INSTRUCTIVE ELEMENTS FOR *BONE TISSUE* ENGINEERING

Hugo Fernandes| 2009

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HUGO FERNANDES

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Hugo Fernandes
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INSTRUCTIVE ELEMENTS FOR BONE TISSUE ENGINEERING

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to obtain
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on the authority of the rector magnificus,
Prof. Dr. H. Brinksma
on account of the decision of the graduation committee,
to be publicly defended

on Thursday, November 26th 2009, at 13.00

by

Hugo Fernandes

Born on October 16th, 1977
In Joane, Portugal

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INTRODUCTION

"Courage is the discovery that you may not win, and trying when you know you can lose"

Tom Krause

A bit of history

For thousands of years Man struggled with nature in order to survive. Survival was not only an evolutionary pressure in which the stronger would survive and perpetuate the species, but a daily fight. Evolution brought us a long way and today, either because some of the selective pressures of the past are not present anymore or because we have learnt how to live with them, we can spend more time doing other things than survive. Although it is prone to discussion whether our relation with nature was symbiotic or parasitic, one thing we know: Nature has been generous to us. Nature gave us oxygen. Nature gave us fire. Nature gave us animals that we domesticate and use for our own benefit. Using animals to help us in our tasks gave us time for other activities. Obviously, living as much as possible and with the highest quality of life possible was something appealing.

Many ancient civilizations had put a lot of effort in learning what is life and how can we extend it. Many disciplines were created with that goal in mind but one above all had a prominent role in comprehending life and how to extend it: Medicine.

Greece was the birthplace of the father of modern Medicine: Hippocrates. Since then, Medicine has tremendously changed so that nowadays modern Medicine is something more elaborate, comprising new advances in diagnostic technologies and regenerative medicine. Interestingly, Greeks were not only interested in Medicine but they were also great engineers, besides many other predicates. The first example of organ regeneration can be found in the Greek mythology. After stealing the fire from the gods and giving it to the mortals, Prometheus was severely punished by the gods. He was bound to a rock where birds would eat his liver during the day and the liver would grow back during the night, only to be eaten again on the next day (Figure 1). The regenerative capacity of the liver is well known in our time but this is probably one of the first reports of regenerative medicine as we aim for today. Although still in its infancy compared with the Greek civilization, tissue engineering arose in the early 90's as a metaphor of the Greek civilization. The definition of tissue engineering is: "an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function" [1]. In Greece, the principles of engineering and life sciences were used to develop a society.

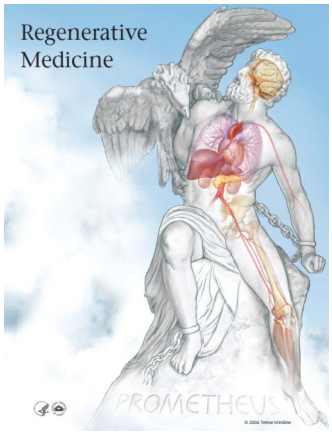

Figure 1 – First report of organ regeneration: Prometheus is punished after stealing fire from the gods and giving it to the mortals. As punishment, birds would eat his liver during the day and during the night it would grow again (© 2006 Terese Winslow).

Concomitant with the increase in life expectancy we saw a surge in chronic diseases such as cancer, osteoporosis and other diseases that severely affect our quality of life and threaten our elderly. Due to this, Man had the need to restore or replace tissues or even entire organs to survive. Unfortunately, somewhere during the course of evolution we lost the regenerative capacity which some organisms, such as salamanders, have retained (Figure 2A) [2]. More strikingly, early during foetal development we still have the capacity to regenerate. Foetal wound healing does not lead to scar tissue formation and the skin is fully regenerated (Figure 2B) [3,4]. Nevertheless, due to the loss

