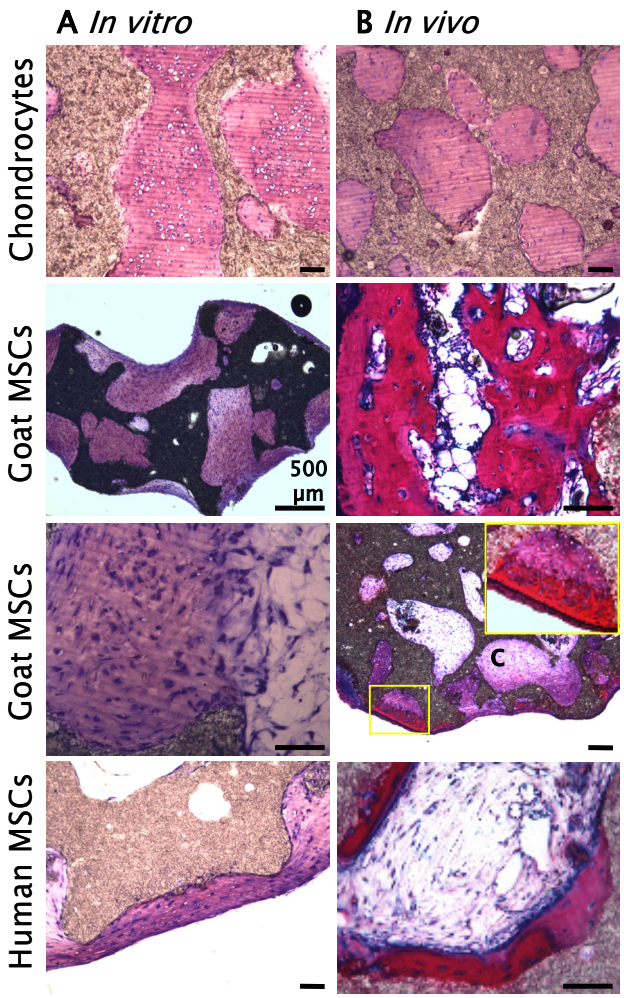


Figure 4. A cartilage template is not sufficient to induce endochondral bone formation. *(A) In vitro cartilage formation by bovine chondrocytes, goat MSCs and human MSCS, stained by thionin. (B) Subsequent implantation of the in vitro samples as displayed in (A). No bone was formed in the bovine cartilage samples. Bone was observed in both goat MSCs and human MSC samples as indicated by basic fuchsin staining. In some regions, bone aligned cartilage (inset), but hypertrophic chondrocytes were not observed. Cartilaginous tissue (c) was still present in goat MSC samples. Scale bars represent 100 µm.*

Bone formation by human MSC remained limited to an average of 1 bone nodule per sample, similar to the rather inefficient chondrogenic differentiation *in vitro*. In studies with mouse ESCs, hardly any cartilage was observed after 21 days *in vivo* (Figure 2C). However, large regions of cartilage were still present in the implanted goat MSCs samples (Figure 4B), but this cartilage did not show typical morphology of maturation or hypertrophy. Although in some regions bone did align cartilaginous tissue, and some basic fuchsin positive areas were observed in cartilaginous tissue, we did not observe the typical replacement of cartilage by bone, as observed with mouse ESCs (Figure 2B). Based on these experiments, we conclude that a cartilaginous matrix does not automatically lead to endochondral bone formation, but rather that ES-derived cartilage has a tendency to mature and enter the process of endochondral ossification.

### *Bone tissue engineering in an orthotopic defect*

The above-mentioned *in vivo* studies were restricted to ectopic implantation sites. To study bone formation in an orthotopic defect, we implanted mouse ESCs in an 8 mm critical size cranial defect in an immuno-deficient rat. Ceramic discs were seeded with mouse ESC-derived EB cells and cultured *in vitro* in chondrogenic medium for 21 days. As a control, empty discs were implanted. Bone formation was visualized by staining with methylene blue and basic fuchsin. Cartilage was observed in all *in vitro* discs (CTECs, data not shown). Bone tissue was observed *in vivo* in all discs, both in CTECs (Figure 5A) and sham implants. To distinguish between bone in-growth from the cranium, and bone formation by ESCs, the discs were divided in an outer and inner ring (Figure 5A). The percentage of bone in the inner circle of both sham implants and CTECs (Figure 5C) was determined by histomorphometry (Figure 5B). Significantly more bone was observed in the inner circle of CTECs, as compared to sham implants. Hereby, we show efficient bone formation by ESCs in an orthotopic defect.

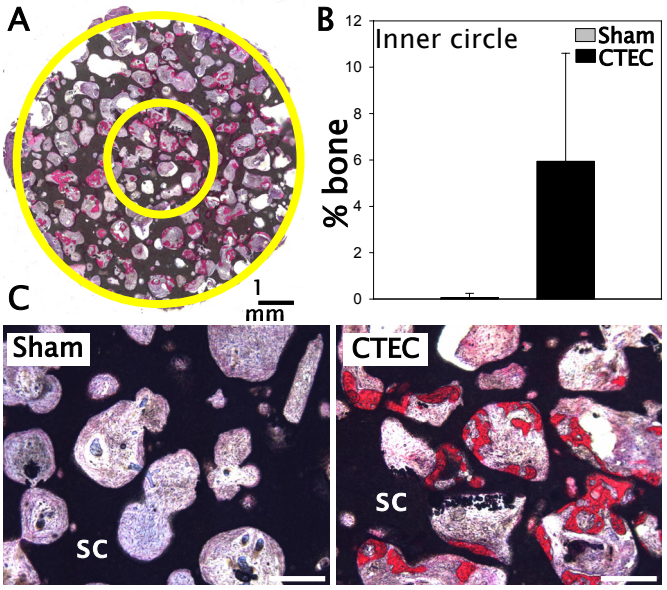


Figure 5. Orthotopic bone for-mation by ESCs in a rat cranial defect. *(A) Bone formation in an 8 mm CTEC implanted for 21 days in a critical size cranial defect in a rat, visualized by methyllene blue and basic fuchsin staining.*

*For subsequent analysis, the sample was divided in an outer and inner ring, indicated by yellow circles. (B) Histomorphometric analysis bone tissue formed in the inner circle of sham implants and CTECs. (C) Higher magnification view of the inner circle (2.7 mm) of a sham implant and a CTEC. Bone tissue was visualized by basic fuchsin staining. Scale bar represents 500 µm, scaffold is indicated with sc.*

## Discussion

In this paper, we describe *in vivo* bone formation by mouse ESCs. We first attempted to directly differentiate ESCs into the osteogenic lineage, based on differentiation protocols and media established for adult stem cells [3] and osteoblasts. Although *in vitro* results were satisfying, *in vivo* experiments did not result in bone formation, by either mouse or human ESCs. We opted for an alternative approach by first forming a cartilage template and subsequently allowing the cartilage to be replaced by bone. Using this approach, we demonstrated for the first time directed and reproducible *in vivo* bone formation by ESCs in ectopic and orthotopic sites. The process is very robust, as we observed bone in all experiments where an ESC-derived cartilage template was formed *in*

*vitro*. The amount of bone formed in these experiments was comparable to the amounts formed by rat and goat MSCs (data not shown). We also observed endochondral bone formation on polymeric scaffolds, indicating that *in vivo* bone formation was not induced by and is not exclusive to ceramic scaffolds (data not shown).

Most, if not all, differentiation protocols result in a heterogeneous population of ESCs differentiated into various lineages, but enriched for the desired cell type. Cartilage and bone were formed in all experiments, but ESCs also differentiated into other less or more advanced tissue types, like squamous and cylindrical epithelial cells lining cyst-like spaces and tubules, endothelial cells lining (blood) vessels, fat and stroma. In some experiments we even observed teratoma formation. Cartilage, hypertrophic and calcified cartilage and some bone were observed in the teratomas, but in contrast to our directed differentiation, these tissues did not align the scaffold material. For future application, the constructs should be purified from residual undifferentiated ESCs to avoid teratoma formation *in vivo*. In addition, purification could result in more homogeneous cartilage formation, which might result in improved bone formation.

The next step will be to show *in vivo* bone formation by human ESCs. When human ESCs were differentiated into the osteogenic lineage, small fragments of mineralized tissue were observed *in vivo* [11], but no osteocytes and osteoblasts were observed. The first step in endochondral bone formation by human ESCs would be the formation of a cartilage template. The chondrogenic potential of human ESCs has been demonstrated in indirect co-culture experiments with primary chondrocytes [22]. Whereas mouse ESCs show consistent cartilage formation, we did not observe cartilage formation when the chondrogenic protocols were transferred to human ESCs (data not shown). Serum-free chondrogenic medium containing TGFß3 did not only result in heterogeneous cartilage formation by mouse ESCs, but the cartilaginous tissue that was formed by human MSCs in our studies was also not homogeneous (data not shown), even when supplemented with

BMP6. Further optimization of the growth-factor regime for both adult and embryonic stem cells is necessary [23, 24].

Mouse ESC-derived cartilage displayed maturation, calcification and subsequent replacement by bone tissue. Indications of maturation were also observed for human ESC-derived cartilaginous tissue [22, 24] Apparently, ESCs followed the route of embryonic development. This implicates that ESCs can be used as a model system to study endochondral bone formation. Where most current models use chicken eggs, *in vitro* limb cultures, or transgenic mice, our results show that endochondral bone formation can now be studied with ESCs. Transgenic ESCs, rather than transgenic mice can be used to investigate the influence of several genes in the process of endochondral bone formation. Besides *in vitro* assays, *in vivo* bone formation can be studied in ectopic and orthotopic models. The orthotopic defect used in our studies might not be the most logical model for endochondral bone formation, as it is well known that the bones of the craniofacium form through intramembranous ossification. However, this study does demonstrate that that the implanted mouse ESCs form bone tissue in an orthotopic defect in a rat.

In conclusion, our data show that mouse ESCs readily undergo endochondral ossification after deposition of a cartilage matrix, which can benefit both bone tissue engineering and the genetic dissemination of endochondral bone formation.

## Acknowledgements

We would like to thank Jan de Wit from Erasmus MC Rotterdam for supply of IB10 mouse ESCs, Hugo Fernandes from the University of Twente and the GDL in Utrecht for assistance with rat cranial defect surgery and Pamela Habibović from the University of Twente for assistance with statistical analysis. JMJ was supported by STW grant TPG 5923, JdB and SB were supported by a Senter/Novem grant.

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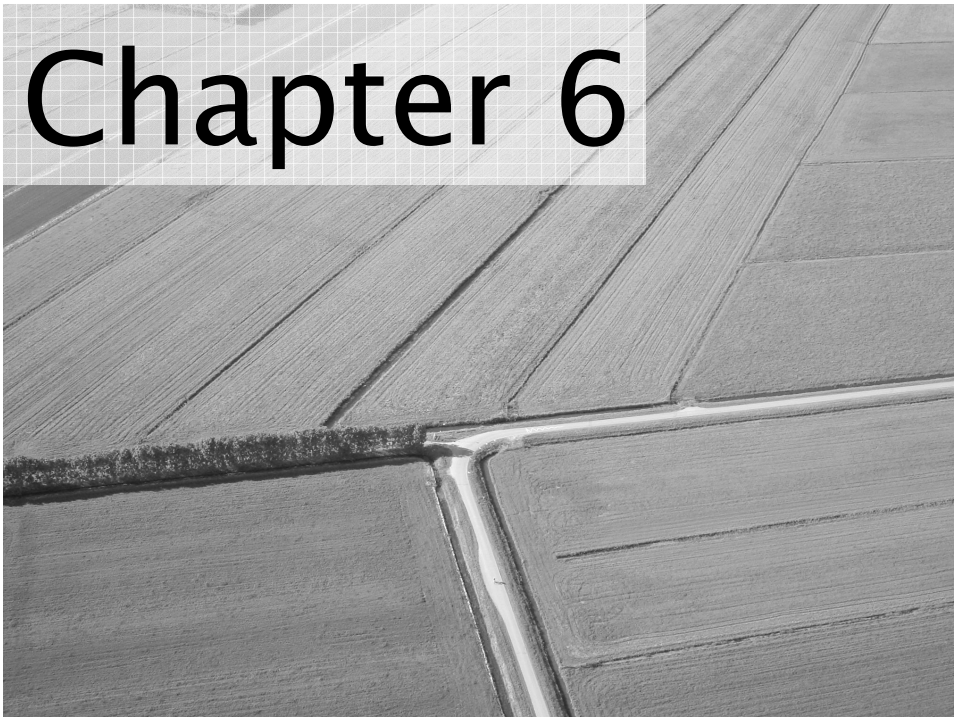
# Chapter 6



# Chapter 6

# Bone tissue engineering in a rat cranial critical size defect with embryonic stem cells

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## Abstract

Bone tissue engineering is typically approached using autologous adult stem cells, but we have recently demonstrated very efficient bone formation by mouse embryonic stem cells. Using a cartilage intermediate, we observed endochondral bone formation on ceramic scaffolds in the subcutis of immune-deficient mice. To extend this observation to osseous defects, we analysed bone formation both subcutaneously and in a cranial defect in immune-deficient rats. Subcutaneous implantation of chondrogenical differentiated mouse embryonic stem cells showed that the process of subcutaneous endochondral bone formation occurs equally efficient in immune deficient rats as previously described in mice. Next, mouse embryonic stem cells were seeded on 8 mm calcium phosphate disks and implanted in a critical size cranial defect in immune-deficient rats for four weeks. In control disks without cells limited bone was observed at the periphery of the disks as a result of bone ingrowth from the skull. In contrast, extensive bone formation throughout the scaffold was observed in the rats treated with tissue engineered implants. We conclude that bone tissue engineering using embryonic stem cells can be achieved through endochondral bone formation, even at an osseous site where bone is normally not formed in this way but rather through endosseous process.