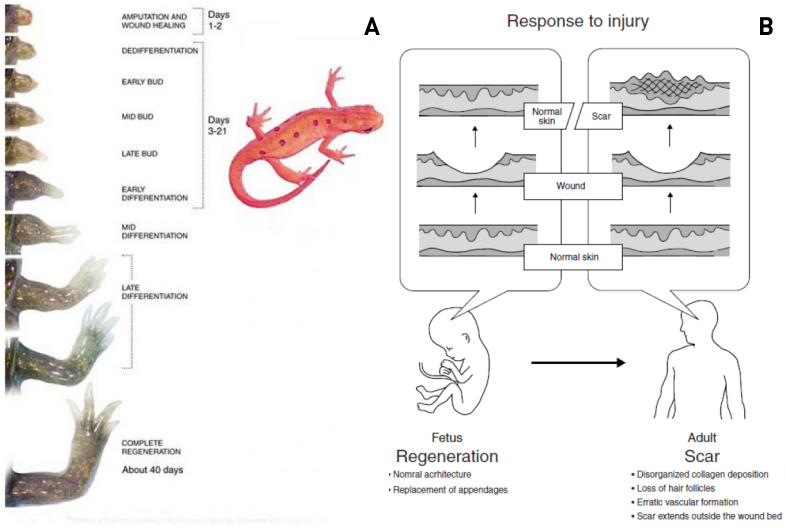

Figure 2 – Regenerative potential: A. Salamander regeneration capacity after amputation. B. Fetal versus adult skin wound healing. Fetal skin repairs without scar tissue formation and the newly deposited tissue has a normal ECM architecture. In the case of adult wound healing, there is formation of fibrotic tissue and the newly deposited ECM shows abnormal architecture (adapted from [3]).

of regenerative capacity in the adult organism, we have to rely on other options in case of injury or lost function of an organ. Organ transplantation is a possibility, but limited availability, graft versus host disease due to incompatibility, and the risk of disease transmission, makes it far from the ideal situation. The multidisciplinary field of tissue engineering was born as a potential alternative to organ transplantation. One of the main goals of tissue engineering was, and is, to enhance the natural regenerative capacity of the adult organism. Nature does this every day and good examples are haematopoiesis, skin and bone remodelling. In all these examples, cells die and/or are remodelled in order for new ones to arise, contributing to maintenance of tissue homeostasis. Unfortunately, Nature either forgot to provide us with the "regenerating instructions" or wrote them in a language we find only partly comprehensible, leaving us to search for those instructions and seek new tools that allow us to read those instructions.

In that sense, much progress has been made. One lesson from these efforts was that we should combine our efforts and expertise to find Nature's hidden secrets. A project such as the Human Genome Project is a good example to be followed. From the discovery of the DNA structure by Watson and Crick in 1953 to the "reading" of the full genome of a human being in 1998, a lot changed in the world of biology. Nevertheless, the information gathered from this project will keep generations of scientist busy and probably the Rosetta stone that will allow us to read the human genome will take its time to be found.

Bone composition and structure

Biological systems have an intrinsic complexity derived from their composition. As a useful analogy, we can compare a biological system to a car. A car is composed of different parts working as a whole for a final goal. If one part is missing, providing it is not a critical component, the car can still work but its overall performance will be affected. Although a car is composed of simple systems, the integration of its parts results in a complex system with multiple connections susceptible to failure due to "fatigue". In biological systems the same complexity can be observed. If we con-

sider a cell a complex system composed of different units we can easily imagine that a group of different cells will have added complexity. Moreover, in most cases, a tissue is composed of different cell types that need to communicate and survive in the same environment. In this thesis I will focus on one of the many tissues in the adult organism that can be damaged or worn-out: bone.

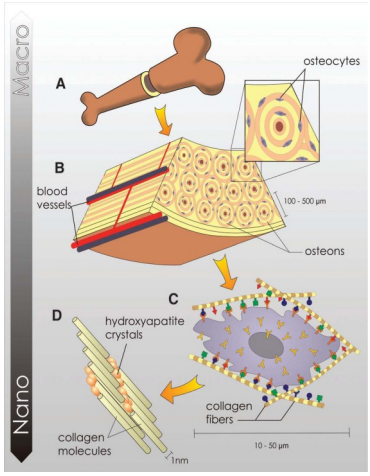

Figure 3 – Bone architecture from a macro to a nano level: The figure shows the bone composition from the macro level followed by a scheme of the bone functional unit (osteon). Collagen fibers are also depicted as they are the main organic component present in the osteon. The mineralization of this fiber, a typical characteristic of bone tissue, occurs between the different collagen molecules as shown (adapted from [86]).