## Introduction

Ever since embryonic stem cells (ESCs) were isolated by Evans and Kaufman in 1981 [1] they have intrigued the scientific community. But it was not until Thomson et al [2] in 1998 described the isolation and culture of human embryonic stem cells (hESCs) that the interest in the use of embryonic stem cells for therapeutic purposes surged. Although clinical applications with hESCs have not yet been achieved, researchers have succeeded in differentiating embryonic stem cells towards many different lineages *in vitro*, such as cardiomyocytes [3], neural precursors [4, 5], insulin-producing cells [6], liver [7] and blood vessels [8].

We focus on the potential application of ES cell in bone replacement surgery, because there is a large group of patients which requires a surgical intervention where a bone graft is needed for recovery of the worn-out or damaged tissue. The most frequently used sources of grafting material, in large bone defects, are autologous and allogeneic bone, but both methods have their drawbacks. Autologous bone harvesting is accompanied with donor site morbidity and post-operative pain and it has limited availability [9, 10]. Allogeneic bone carries an immunological risk, potential disease transmission and it is not as potent as autologous bone. Therefore, the field of cellular skeletal tissue engineering focuses on the use of human mesenchymal stem cells (hMSCs) for bone repair. By combining bone-forming cells with a biomaterial, a hybrid construct is created which could function as an alternative for autologous bone. However, hMSCs have a number of drawbacks. During expansion, hMSCs display loss of multipotency [11], large donor variations occur and the bone forming capacity of hMSC-seeded grafts is currently not sufficient to bridge an osseous defect [12]. Recently, we focused on ESCs as an alternative cell source for bone tissue engineering because they can proliferate indefinitely and differentiate into all cell types of the body. So far, *in vitro* osteogenic differentiation of both mESCs [13, 14] and hESCs [15] was accomplished via the culture method previously used for hMSC osteogenesis. The ESCs are hereby directly differentiated into the osteogenic lineage by using

osteogenic stimuli provided in the culture medium. However, given that most studies were done *in vitro* we recently compared the osteogenic performance of mESCs and hESCs versus hMSCs both *in vitro* and *in vivo* [16]. This study demonstrated that at least part of the embryonic stem cell population is differentiating towards mineralising osteoblasts, but upon implantation these cells *in vivo* it do not result in the deposition of mature bone tissue even though ESCs do have the ability to form bone in teratomas. As an alternative strategy, we first differentiated ES cells into cartilage-like tissue on ceramic scaffolds and next implanted the cartilage graft into immune-deficient animals. We observed a process of endochondral bone formation whereby bone tissue was first observed after 14 days but was more pronounced after 21 days. To demonstrate that embryonic stem cells have the potential to be used for tissue engineering and that the process of endochondral bone formation by mESCs does not only occur subcutaneously, we investigated the use of ES cells in a critical size osseous model.

There are various osseous animal models, but because we are investigating mESCs, we are restricted to the use of immune-deficient animals like rats and mice. Evaluation of autologous bone graft substitutes with a cranial defect model is over a 100 years old [17]. In 1986, Schmitz and Hollinger [18] examined the use of a cranial defect model and defined a critical size defect as “the smallest size intra-osseous wound in a particular bone and species of animal that will not heal spontaneously during the life time of the animal”. Other investigators have shown that in a rat cranium the critical defect size is 8 mm [18-20]. To this end, we made calcium phosphate disks with a diameter of 8mm, seeded them with mESCs and differentiated these into cartilage-like tissue. These disks were then implanted into the cranium of immune-deficient rats to investigate if the process of endochondral bone formation also occurs in an orthotopic defect.

## Materials and methods

### *Culture of mouse embryonic stem cells.*

The mouse embryonic stem cell (mESC) line, IB10 [21] was grown on tissue culture polystyrene, coated with 0.1% gelatin (Sigma) and maintained in basic medium consisting of DMEM (Cambrex, Biowhitakker) medium supplemented with 10% FBS (Greiner, selected batch for mESCs), 1% non-essential amino acids 100x (Sigma), 4mM L-glutamine (Sigma), penicillin G (100 u/ml, Invitrogen); streptomycin (100 μg/ml, Invitrogen) and 100 μM 2-mercaptoethanol (Invitrogen). To maintain the undifferentiated state, cells were cultured in the presence of Buffalo rat liver cell-conditioned (BRL) medium and 1000 units/ml of Leukemia inhibiting factor (Esgro, Chemicon international). The cells were grown in a 37$^0$C humid atmosphere at 5% $CO_2$ and upon reaching near confluence (80-90%), they were washed with phosphate buffered saline (PBS, Sigma) and detached with 0.05 % trypsin (Gibco), containing 1 mM ethylenediaminetetraacetic acid (EDTA).

### *Embryoid body formation and differentiation on calcium phosphate scaffolds.*

For formation of so-called embryoid bodies (EBs), 175,000 cells were cultured in 4 ml suspension for 4 days, in 10 cm$^2$ non-adherent plates in basic medium. Next, cells were dispersed with trypsin, and seeded onto a calcium phosphate disk with a diameter of 8 mm and a thickness of 1.5 mm. One million cells were seeded statically on both sides of the disk. For subcutaneous control implants, cells were seeded on calcium phosphate scaffolds of 2-4 mm [22]. The tissue engineered constructs (TECs) were then grown in chondrogenic medium consisting of DMEM high glucose (Gibco), 100 nM dexamethason (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, insulin-transferrin-selenite (ITS+1, Sigma), 0.2 mM ascorbic acid 2-phosphate (Sigma), 100 μg/ml sodium pyruvate (Sigma), 40 μg/ml proline (Sigma) and 10 ng/ml TGFβ3 (R&D Systems or Biovision) [21, 23]. Medium was refreshed every 3-4 days and

after 21 days of culture the samples were used for *in vivo* implantation or fixated for *in vitro* analysis.

### *Implantation in the rat cranium and subcutaneous.*

Thirteen immune deficient rats (Crl:NIH-*Foxn*$^{rnu}$, Charles River) were used as recipients for the TECs. Prior to implantation all rats received a subcutaneous injection with buprenorfine (0.02 mg/kg, Tempgesic) for analgesia. The rats were anaesthetized with Isoflurane and the skin was cleaned with iodine. During the operation they were maintained with a mixture of Isoflurane (1.5-3%), $O_2$ (200-300 ml/min) and $N_2O$ (50-200 ml/min). An incision in the skin was made over the cranium from the middle of the nasal bones to the posterior nuchal line. The periostium on the cranium was sedated with lidocaine (2%) and then removed. An 8 mm trephine dental bur (ACEuropa, Lda) was used to mark the defect side and next the bone was further removed with a 0.7 mm drill (Synthes) until the 8 mm craniotomy was realised. The bone was gently removed and six rats received a press fit sham implant without cells, and 7 rats received a TEC. The overlaying tissue was them sutured back in layers with resorbable 5-0 vicryl. Secondly, the skin on both lateral sites of the spine was cleaned with iodine and 2 subcutaneous pockets were created in each rat that had also received a TEC in the cranium. In each pocket, three samples were inserted. All wounds were sutured with 5-0 vicryl. After surgery the rats were housed individually and received subcutaneous injections for analgesia (buprenorfine) during a period of three days. They were monitored daily for complications and had free access to water and chow. Four weeks post-operatively, the animals were euthanized by $CO_2$ inhalation and the implants were removed. In accordance with the animal health guide lines, two rats were euthanized one day prior to the planned explantation due to poor health. The animals were housed at the Central Animal Laboratory institute and the experiments were approved by the local animal experiment committee.

### *Micro-CT analysis of the calcium phosphate disks*

Prior to embedding, 13 calcium phosphate disks were micro-CT scanned (Skyscan 1072, Skyscan, Belgium) resulting in reconstructed 3D datasets with an isotropic voxel size of 12 µm. The reconstructed datasets were filtered using a 3D Gaussian kernel with a radius of 18 µm to reduce noise, after which the datasets were segmented to separate the scaffold from background using a global threshold value that was selected visually. Porosity was calculated as the pore volume (background voxels) divided by the total volume (background + scaffold voxels).

### *Analysis of the in vitro and in vivo samples*

The *in vitro* and *in vivo* explanted samples were fixated in 1.5% glutaraldehyde (Sigma) in 0.14M cacodylic acid buffer, dehydrated by increasing ethanol steps and embedded in methylmethacrylate (LTI, Bilthoven, The Netherlands). Sections were made using a diamond saw (Leica SP1600), the in vitro samples were stained with 0.04 % thionine (Sigma) in 0.1 M sodium acetate (Merck) and the *in vivo* samples were stained with 1 % methylene blue (Sigma) and 0.3 % basic fuchsin solution (Sigma) [24]

### *Histomorphometry*

Histomorphometry was performed on three sections of each cranial implant. From each section, low magnification digital images were made. All images were pseudo colored and measurements were performed with KS400 software (Carl Zeiss Vision, Oberkochen, Germany). Prior to measurement the system was calibrated with an image of known dimensions. To analyze the growth and in-growth of bone more precise, the implant was divided in three round sections.

### *Statistical analysis*

In order to account for the nested nature of the data (three measurements per rat), we chose a mixed effects model with a random intercept for rats and a fixed effect for treatment (TEC versus sham). Nearly all samples of the Sham group had little or no bone in growth in

the inner circle and all TEC did show bone in growth. Due to this lack of variation in the sham group it was impossible to apply the mixed effects model used for the other outcomes. Instead the mean of the three samples was determined for each rat and a Mann-Whitney U test was used to detect a difference between the two groups.

## Results

### *Subcutaneous endochondral bone formation in rats*

We recently established an efficient strategy for bone formation by embryonic stem cells *in vivo* via the process of endochondral bone formation [25]. Thereby a construct with *in vitro* formed cartilage was implanted subcutaneously in immune-deficient mice where bone was formed via the process of endochondral bone formation. Because we wanted to use the rat cranium as a model for osseous defects, we first assessed whether endochondral bone formation could be observed subcutaneously in rats. Therefore, we seeded the mESCs onto 2-4 mm calcium phosphate scaffolds and cultured them for 21 days in chondrogenic medium. Light microscopy showed that the scaffolds were surrounded by an immature and variably edematous stroma (connective tissue), composed of loosely arranged myofibroblast-like cells with a stellate stromal cell phenotype. Amid this stroma, isles of cartilage cells with variable grades of differentiation, including hypertrophic cartilage were formed (Figure 1A).

Next, we implanted these chondrogenic scaffolds subcutaneously in the back of immune-deficient rats for 4 weeks. After explantation the histology of tissue on the constructs was studied by light microscopy (Figure 1 B,C). *In vivo* the stroma had become denser than in the *in vitro* situation as previously observed by Jukes et al. indicating that the mESCs had continued to proliferate *in vivo*. Furthermore, many blood vessels filled with erythrocytes were seen. In stead of the isles of cartilage observed *in vitro*, *in vivo,* only isles of bone were seen. Hereby we showed that the process of subcutaneous endochondral bone formation

can not only occur in mice, as we have previously shown, but also in in rats.

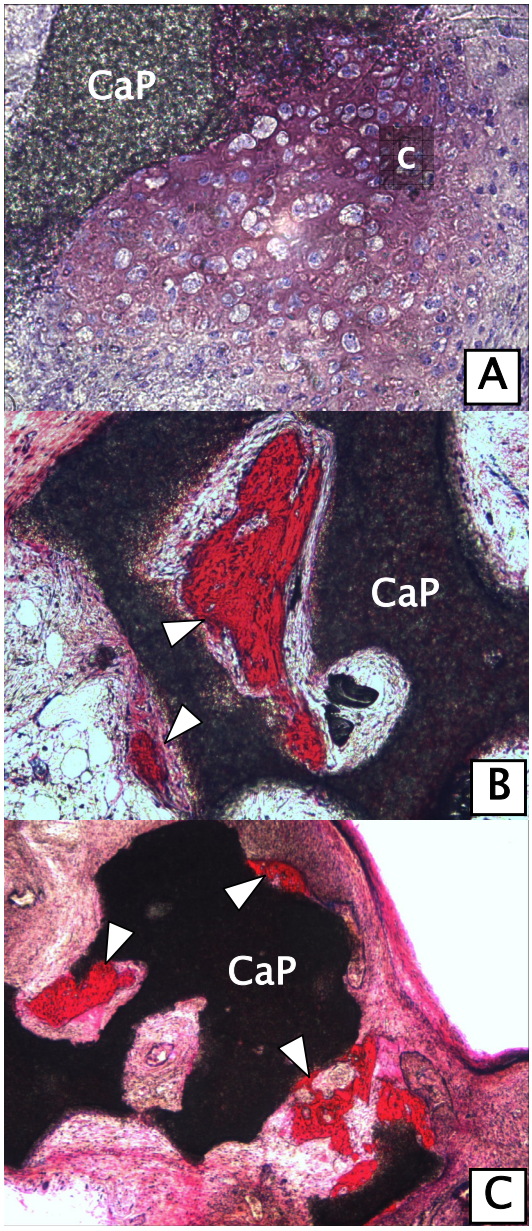


Figure 1. *In vivo* bone formation by embryonic stem cells. *Isles of cartilage cells (c), stained with thionine, with variable grades of differentiation deposited against the calcium phosphate scaffolds after three weeks of in vitro chondrogenic differentiation (A). Bone was formed by mESCs on porous biphasic calcium phosphate particles after 4 weeks of subcutaneous implantation in immune-deficient rats (B,C). The newly formed bone (arrowheads) was deposited against the material (CaP) surfaces.*

*Tissue engineered cranial grafts*

For the rat cranial defect, we prepared porous calcium phosphate disks with a diameter of 8 mm and a thickness of 1.5 mm. Micro-CT analysis showed that the scaffolds had a porosity of 60 % (±2.7% n=13) (Figure 2A). The TECs were created by seeding mESCs onto the disks and subsequent culturing for 21 days in chondrogenic medium. Five extra TECs were used for *in vitro* evaluation and were stained with methylene blue to visualize cell adherence, as shown in figure 2B. Each side of the stained disks contained cells but there was variation in the amount of cells adherent to the disks. Light microscopy showed similar cell morphology for the TECs including cartilage formation as described earlier for the subcutaneous implants (Figure 2C). About a tenth to a third of the cells on the TECs had differentiated into chondrocytes.

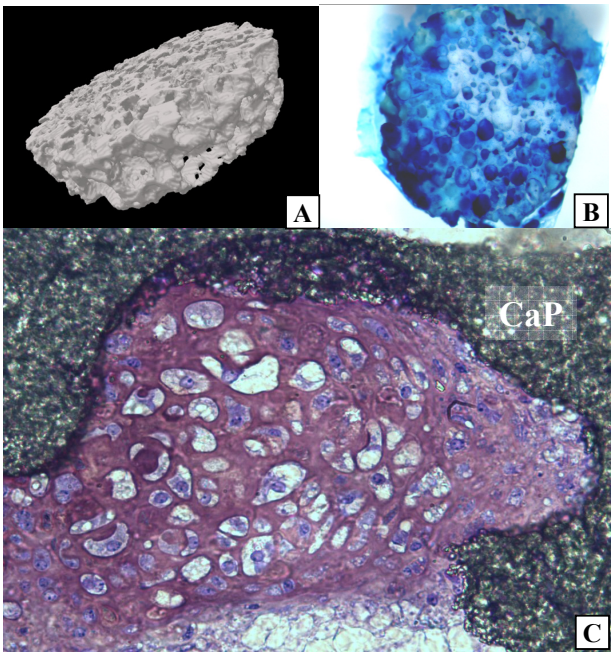


Figure 2.
Tissue engineered constructs for cranial defect. A micro-CT image of the 8 mm calcium phosphate disk used for the cranial implantations *(A). The presence of mESCs on a disk after 21 days of culture is visualised by methylene blue staining (B). Isles of cartilage cells, stained with thionine, with variable grades of differentiation deposited against the calcium phosphate (CaP) disk (C).*

*Light microscope evaluation of the cranial defect*

A cranial defect was created in immune-deficient rats by removing an 8 mm circle from the skull. Six rats received a sham disk, without pre-seeded cells, and seven received a TEC that was press fitted into the defect. After 4 weeks the rats were killed and the disks removed, embedded and stained with methylene blue and basic fuchsin for microscope analysis. In all six sham disks limited bone formation was seen only and at the periphery of the disks, whereas no bone was detected further inwards (Figure 3A,B). We did not observe cartilage tissue, which suggests that bone from the surrounding cranium had grown into the sham disks by osteoconduction. Because bone was only observed at the periphery of the disks we thereby demonstrated that our defect is of a critical size at a time point of 4 weeks.

The TECs were created by seeding mESCs on both sides of the disks and subsequent culture for three weeks in chondrogenic medium prior to implantation. Four weeks after implantation they were processed similar to the sham disks. As observed before in the subcutis, all TECs showed extensive bone formation (Figure 3C,D). Bone formed by the mESCs was photographed with polarized light, which shows that lamellar bone had formed (Figure 3E). Two of the seven rats had respectively one and two pieces of bone that still showed remains of cartilage differentiation, highlighting endochondral bone formation (Figure 3F). Normally, when a lesion occurs in the skull bone, healing will start with an inflammatory response, thereby cleaning the area. This is followed by the production of an extracellular matrix and the ingrowth of new blood vessels (angiogenesis). Ultimately bone formation is brought about via intramembranous ossification; thereby mesenchymal stem cells differentiate into osteoblasts which in turn form the bone. Except for mature cartilage, also islets of immature cartilage were seen. This diversity of maturation with chondrocyte and osteoblast differentiation seems to be an ongoing process. Both TECs and sham disks were fully surrounded by the same stroma, but only in the TECs bone formation was seen throughout, while in sham disks, without pre-seeded cells, bone formation was only limited to the periphery of the constructs.

Cartilage was only seen in some of the TECs and not in the sham disks at all. Apparently it takes about 4 weeks for the *in vitro* formed cartilage to be replaced by bone, since no cartilage was seen in the subcutaneously implanted samples and only a few spots in the TECs that were implanted in the cranium. In addition, in four out of seven rats that obtained a TEC, teratoma formation was seen at the time of explantation. However no correlation was found between the process of endochondral bone formation and teratoma formation.

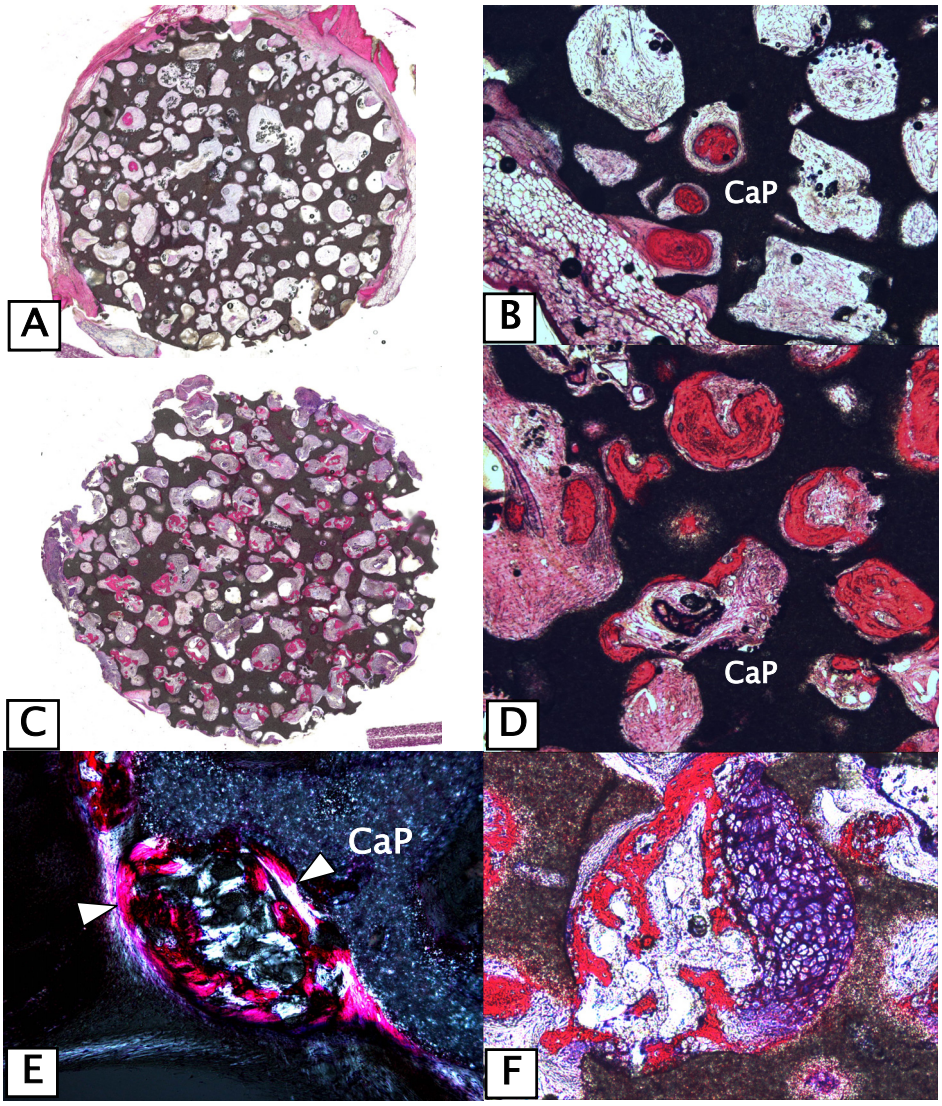


Figure 3. Bone formation in cranial defect. *Bone formation in the sham disks, without cells, was limited and only seen at the periphery of the disks after 4 weeks of implantation in a cranial defect (AB). All TECs showed extensive bone formation throughout the entire TECs after 4 weeks (C), also in the inner part of the TECs (D). Bone formation was photographed with polarized light (E), which shows that lamellar bone (arrowhead) is formed. Some pieces of bone that still showed remains of cartilage differentiation, highlighting endochondral bone formation (F).*

*Histomorphometry*

For quantification of the percentage of bone that was formed in the cranial implants after 4 weeks of implantation, we divided the images of the 8 mm disks in 3 rings (Figure 4). These 3 rings were chosen to ensure that we could distinguish between bone ingrowth and bone formed by the implanted cells. The percentage of bone was calculated by the total amount of bone measured divided by the total surface area. It is expected that bone will grow into the disks but not fully infiltrate the entire disk. After pseudo coloring, the percentage of bone in all scaffolds and in their separate rings was measured.

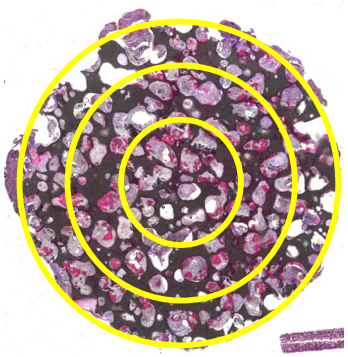

Figure 4. Visualization of criteria for quantification of bone percentage. *To quantify the percentage of bone formed in the cranial TECs and control disks after implantation, the images of all disks were divided in 3 evenly distributed rings.*

As a control we implanted disks without cells (sham) where bone was mainly observed in the outer and middle ring, respectively 1.6% and 1.2 % of bone was measured (Figure 5C,D). In the inner ring almost no bone was present (0.1%). Via the process of osteoinduction bone has grown into the disk and gradually diminishes from the outside to the inside, thereby proving we have a critical size defect. In contrast to the sham implants we find an overall equivalent percentage of bone formed of 5.1% in all rings of the TECs (figure 5A), which is significantly higher than the 1.4 % bone observed in the sham disks. If we closely examine the outer (5.6%), middle ring (4.9%) and inner (6%) (Figure 5B) we can see that in all three rings the amount of bone formed is significantly higher compared to the rings of the sham implants. Since bone formation in the TECs is consistent throughout the disks, it indicates that most of the

bone formed originates from mESCs. Thereby demonstrating that the process of endochondral bone formation by mESCs also occurs in a critical size defect.

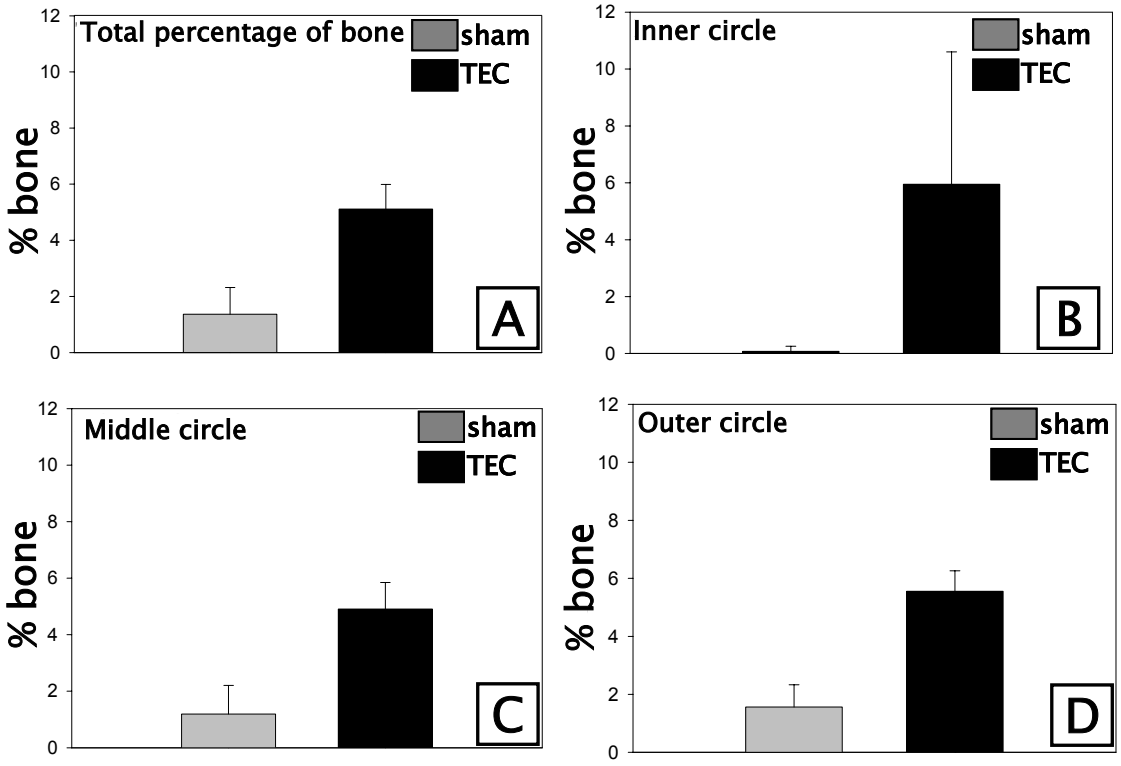


Figure 5. Percentage of bone in cranial TECs and control disks. *The percentage of bone in the TECs is significantly higher compared to the sham disks (A). There is almost no bone presence in the inner circle of the sham disks, while a significantly higher amount is presence in the TECs (B). In both the middle and outer ring (C, D) a significant difference, in the percentage of bone, can be detected between the sham disks and the TECs.*

## Discussion

We have succeeded in making bone through mESCs in an orthotopic model for the first time. Previous studies have shown osteogenic

differentiation of mESCs and even hESCs but almost all work was done *in vitro* and not *in vivo*. In our earlier study we demonstrated the formation of functional bone *in vivo* by mESCs in an ectopic model. With this study we proved that orthotopic bone formation by mESCs is also feasible. Simultaneously we showed that the process of endochondral bone formation by mESCs is not restricted to one species; bone is formed in rats similar to bone formed in mice. Although these results are obtained with embryonic stem cells isolated from a mouse, it indicates that there is a possibility of skeletal tissue engineering with embryonic stem cells.

At present human mesenchymal stem cells (hMSCs) are the preferred choice in bone tissue engineering, although the average amount of bone formed in ectopic model is currently insufficient for clinical applications. In this respect mesenchymal stem cells obtained from animals, like goat, mouse or pig have superior osteoinductive capacities as compared to human cells. Also our mESCs surpass hMSCs with respect to *in vivo* bone formation, but we need cells of human origin for treatment of patients.

Certainly there can improvements on our experimental setting, like optimizing the chondrogenic differentiation of the mESCs. With the current medium we do not succeed in differentiating the mESCs for a 100% into the chondrogenic lineage. In previous studies by Johnstone et al and Mackay et al [23, 26] the same medium stimulated an almost homogeneous cartilage tissue formation with rabbit and human mesenchymal stem cells. After three weeks of *in vitro* culture with our mESCs, we see the main type of tissue present is chondrogenic like tissue but it is not the sole tissue formed by these cells (as described earlier). Also i*n vivo*, besides bone tissue, we observed other tissue types. As observed in Figure 1A, the cell seeding is not homogeneous over the disk; this is mainly due to the fact that the chondrogenic culture medium does not contain fetal bovine serum (FBS). FBS contains a complex cocktail of proteins which improves the adhesion between the cell and the material, like fibronectin and vitronectin. In cartilage tissue engineering, polymers mainly are used as substrates and from such a study it was determined that fibronectin is the best substrate for chondrocyte attachment [27]. Soaking prior to cell adhesion in a

fibronectin solution could contribute to a more homogeneous cell layer and thus for consistency in the amount of bone formed in the disks. Obviously the effect of fibronectin on our differentiation protocol should be tested. Currently we seed our cells statically, which also not contributes to a homogeneously distributed cell layer. The use of a perfusion bioreactor could solve this problem. With such a bioreactor, Janssen et al [28] were able to create a viable, homogeneous cell layer of goat mesenchymal stem cells on calcium phosphate scaffolds. If we would succeed in solving the seeding problems it is very likely that the amount of bone present in the scaffolds would increase.

Despite the fact that our TECs were cultured for three weeks in differentiation medium, teratoma formation was observed in some rats. Teratomas contain tissue or organ components resembling normal derivatives of all three germ layers. They can be fully differentiated and are therefore benign, or they can (partly) be undifferentiated and therefore will behave in a malignant manner with ongoing growth and potency to metastasize. The presence of different cell types and tissues shows that the pre incubation with chondrogenic medium for three weeks is not sufficient to force the mESCs to a stable differentiation process. It appears that an undifferentiated population of embryonic stem cells is not fully committed to the chondrogenic lineage after a three week treatment with the chondrogenic medium and is still capable of teratoma formation. If ESCs are to be used for skeletal tissue engineering, the formation of a teratoma is undesired, thus we will further investigate this problem.

Because we work with embryonic stem cells, we are limited in the animal models for implantation studies. Thereby we are also restricted in the critical size defects that can be made in these animals. The bone in the cranium of the rat is formed via intramembranous ossification and not through endochondral ossification. Another option would be a critical size defect in tibia, in this model bone is formed via endochondral ossification [29, 30]. A bone piece of approximately 7 mm is removed from the tibia and replaced by a bone filler or left open, the defect needs to be fixated from the outside since it is load bearing. Our aim was to

demonstrate that tissue engineering with embryonic stem cells could be possible and therefore we choose for the less invasive cranial defect. However this model system is also far from perfect. From other studies with cranial defects in rats it is known that at a certain age the main blood vessel (sinus) will adhere to the skull. This vessel runs directly underneath the implantation site and drilling the skull needs to be performed with tremendous precision. At the time of implantation there were no reports on the use of this model in immune-deficient rats. We discovered that with these rats the sinus will start adhering to the skull when they are 12 weeks of age. Secondly these rats are more sensitive to isoflurane used for sedation compared to non-immune deficient rats.
Endochondral bone formation by human ESCs seems the next most logical step. We have tried to use the chondrogenic differentiation protocol for mESCs on human ESCs unfortunately without succes (data not shown). Apparently, different concentrations of the media supplements or other stimuli are needed since the hESCs were not susceptible to chondrogenic differentiation. Culturing hESCs is currently still very time consuming and labor intensive. From isolating the inner cell mass of a blastocyst for ESC culture to proliferating a sufficient amount of cells, usable for a clinical application, would already take 2-3 months. The next step, formation of embryoid bodies and differentiation would likely take another month. Besides technical problems there are still the ethical problems with the use of hESCs. However in a recent breakthrough several researchers succeeded in creating ESCs from somatic cells [31-33]. They demonstrated that certain factors play an important role in the maintenance of the ESC identity and induce the somatic cell into an embryonic state. These factors are Oct-4, Sox2 (which have a function in maintaining pluripotency in ES cells as well as early embryos) and c-Myc and Klf-4 (which contribute to the maintenance ES cell phenotype and their rapid proliferation). These four transcription factors were introduced in mouse fibroblast by retroviral introduction, thereby reprogramming these somatic cells into a pluripotent state. They named the cells induced pluripotent stem (iPS) cells. The iPS cells displayed a similar growth pattern and morphology as

ESCs, as well as expressing ES cell markers. Upon subcutaneous implantation in immune-deficient mice, teratomas were formed containing tissues originating from of all three germ-layers. Although iPS cells were not fully identical to ESCs, they could overcome the problems that ESCs face in future clinical applications. The latest discovery in this field are the iPS from human somatic cells by Yu and co-workers. [34] The human iPS cells exhibit all the characteristics found in the iPS obtained from mouse cells. When problems related to culturing of ESCs are solved, it seems likely that the endochondral differentiation process can be used for skeletal tissue engineering with either hESCs or human iPS.

## Acknowledgements

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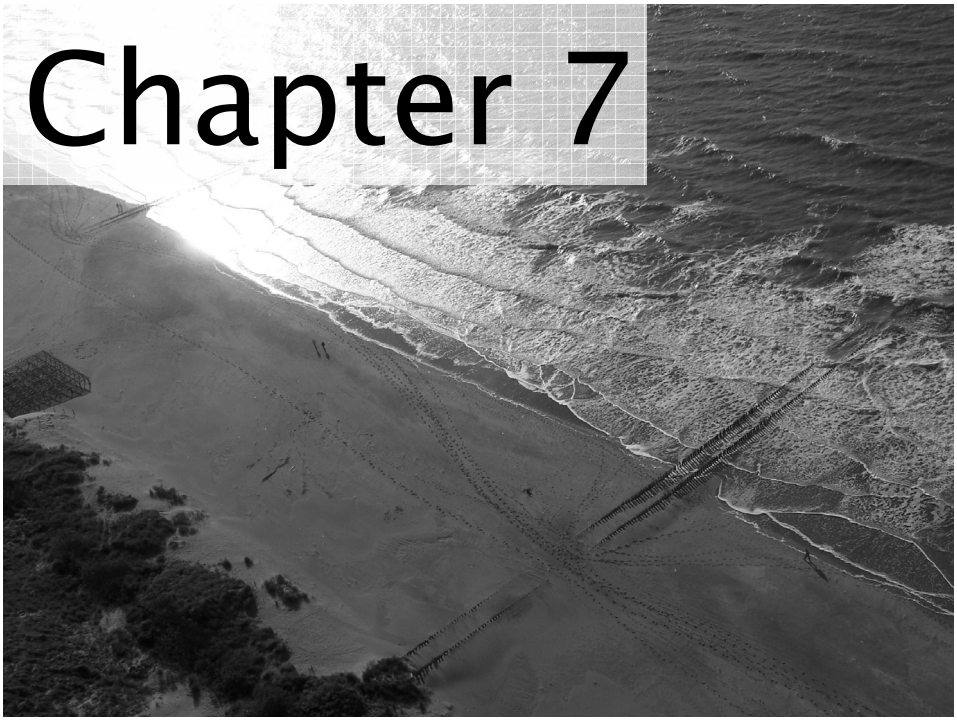
# Chapter 7



# Chapter 7

# Survival of a tumorigenic population of embryonic stem cells despite an intensive differentiation protocol

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## Abstract

Recent years have seen a surge in research regarding the potential use of embryonic stem cells (ESCs) for tissue replacement. ESCs can differentiate into all lineages, which make them very attractive for cell therapy, but a disadvantage is their potential to form teratomas. We recently established a strategy for ESC-based bone tissue engineering which uses a cartilage intermediate on which bone is formed *in vivo* via the process of endochondral ossification. Although we exposed the ESCs to a comprehensive differentiation scheme *in vitro*, we did observe teratoma formation upon implantation *in vivo*. To this end, we investigated the correlation between ES differentiation and teratoma formation. We observed an inverse correlation between the expression of the cartilage marker collagen type II and Oct-4, a marker for undifferentiated cells, upon differentiation *in vitro*. Nevertheless, regardless of the duration of *in vitro* differentiation, teratoma-formation is observed around the tissue engineered grafts upon implantation, suggesting that a population of Oct-4 positive cells is still present in the graft. This was confirmed by Oct-4 staining 7 days after implantation of ESCs which had been exposed to an *in vitro* chondrogenic differentiation protocol for 21 days. Our data demonstrates that our tissue engineered grafts are a heterogeneous population of committed and pluripotent cells, which warrants the establishment of robust strategies to exclude the tumorigenic population.

## Introduction

Ever since Evans and Kaufman established mouse embryonic stem cell (mESC) cultures in 1981 [1], the cells have been widely studied by the scientific community to understand their unique features. However, it was not until Thomson *et al.* [2] in 1998 described the isolation and culture of human embryonic stem cells (hESCs) that the scientific community focused its attention on the use of embryonic stem cells (ESCs) for therapeutic purposes. Although clinical applications with hESCs have not yet been achieved, researchers have succeeded in differentiating ESCs towards many different lineages *in vitro*, such as cardiomyocytes [3], neural precursors [4, 5], endothelial cells [6] and insulin-producing cells [6, 7]. Considering this, ESCs have been suggested as a potential source for cell replacement therapy in e.g., the treatment of diabetes. In recent research, ESC-derived insulin-producing cells were transplanted into diabetic mice, where they reversed the hyperglycemic state for approximately 3 weeks. However, overall the treatment failed due to the formation of teratomas, tumors composed of different cells and tissues derived from all three germ layers. In fact, teratoma formation is one of the quality control tests used to prove ESC pluripotency [7]. The diabetes experiment clearly demonstrates the downside of the acclaimed pluripotency of ESCs, which is their ability to form teratomas.

For mESCs, self-renewal is dependent on signals from the cytokine leukemia inhibitory factor (LIF) and from either serum or bone morphogenetic proteins (BMPs). In addition, a number of transcription factors are required for maintenance of the undifferentiated state, including Oct-4 and Nanog [8]. Expression of Oct-4 can be detected throughout early embryonic development until the epiblast stage. Afterwards, Oct-4 can still be detected in primordial germ cells where it persists through the formation of the genital ridges in both sexes. An experiment by Nichols *et al.* demonstrates the essential role of Oct-4 in the maintenance of pluripotency [10]. Oct-4-deficient embryos developed to the blastocyst stage, but the inner cell mass cells were not

pluripotent. Instead, they were restricted to differentiation along the extraembryonic trophoblast lineage. The importance of Oct-4 in self renewal and maintaining pluripotency in embryonic stem cells was further strengthened by the recent generation of induced pluripotent stem cells [11-13], in which it was shown that four genetic factors alone are sufficient to induce somatic cells into an embryonic state. These factors are Oct-4 and Sox-2, which have a function in maintaining pluripotency in ES cells as well as early embryos, and c-Myc and KLF4, which contribute to the maintenance of the ES cell phenotype and their rapid proliferation.

We have used ESCs as an alternative cell source in bone tissue engineering to treat large bone defects. There are many patients that require bone grafts to repair an injury or defect. However there are limitations on the application of autologous and allogeneic bone, which are the most commonly used bone grafts. Tissue engineering of bone with ESCs could provide an alternative. In this strategy, osteogenic cells are combined with an appropriate biodegradable scaffold, which can be implanted into the osseous defect. Recently, we have established an efficient strategy for bone tissue engineering with ESCs via endochondral ossification of an ESC-derived cartilage template [14, 15]. Bone was formed both ectopically and orthotopically by inducing mESCs into the chondrogenic lineage *in vitro* resulting in bone formation *in vivo* through a process of endochondral bone formation. Although this is a very promising approach we were interested to see whether our differentiation protocol was sufficient to drive all cells into the chondrogenic lineage and to eliminate all pluripotent, undifferentiated cells and thereby prevent teratoma formation.

## Materials and Methods

### *Culture of mouse embryonic stem cells and embryoid body formation.*

The mouse embryonic stem cell (mESC) line, IB10 [16] was grown on tissue culture polystyrene, coated with 0.1% gelatin (Sigma) and maintained in basic medium consisting of DMEM (Cambrex, Biowhitakker) medium supplemented with 10% FBS (Greiner, selected batch for mESCs), 1% non-essential amino acids 100x (Sigma), 4 mM L-glutamine (Sigma), penicillin G (100 u/ml, Invitrogen); streptomycin (100 μg/ml, Invitrogen) and 100 μM 2-mercaptoethanol (Invitrogen). To maintain the undifferentiated state, cells were cultured in the presence of Buffalo rat liver cell-conditioned (BRL) medium and 1000 units/ml of Leukemia inhibiting factor (Esgro, Chemicon international). The cells were grown in a 37°C humid atmosphere at 5% $CO_2$ and upon reaching near confluence (80-90%), they were washed with phosphate buffered saline (PBS, Sigma) and detached with 0.05 % trypsin (Gibco), containing 1 mM ethylenediaminetetraacetic acid (EDTA). For formation of so-called embryoid bodies (EBs), 175,000 cells were cultured in suspension for 4 days, in 10 $cm^2$ non-adherent plates in 4 ml basic medium. Next, cells were dispersed with trypsin, to obtain a single cell suspension.

### *Chondrogenic differentiation of mouse embryonic stem cells.*

Differentiation into the chondrogenic lineage was performed in chondrogenic differentiation medium consisting of DMEM high glucose (Gibco), 100 nM dexamethason, 100 U/ml penicillin, 100 μg/ml streptomycin, insulin-transferrin-selenite (ITS+1, Sigma), 0.2 mM ascorbic acid 2-phosphate (Sigma), 100 μg/ml sodium pyruvate (Sigma), 40 μg/ml proline (Sigma) and 10 ng/ml TGFβ3 (Biovision) [17, 18]. For pellet culture, aliquots of $0.5x10^6$ single EB cells in 1 ml medium were centrifuged at 500 x g for 2 minutes in 12 ml polypropylene conical tubes. The pellets were cultured for 3, 7 and 21 days in chondrogenic medium. For subcuteanous implantation studies 1.5 million single EB cells were resuspended in 150 μL basic medium and seeded statically

onto 3 porous bi-phasic calcium phosphate (CaP) particles (2-4mm) and incubated for 2 hours. Subsequently all samples were cultured in chondrogenic medium or basic medium for 3 to 21 days as indicated per experiment. For all experiments, the medium was changed every 3 to 4 days.

*RNA isolation and quantitative PCR of hESCs and mESCs.*

To analyze the expression of Oct-4 and Collagen type II we isolated total RNA from the pellet cultures using the TriZol method according to the manufacturer's protocol (Invitrogen). The quality and quantity of RNA was analyzed by gel electrophoresis and by spectrophotometry. One µg of RNA was used for first strand cDNA synthesis using Superscript II (Invitrogen) according to the manufacturer's protocol. One µl of 100x diluted cDNA was used for GAPDH (5'-AACGACCCCTTCATTGAC-3'and 5'-TCCACGACATACTCAGCAC-3') amplification and 1 µl of undiluted cDNA was used for Oct-4 (5'-GGCGTTCTCTTTGGAAAAGGTGTTC -3' and 5'-CTCGAACCACACATCCTTCTCT -3') and for Collagen type II (5'-CAAGGCCCCCGAGGTGACAAA-3' and 5'-GGGGCCAGGGATTCCATTAGA GC-3') amplification. PCR was performed on a Light Cycler real time PCR machine (Roche) using a SYBR green I master mix (Invitrogen). Data was analyzed using Light Cycler software version 3.5.3, using fit point method by setting the noise band to the exponential phase of the reaction to exclude background fluorescence. Expression of marker genes were calculated relative to GAPDH levels by the comparative $\Delta$CT method and the statistical significance was found using Student's t test at $P<0.05$.

*In vivo implantation in mice.*

To assess the *in vivo* bone forming and tumorigenic capacity of the cells, they were implanted in immune-deficient mice. Prior to implantation, the particles were placed in serum-free medium for approximately 1-2 hours and washed 3 times with PBS. Immune-deficient mice (HsdCpb:NMRI-nu, Harlan) received a subcutaneous injection with Temgesic for pain relief and were anaesthetised by Isoflurane. Before surgery, the skin on both lateral sites of the spine was cleaned with 70%

alcohol and 4 subcutaneous pockets were created in each mouse. In each pocket, three tissue engineered samples were inserted and each culture condition was divided over six mice. The samples were explanted 21 days post-operatively or as indicated; the animals were euthanized by $CO_2$. The samples where then processed according to the method for the specific analysis. The animals were housed at the Central Animal Laboratory institute and the experiments were approved by the local animal experiment committee.

*Analysis of tissue formation in the in vivo samples.*

The *in vivo* explanted samples were fixated in 1.5% glutaraldehyde (Sigma) in 0.14 M cacodylic acid buffer, dehydrated by increasing ethanol steps and embedded in methylmethacrylate (LTI, Bilthoven, The Netherlands). Sections were made using a diamond saw (Leica SP1600), the sections were stained with 1 % methylene blue (Sigma) and 0.3 % basic fuchsin solution (Sigma).

*Oct-4 immunostainings.*

The explanted samples were fixated in 4% paraformaldehyde and incubated for three days in a decalcification solution at 37°C. The decalcification solution consisted of a 25% Titriplex solution in 2% sodiumhydroxide at pH 7.4. Next, they were dehydrated by increasing ethanol steps and embedded in paraffin. Two µm sections were made which were attached on a Super Frost plus slide (Menzel Glazer) and dried for one hour at 60°C. The paraffin was removed and for antigen retrieval sections were treated by heating in a sodium citrate buffer (pH 6.0). Subsequently, after cooling to room temperature, they were treated with 3% $H_2O_2$ and stained with the Oct-4 antibody (sc-5279 Tebu-Bio) for 60 minutes. Next, they were treated for 15 minutes in postblock Powervision plus (Immunologic) which was followed by a 30 minute treatment with Powervision Plus HRP (Immunologic). Then the samples were treated twice for 5 minutes with Ultra DAB (Immunologic). For mild counterstaining, Mayer's hematoxylin solution was used for light microscope observation.

## Results

### *In vivo survival of an undifferentiated population of cells amidst differentiated cells.*

As reported previously, mESCs can form bone *in vivo* via endochondral ossification. In the course of the study, we observed teratomas in and around the tissue engineered implant, despite intensive differentiation *in vitro*. Therefore we investigated the effect of *in vitro* differentiation on teratoma formation. To this end, we seeded single EB cells on calcium phosphate (CaP) scaffolds and cultured them for 3, 7, 14 and 21 days respectively, in chondrogenic medium. After subcutaneous implantation in immune-deficient mice for 21 days we observed teratoma formation surrounding the CaP scaffolds, in all conditions and in all mice, with light microscopic analysis. Teratoma formation was most profound in the conditions where the cells were differentiated *in vitro* for 7 days. Whereas the amount of tissue in and around the scaffold in the 3, 14 and 21 day groups was similar to what was implanted (a sample size of about 0.4 cm), we observed a drastically increased samples size (0.8 cm) in 4 out of the 6 mice in the 7 days group. Besides sample size, also from a histological point of view we did observe some differences in the tissue formed from the different groups. Teratomas formed by cells that were differentiated for 3 days (Figure 1a) consisted of a variably edematous and highly vascularised stroma (connective tissue) and cells with epithelial differentiation. Both mature and immature stroma derived from mesoderm, and epithelial elements derived from ecto- and endodermal origin was formed. No bone formation was observed in these implants. After 7 days (figure 1b) of *in vitro* differentiation, a denser stroma, sometimes resembling blastema (a group of immature cells from which during embryogenesis organogenesis takes place) was formed. Also rosette formation, an indication of possible neurogenic differentiation, was detected in several mice. Compared to day 3, there was much more tubule formation and several hair follicles were observed. Similar to the 3 day sample, no bone formation was observed in the teratoma surrounding the CaP scaffold. Samples of 14 days (figure

1c) *in vitro* culture displayed a dense stroma and some tubule formation although less compared to 7 days of *in vitro* differentiation. Bone formation was observed adjacent to the CaP scaffolds. The 21 days (figure 1d) *in vitro* differentiated samples had a similar appearance as the 14 days differentiated although more bone tissue was seen. In one of the 21 day samples we observed remains of blastema formation within the onset of tubule formation.

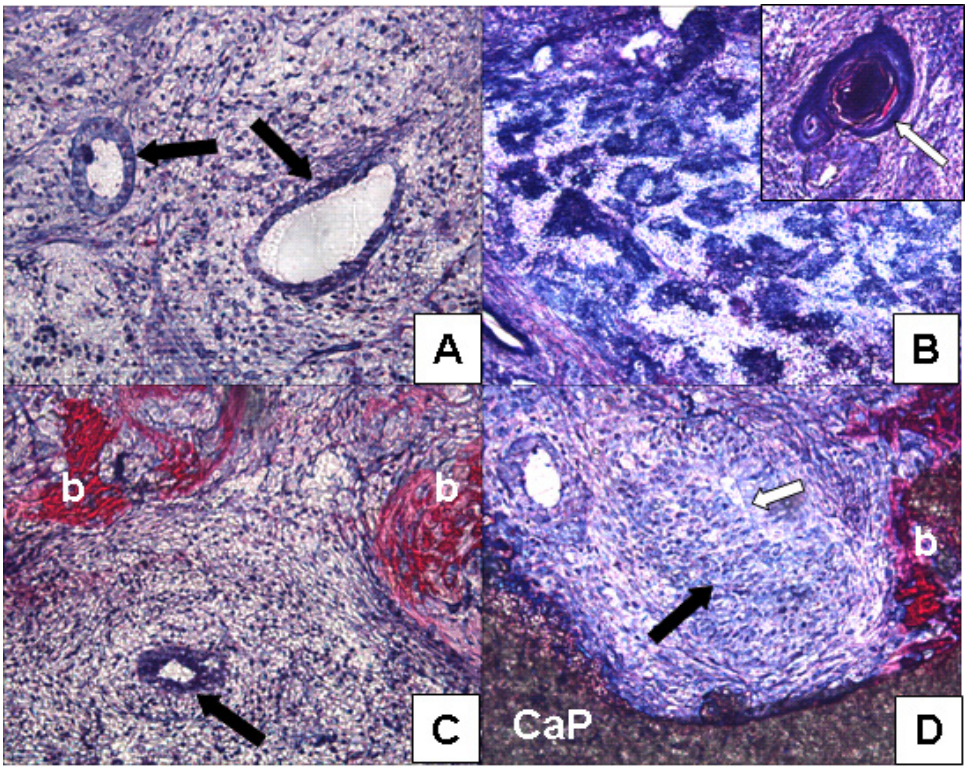


Figure 1. Teratoma formation by mESCs. *To observe whether prolonged in vitro differentiation has an effect on teratoma formation, cells were cultured in vitro for 3, 7, 14 and 21 days prior to 21 days of in vivo implantation. (A) After 3 days of in vitro culture an immature and variably edematous stroma was observed containing tubule formation (arrows). (B) A denser edematous stroma containing rosette formation (7d) and hair follicles (small picture, white arrow) is observed after 7 days of in vitro culture. (C) After 14 days dense stroma and some tubule formation (arrows) are observed. Bone (b) was formed adjacent to*

*the calcium phosphate scaffolds (CaP). (D) The 21 days in vitro differentiated samples had extended bone formation. In one of the 21 day samples we saw remains of blastema formation (arrow) and the onset of tubule formation (white arrow).*

### *Effect of differentiation on mESCs in time monitored by qPCR*

We suspect that, despite *in vitro* differentiation, a small population of cells on the tissue-engineered graft remains undifferentiated and causes teratoma formation. To test this hypothesis, we used quantitative PCR to monitor the expression of the stem cell marker Oct-4 during chondrogenic differentiation of mESCs in vitro. EBs were created from mESCs and dispersed to form micromass cultures in chondrogenic and control medium for up to 21 days. QPCR analysis was performed on undifferentiated mESCs, EBs, and after 7 and 21 days of culture in control and differentiation medium. To confirm that chondrogenic differentiation occurred, we performed qPCR on the cartilage marker collagen type II.

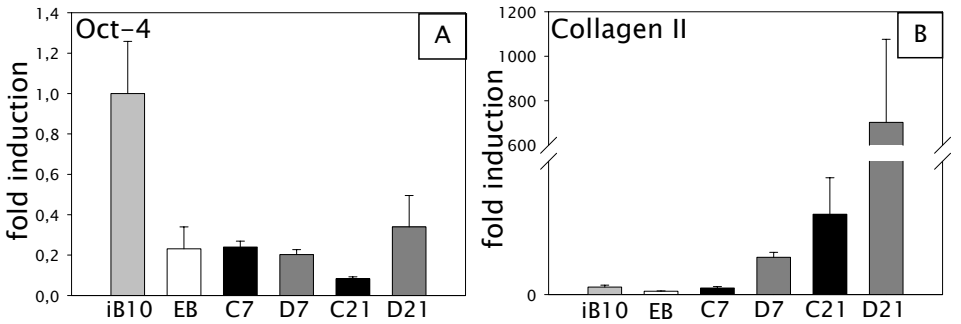


Figure 2. Oct-4 and collagen type II expression during in vitro differentiation. *Oct-4 and collagen type II expression of mESCs in micromass cultures in control (C) and differentiation medium (D) on several time points. Oct-4 expression decreases in time although after 21 days a small increase can be observed in the differentiation medium versus the control. The collagen type II expression increases in time especially in the chondrogenic condition. Expression was normalized to GAPDH levels. Standard deviation is represented by error bars.*

At day 7 we detected a 2.5-fold increase in collagen type II expression between differentiated cells versus the control condition. Although we see an increase in collagen type II expression in the control medium at day 21 compared to day 7, it does not compare to the 70 fold increase in expression observed in the differentiation medium compared to control medium. The persistence of undifferentiated cells was assessed by qPCR of the stem cell marker Oct-4. The initiation of differentiation in EBs resulted in a 4-fold decrease in the expression of Oct-4 compared to mESCs (Figure 2a). However this level of Oct-4 expression persisted seven days after EB formation both in control and chondrogenically-induced mESCs. At day 21, we even observe a three-fold higher expression of Oct-4 in ESCs grown in chondrogenic medium compared to control medium.

Our qPCR data suggest that Oct-4 positive cells are still present in the bone tissue engineering graft and to test this, we seeded single EB cells on calcium phosphate scaffolds and cultured these for 21 days in chondrogenic medium. The samples were implanted subcutaneously in immune-deficient mice for 7 days, then explanted and stained for Oct-4 expression by immunostaining. Indeed, we observed Oct-4 positive cells (figure 3) in the tissue engineered samples in an area where no teratomal tissue was observed.

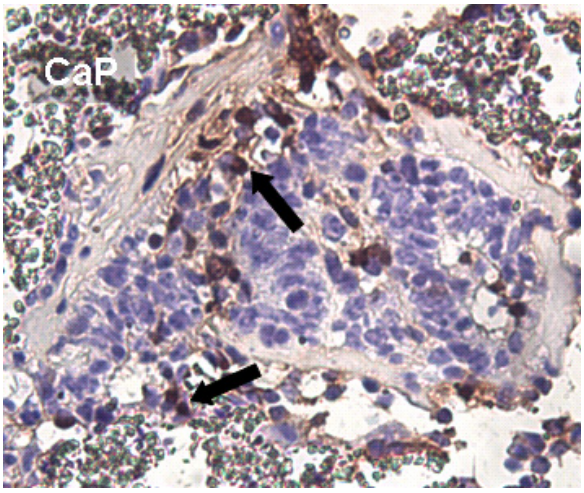


Figure 3. Oct-4 presence after *in vivo* implantation. *After three weeks of in vitro chondrogenic culture on calcium phosphate scaffolds (CaP) the cells were implanted for 7 days in immune-deficient mice. Some cells display positive staining for Oct-4 (arrows).*

## Discussion

This study demonstrates the importance of ensuring that no undifferentiated or Oct-4 positive cells are present when ESCs are used for therapeutic purposes. Despite a rigorous *in vitro* chondrogenic differentiation protocol, an Oct-4 positive population maintained itself amid chondrogenically differentiated cells. Prolonged differentiation only seems to have an overt effect on the amount of bone formed and not on the presence of tumorigenic cells in the graft. Although we do not know the amount of cells expressing Oct-4, we do observe their survival outside a teratoma following *in vivo* implantation.

We did not only observe teratoma formation with the IB10 cell line used in this study, but also with ES cells derived from C57Bl/6 mice (data not shown). Furthermore, ES-derived bone tissue engineering grafts were prepared using several osteogenic differentiation protocols, we used polymeric scaffolds and hydrogels and the grafts were implanted in rats, in both an orthotopic and ectopic site, but teratoma formation was observed in all studies and in all conditions (data not shown). We conclude that teratoma formation is an inherent problem of ES cells and not just in our specific experimental conditions. There are also other studies reporting teratoma formation by mESCs after in vitro differentiation [21]. Teratoma formation was observed by implanted mESCs that were differentiated into hepatocytes in cultured embryoid bodies[21]. Other researchers found that implantation of high numbers of cells stimulates teratoma formation [22, 23]. In our studies we have observed differences in the amount of cells adhering to the scaffolds, with more cells on scaffolds grown *in vitro* for 7 days than for 3 days and this difference in cell number may account for the difference in teratoma formation between both time points.

In light of possible application for bone tissue engineering with ESCs we have also performed several *in vivo* studies with human ESCs. Unfortunately bone formation was never observed and remarkably also no teratoma formation on the osteogenic implants, in contrast to undifferentiated hECS (see chapter 4). Many reports focus on the differentiation of ESCs into a specific lineage. The question whether or

not these cells still posses the potential to induce teratomas has to be determined empirically. For human ESCs it was reported that when induced to form ectodermal cells [24] or cardiomyocytes [25], teratoma formation was not observed. In contrast, other reports describe teratoma formation by cells differentiated into pancreatic islet-like cells for treatment of type I diabetes [7].

Evidently, we are not able to completely reproduce the complexity of embryonic development *in vitro,* and it is therefore likely that we miss certain factors to achieve uniform and mature tissue differentiation from ES cells. During embryonic development the pluripotency and plasticity of the cells declines when cells develop into more mature cell types and sometime during this process the cells loose their potential to form teratomas. Indeed, some studies suggest that organogenesis without teratoma formation from embryo-derived cells is depending on a distinct time window [19]. The extend of differentiation within the tissue will determine whether teratoma formation occurs and this window differs for each tissue type. Moreover, the loss of cellular proliferation and differentiation over time is not only observed during embryonic development. For instance, human mesenchymal stem cells obtained from paediatric and young adult donors have a significantly higher proliferation rate and differentiation capacity compared to hMSCs from older donors [20]. This transition illustrates the difference between development, in a young body we need pluripotent and replicating cells in order to meet the requirements of the rapidly growing body compared to adulthood, when pluripotent cells are mostly needed for maintenance. The maturation process of a tissue can take months to years, but in ESC-based tissue engineering, the process, or a part of this process needs to be achieved within a number of weeks. As such, it seems logical that we will not achieve proper tissue formation until we find the clue to proper maturation of the designated tissue. Differentiated ESCs all contain the same genetic material but during differentiation cell morphology and function changes. The differentiation of cells to various lineages is caused by differential gene expression patterns. The genes by which stem cells renew themselves have to be silenced during differentiation, and others involved in differentiation are activated. This expression

pattern is passed onto the daughter cells upon cell division and coordinated by epigenetic mechanisms. We hypothesize that controlling the epigenetic program is essential in controlling tissue engineering using ESCs and it would be interesting to compare epigenetic processes of ESC-derived cells to cells derived from adult stem cells.
There are several ways by which the teratoma issue might be solved. First of all, a robust differentiation and maturation procedure could be established which eradicates all tumorigenic cells. For instance, it is known that expression of Oct-4 in embryonic stem cells can be down-regulated rapidly following retinoic acid (RA) treatment [26]. By adding RA during EB formation or even during chondrogenic culture it could be possible to initiate a differentiation process in all Oct-4 positive cells. However, we then have to ensure that the cells will differentiate into the chondrogenic lineage and that the RA does not trigger other differentiation pathways. Another option might be to isolate a specific cell type from a heterogeneous population of differentiated ESCs. After initiating differentiation towards a specific cell lineage the desired cell type can be labeled and sorted with a lineage-specific antibody. For example, Levenberg and co-workers used flow cytometry to isolate human embryonic stem cell-derived endothelial cells using anti-platelet endothelial cell-adhesion molecule-1 (PECAM1) antibodies [27]. A third option would be to derive a non-teratoma forming cell line from hESCs, as described recently [28].
To conclude, even though we do see an effect of *in vitro* differentiation on teratoma formation in vivo, our data strongly argue for a robust strategy to preclude teratoma formation by ESCs for cell therapeutic use.

## Acknowledgements

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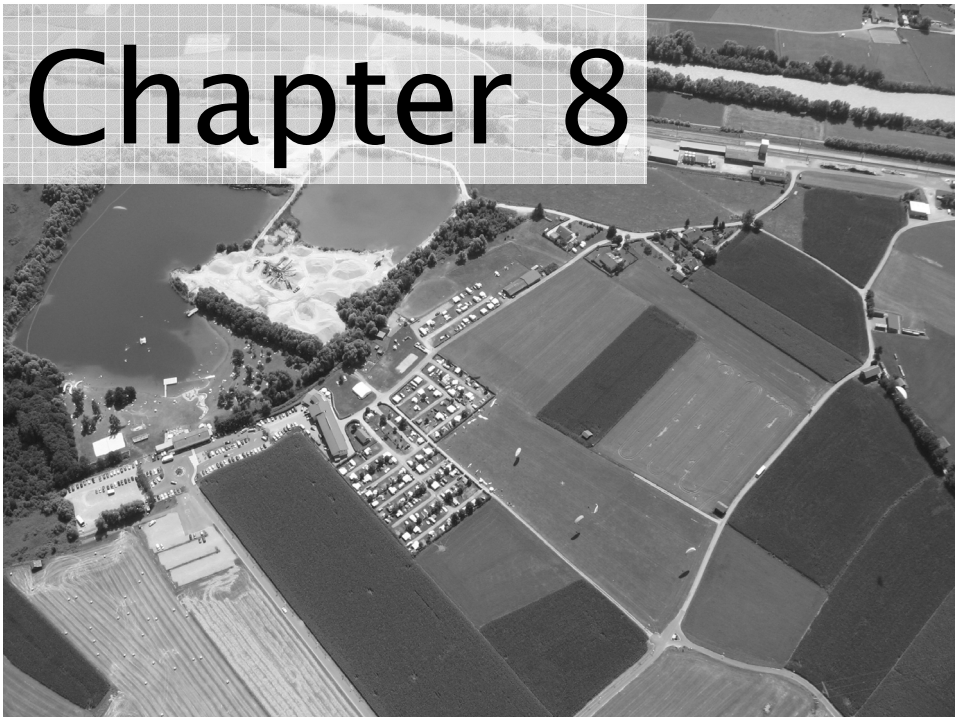
# Chapter 8



# Chapter 8

# General discussion and conclusions

Bone tissue engineering is an interdisciplinary field that addresses the need for bone substitutes by combining the biology and engineering fields. There are many patients that require bone grafts to repair an injury or defect. However there are limitations on the application of autologous or allogeneic bone, which are the most commonly used bone grafts, as discussed in chapter 1. Tissue engineering of bone could provide an alternative. Generally, bone tissue engineering approaches are based on the combination of bone forming cells with a biomaterial. Mesenchymal stem cells (MSCs) are an easily accessible cell source that possess the ability to differentiate into bone, cartilage, fat and muscle [1, 2]. MSCs can be proliferated extensively, differentiated towards the osteogenic lineage *in vitro* and form bone upon implantation *in* vivo. Although proof of principle for the creation of an autologous tissue engineered implant has been demonstrated [3-5], MSC loss of multipotency and proliferation capacity upon expansion and incomplete osteogenic differentiation are poor advocates of this cell type [6, 7]. In addition, MSCs do not succeed in producing a sufficient amount of bone *in vivo* to provide stability in an orthotopic site [4]. These problems need to be solved before MSCs can be used for successful application in bone tissue engineering. Embryonic stem cells (ESCs) have been considered as an alternative cell source for bone tissue engineering. ESCs are pluripotent cells that can proliferate indefinitely and differentiate into all tissues types.

*Heterogeneity of differentiated embryonic stem cells cultures*

Some of the issues we encountered with mouse ESCs where previously observed with MSCs. For instance, from hMSCs it is known that osteogenic differentiation does not commit all cells to this lineage and varies among donors. This is exemplified by the large variation in ALP expression *in vitro* between donors as demonstrated in chapter 2. Combining this fact with the relative poor performance *in vivo,* we conclude that we know little about the heterogeneous population we implanted. To succeed in producing a homogeneously seeded implant which displays extensive bone formation *in vivo*, it seems obvious that we have to implant a pool of cells that is fully committed to the osteogenic lineage. Although we have shown in chapter 5 and 6 that ESCs form an extensive amount of bone when implanted ectopically and orthotopically, we know it is formed from a heterogeneous population of cells. Upon implantation we observed isles of cartilage cells. After implantation we observed teratoma formation in our chondrogenicaly-induced mouse ESC studies on several occasions (chapter 7). Obviously this is an undesired effect and we have to rule out the possibility of teratoma formation if we are ever to use ESCs in clinical applications. Although teratoma formation is one of the characteristics defining ESC pluripotency we had hoped that the 21 days differentiation period in chondrogenic differentiation medium would have eliminated this potential. However it appears that an undifferentiated population remains amidst our differentiated cells. This shows that better characterization of the implanted cells is of great importance for both ESCs and MSCs.

For the creation of a tissue engineered construct somehow all ESCs have to be stimulated into the chondrogenic lineage by either optimizing the differentiation protocol or by selection of a chondrogenic cell type. In this thesis we differentiated ESCs into the chondrogenic lineage within a pellet culture or in multi-layers of cells, attached to a calcium phosphate ceramic. A possibility to optimize chondrogenic differentiation could be the use of small interfering RNA (siRNA), since siRNA can interfere with the expression of a specific gene [8, 9]. To achieve this we should

thoroughly understand the chondrogenic differentiation process and knockdown the genes expressed which are inhibiting chondrogenesis. For instance siRNA could be used as a tool to silence the expression of Oct-4 to prevent teratoma formation. The feasibility of such an approach is described in a study about differentiation of human embryonic stem cells into the cardiac lineage. It was reported that Oct-3/4 siRNA injected into the inner cell mass of blastocysts impairs cardiogenesis in early embryos [10]. This study demonstrates it is feasible to silence the expression for Oct-4. One complicating aspect in this approach is the fact that the exposure to reagents, such as siRNAs, will be different for cells on either the inside or outside of the pellet or scaffold due to the limited diffusion. As such heterogeneous differentiation will always be an issue.

Isolation of a chondrogenic cell type would therefore be an alternative. In this approach, after initiating differentiation towards a specific cell lineage the desired cell type can be labeled with a specific antibody. Via cell sorting techniques such as fluorescent-activated cell sorting (FACS) or magnetic affinity cell sorting (MACS), the desired cells can be isolated from the pool of undifferentiated cells. Thereby a population of lineage-specific differentiated ESCs can be obtained for further use. The feasibility of such an approach was demonstrated by Levenberg and co-workers. They used FACS to isolate human embryonic stem cell-derived endothelial cells using platelet endothelial cell-adhesion molecule-1 (PECAM1) antibodies [11]. There are several chondrogenic markers that could be used to accomplish this task. However isolation of single cells by antibody labeling from either a cell pellet or from cells adhered to the calcium phosphate scaffold will prove a difficult task. Another approach to guide the differentiation process more specific into the chondrogenic lineage could be the design of biomaterials with pro-chondrogenic properties. Mahmood *et al.* in our research group demonstrated that modulation of the chondrocyte phenotype can be achieved by designing the cell-polymer interface [12]. The surface chemistry of poly (ether ester) segmented copolymers was modulated by their chemical composition. This led to differential competitive protein adsorption of fibronectin and

vitronectin from serum and consequently into different cell attachment modes. These adhesion differences influenced the cell phenotype (spread or spheroid) and the subsequent expression of mRNA transcripts (collagen types II, I) characteristic of phenotypically differentiated or dedifferentiated chondrocytes, respectively. In chapter 3 we have observed that embryonic stem cell differentiation is influenced by CaP materials. We used CaP ceramics with similar chemical structure but with a difference in micro porosity. This difference was somehow enough to provide osteogenic stimulation of the mESCs. Another example of material-driven differentiation is seen in an article by McBeath and co-workers who demonstrated that cell shape influences commitment of hMSCs into the adipocyte or osteoblast lineage [13]. In yet another publication it was shown that the use of nanoscale surface disorder can stimulate hMSCs osteogenesis in the absence of osteogenic supplements [14]. These studies demonstrate that cell differentiation can be further optimized throughout the scaffold by choosing the right biomaterial. Therefore we need to screen biomaterials and one way to accomplish this task is by high throughput screening. Anderson *et al*. have described an approach for rapid, nanoliter-scale synthesis of biomaterials and characterization of their interactions with cells. In this study over 1,700 human embryonic stem cell-material interactions were simultaneously characterized, which led to the identification of a host of unexpected material effects that offer new levels of control over human embryonic stem cell behaviour [15]. Such an approach would allow us the optimize biomaterials for bone tissue engineering for both hMSCs and ESCs.

### *Biomaterials and cell distribution of embryonic stem cells*

Besides a role in stimulating differentiation, biomaterials provide the stability and strength for the cells upon implantation. A biomaterial fully covered with bone-forming cells could be an ideal bone graft substitute. Calcium phosphate ceramics (CaP) closely resemble the mineral composition, properties, and micro-architecture of human cancellous bone. In addition they have a high affinity for proteins, and cells can

deposit an extra cellular matrix onto these materials [16]. When hMSCs or mouse ESCs proliferate or differentiate on CaP ceramics we observe that cells fully cover the ceramics after migrating and spreading, as shown in chapter 3. With chondrogenic cell cultures however there is a complicating aspect, being that serum proteins are necessary for cell attachment. But the addition of serum to the medium is detrimental for chondrogenic differentiation. Although serum is present during the initial seeding phase, the lack of serum during the following culture time results in a heterogeneous distribution of the cells over the ceramics. The large standard deviations observed within the tissue engineered cranial implants in chapter 6 are likely caused by this adherence predicament. Pre-culturing of the cells with serum prior to the addition of the serum-free chondrogenic medium could ensure a more homogenous distribution of cells. Coating the ceramics with serum proteins such as fibronectin or vitronectin could provide a basis for cell adherence, although these proteins might have an adverse effect on the differentiation pathway of the ESCs. In-depth investigations of the interaction between chondrogenic ESCs and extracellular matrix proteins could be a fruitful line of research.

The seeding experiments described in several chapters were all performed statically; in which a cell suspension was added to the ceramics and the cells were left to adhere for 2 to 3 hours prior to the addition of culture medium. In this way, gravity will have a vast impact on the location were cells will adhere, which is mostly on the bottom part of the porous ceramics as we frequently observed. From previous experiments we know that only 15 to 20% of the cells adhere (data not shown), and the rest is lost during medium refreshment. Bioreactors have been designed that will allow homogeneous cell proliferation, by seeding the cells dynamically under a constant fluid flow. Our lab provided proof of concept for this approach in a recently published article in which it is described that goat-derived MSCs produce clinically relevant volumes of tissue-engineered bone products using a direct perfusion bioreactor system [17]. Combining a bioreactor with a biomaterial which allows attachment of chondrogenicly differentiated

ESCs may perhaps be the ideal circumstance to create a homogenously seeded bone substitute.

### *Human embryonic stem cells and tissue engineering*

Our results so far are mainly based on experiments with mouse embryonic stem cells, but for future applications extrapolation of our results to human ESCs is required. However, there are several obstacles that hinder the use of ESCs for future tissue engineering purposes besides the ethical questions raised. Creation of autologous human ESC lines involves techniques which are currently expensive and require a large number of blastocysts to create a single cell line. Another aspect is that expansion of human ESCs is currently achieved through a mechanical and intensive culture method that prohibits fast progress towards clinical application because *in vitro* proliferation of human ESC is very time consuming. To illustrate this, it took us several weeks to obtain a sufficient amount of undifferentiated colonies, about 20 million cells, to start a differentiation experiment as described in chapter 4. As described in chapter 2, about 160 million hMSCs are needed for the creation of a bone tissue engineered construct used in spinal surgery. Even though we spend about 10 times the effort in our human ESCs experiment compared to the hMSCs culture, we did not nearly obtain enough cells to create such a construct.

Efforts have been made to simplify human ESC culture by for instance replacing the feeder layer with Matrigel or laminin [18]. In a recent publication it was also reported that human ESCs can be expanded feeder-free and matrix-free directly on plastic surfaces in the presence human fibroblast-conditioned medium, without any supportive coating [19]. Although this would reduce some of the work there is still the problem of the time consuming cell replanting. Another recent publication might provide the solution to this problem. Ellerström and co-workers demonstrated that human ESCs, which were derived and passaged by mechanical dissection, can be rapidly adjusted to propagation by enzymatic dissociation to single cells [20]. With time, several of the problems which hinder the use of ESCs in clinical

applications will be solved. For instance, we have discussed the use of induced pluripotent stem cells as an alternative for the use of human ESCs [21-24] in several chapters, which could solve the issue of the immunological rejection of the allogeneic ES cells. Moreover, since iPS are derived form somatic cells blastocyst are not required. Although these cells would therefore provide a good alternative, from *in vivo* studies it is known that they also form teratomas, similar to ESCs. Even when current bottlenecks are solved, application of human ESCs in the field of bone tissue engineering will meet fierce competition from other alternatives, including bone morphogenetic proteins, inductive materials and MSC-based bone tissue engineering.

### *Future applications of embryonic stem cells*

Although we realize that many steps need to be taken to implement ES cells in bone tissue engineering, this thesis does demonstrate that ESCs have great potential as model system in bone tissue engineering. For instance, the best way to test the osteoinductive properties of ceramic materials is by implanting them intra-muscular in large animals. Although this method of testing gives a clear readout on the bone inductive potential of the CaP ceramics, these models are not very useful to unveil the underlying molecular process by which the bone is formed. Also the population of cells that respond to these materials by producing bone is still unknown. If we are able to unravel this process it could provide the possibility to further optimise the performances of CaP ceramics and their osteoinductive properties. We demonstrate in chapter 3 that *in vitro* osteogenic differentiation of mESCs can be influenced by the properties of ceramic materials and seems to reflect the osteo-inductive properties of the materials *in vivo*. Further investigations into the molecular pathway controlling ESC differentiation on different ceramics is ongoing.

With our discovery of endochondral bone formation by mouse ESCs (chapter 5) we have provided researchers with a tool to study endochondral bone formation. Currently the only method to study this process is during embryonic development or in fracture healing. When

studying for example a genetic mutation causing abnormalities during embryonic development of bone, a mouse can be genetically altered to study this process. This however is time consuming and expensive. With our discovery this process can be studied in an ectopic model, if an embryonic stem cell line of the mutation is available or developed.
In conclusion, we have identified critical steps in bone tissue engineering using ESCs and unexpectedly found a new tool by which the process of endochondral bone formation can be studied. It demonstrates the value of ESC research and may give us a better insight for possible future clinical applications.

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# Summary

Due to increased life expectancy of humans the number of patients with age related skeletal compliciations has increased. These patients but also patients suffering from complications due to trauma or disease often need surgical interventions in which additional bone is required for optimal recovery. Currently the most frequently used bone replacement is autologous or allogeneic bone, but both methods have their drawbacks.

An alternative approach could be the use of tissue engineered bone. By combining autologous cells with a biomaterial, a bone substitute can be created. Mesenchymal stem cells (MSCs) are an option for such an approach. These cells can be expanded in culture to reach an amount needed to fill a defect (Chapter 2). Both *in vitro* and *in vivo* these cells display osteogenic potential. Unfortunately also MSCs have their limitations. During expansion they gradually loose their multipotency and large donor variation is observed with respect to bone forming capacity. In contrast to MSCs, embryonic stem cells can be proliferated indefinitely. They differentiate into derivatives of all three germ layers and are easily genetically manipulated. These aspects make them a potential interesting cell source for bone tissue engineering, but moreover an ideal candidate as a model system since donor variation is excluded.

*In vitro* these cells display all signs of osteogenic differentiation when differentiated in a similar way as MSC (chapter 3 & 4). Osteogenic differentiation of mouse ESCs is even enhanced by calcium phosphate ceramics at least in part by influencing the medium composition (chapter 3). From these studies It appears that mouse ESCs can be used as a cell source for further investigating the osteogenic potential of different calcium phosphate ceramics and the underlying molecular mechanism.

*In vivo* however this osteogenic differentiation protocol which induces MSC to form bone does not induce bone with either human or mouse ESCs (Chapter 4). During embryonic development there are two mechanisms by which bone is formed; intramembranous bone formation

and endochondral ossification. With endochondral ossification a cartilage matrix is formed which is then replaced by bone. Therefore we induced the mouse ESC into forming a cartilage matrix *in vitro* which was then implanted subcutaneously in immune-deficient mice. *In vivo* the cartilage matured calcified and was ultimately replaced by bone tissue in the course of 21 days (chapter 5) We did not only observe this process in mice but also in rats both in an ectopic and osseous site (chapter 6). We hereby report for the first time ESC-derived bone tissue engineering under controlled, reproducible conditions. Our data also indicates that ESCs can be used as a model system to study endochondral bone formation. Unfortunately also ESCs have their drawbacks. One of the things that defines an ESC is its ability to form a teratoma, a tumor composed of different cells and tissues derived from all three germ layers. Even though we exposed the ESCs to a comprehensive differentiation scheme *in vitro*, we did observe teratoma formation *in vivo* (chapter 7). Suggesting that a population of undifferentiated cells is still present when implanted. This is a warrant for the establishment of strategies for using ESC in possible human applications to exclude the tumorigenic population.

# Samenvatting

Door de verhoogde levensverwachting van de mens is er een stijging in het aantal patienten met bot aandoeningen. Deze patienten maar ook patienten met complicaties aan bot door trauma of ziekte hebben vaak een operatie nodig waarbij extra bot vereist is voor een betere genezing. Op dit moment wordt hoofdzakelijk gebruik gemaakt van patiënt eigen of donor bot als botvervanger. Helaas heeft het gebruik van beide opties nadelen.

Een mogelijk alternatief is het gebruik van gekweekt bot. Door cellen van de patiënt te combineren met een drager materiaal (bijvoorbeeld een calciumfosfaat-keramiek) kan een botvervanger worden gecreëerd. Mesenchymale stamcellen (MSC) zijn een optie voor een dergelijk aanpak. Deze cellen kunnen in het laboratorium worden vermeerderd tot het aantal wordt bereikt dat nodig is voor het behandelen van een botdefect (hoofdstuk 2). MSC laten zowel *in vitro* (in het laboratorium) als *in vivo* (in een organisme) zien dat ze over botvormende capaciteiten beschikken. Helaas hebben ook deze cellen nadelen. Wanneer MSC worden vermeerderd verliezen ze het vermogen tot differentiatie en er is veel variatie in de hoeveelheid bot die wordt gevormd tussen de verschillende donoren. In tegenstelling tot MSC kunnen embryonale stemcellen (ESC) oneindig worden vermeerderd. Door differentiatie kunnen deze cellen alle weefseltypes vormen die voorkomen in het menselijk lichaam. Daarnaast zijn ze eenvoudig genetisch te manipuleren. Deze aspecten maken van ESC een interessante celsoort voor het maken van gekweekt bot, ook kunnen ESC gebruikt worden als een modelsysteem voor onderzoek naar botvorming.

*In vitro* tonen ESC alle tekenen van bot differentiatie vergelijkbaar met die van de MSC (hoofdstuk 3 & 4). Bot differentiatie van muizen ESC wordt zelfs verbeterd door calciumfosfaat-keramiek, waarschijnlijk deels door beïnvloeding van de samenstelling van het kweekmedium (hoofdstuk 3). Mogelijk kunnen muizen ESC worden gebruikt voor verder onderzoek naar de bot inducerende potentie van verschillende calcium fosfaten en het daarbij behorende moleculaire mechanisme.

Het gebruikte differentiatie protocol dat bij MSC *in vivo* bot induceert geeft *in vivo* geen botvorming bij zowel humane als muizen embryonale stamcellen. Tijdens de embryonale ontwikkeling wordt via twee verschillende processen bot gevormd, één daarvan is het endochondrale proces. Tijdens dit proces wordt een kraakbeen matrix gevormd die vervolgens wordt vervangen door bot. Om dit proces na te bootsen hebben wij de muizen ESC *in vitro* eerst een kraakbeen matrix laten vormen op calciumfosfaat-keramiek en die vervolgens onder de huid van een muis geïmplanteerd. *In vivo* verkalkte het kraakbeen en werd daarna vervangen door bot in een tijdsbestek van 21 dagen (hoofdstuk 5). Dit proces hebben wij niet alleen waargenomen in muizen maar ook in ratten zowel onder de huid als in een botdefect (hoofdstuk 6). Hierbij doen wij voor de eerste keer verslag van botvorming door ESC onder controleerbare en reproduceerbare condities. Onze data laat ook zien dat ESC gebruikt kunnen worden als een model systeem voor onderzoek naar endochondrale botvorming. Helaas hebben ESC ook nadelen. Eén van de aspecten wat een embryonale stamcel definieert is hun potentie tot het vormen van een teratoma, een tumor die bestaat uit cellen en weefsels die gevormd kunnen worden tijdens de embryonale ontwikkeling. Ondanks ons intensieve *in vitro* differentiatie protocol zagen wij teratoma formatie *in vivo* (hoofdstuk 7). Deze data suggereert dat een populatie ongedifferentieerde cellen aanwezig is tijdens implantatie. Dit is een waarschuwing dat bij eventueel toekomstig gebruik van humane ESC tumorvorming moet worden uitgesloten.

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Sanne.

# Curriculum vitae

Sanne Both was born on the 11th of July 1976 in Gouda, The Netherlands. She grew up in Gouda and graduated in 1995 from high school (HAVO, Chr. Lyceum in Gouda). She then went to the Poly Technical School (HLO Rotterdam) to study Medical Biology for which she received her bachelor degree in 2000. During her studies she performed her traineeship under the supervision of Dr. J.D. de Bruijn at IsoTis NV where she investigated osteogenic differentiation of cultured human bone marrow cells. From 2000 on she continued working at IsoTis NV as researcher in the "bone team". In April of 2003 she started as a PhD student in the department of Tissue Regeneration under the supervision of Prof. Dr. C.A. van Blitterswijk and Dr. J de Boer. The subject of her research was embryonic stem cells in bone tissue engineering. The results are described in this thesis.

Once you have tasted flight, you will forever walk the earth with your eyes turned skyward, for there you have been and there you long to return.

Leonardo da Vinci.