Bone is a dynamic complex connective tissue composed of different cell types with distinct functions [5]. While it is mainly known for its protective and supportive role for muscles and tendons, it also plays a key role in calcium and phosphate homeostasis as the main calcium reservoir in the human body [6,7]. Moreover, it is the main site of haematopoiesis in the human adult [8,9]. On a weight basis, bone is composed of mineral (60%), organic matrix (30%) and water (10%) [10]. The main component of the organic phase is collagen type I. Collagen is the main protein of the human body and can be found in virtually every tissue. Based on its structure and composition, collagen has different roles depending on the tissue where it is found. For example, in the case of bone, collagen fibers are highly mineralized providing elastic properties (the collagen) and compressive strength (the hydroxyapatite crystals deposited on the collagen fibers) which are characteristic of bone [11]. The mineralization of collagen occurs mainly at the gap junctions between collagen fibers (Figure 3).

Three major cell types are involved in skeleton formation and homeostasis: osteoblasts, chondrocytes and osteoclasts. Osteoblasts and chondrocytes are responsible for the formation of bone and cartilage, respectively, and both are of mesenchymal origin. Osteoclasts, on the other hand, are responsible for bone resorption and originate from hematopoietic cells. Terminally differentiated osteoblasts secrete a matrix which, in a later stage, will be mineralized and entrap the osteoblast [12,13]. An osteoblast entrapped in its own deposited matrix (denominated osteoid) is called an osteocyte and it is a post-mitotic cell that can communicate with other osteocytes via small canals called canaliculi. Osteocytes are known to be involved in bone homeostasis. They secrete molecules that can act as negative regulators of bone formation or that can inhibit osteoclastic activity [14-16] (Figure 4).

Bone development can occur via two different mechanisms: intramembranous or endochondral ossification [17]. Intramembranous ossification occurs in the flat bones of the skull and in the addition of new bone to the outer surface of long bones. All the other bones are formed via endochondral ossification. In intramembranous ossification mesenchymal precursor cells differentiate directly into osteoblasts (Figure 5A). In the case of endochondral bone formation, mesenchymal cells condense and differentiate into cartilage. The cartilaginous template consists of chondrocytes distributed in three different zones: a resting zone where chondrocytes do not proliferate, a proliferating zone where chondrocytes are proliferating, and a hypertrophic zone where chondrocytes become

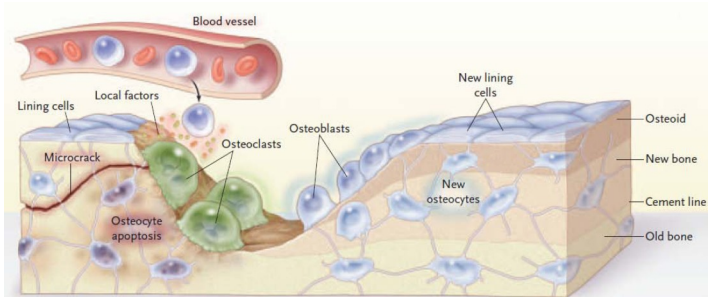

Figure 4 – Scheme of a bone remodeling unit: On the left side of the scheme is a depiction of an inflammatory process due to a microcrack with the concomitant death of the osteocyte and the release of signals that will drive the migration of cells from the blood vessels and marrow into the defect. Osteoclasts will resorb the old matrix and osteoblasts will deposit a new matrix contributing to the repair of the microcrack (adapted from [143])

larger and later die followed by calcification. This process is highly dependent on neovascularisation of the cartilage template. Blood vessels invade the cartilage template bringing along osteoclasts, bone marrow cells and osteoblasts which will replace the remaining cartilaginous template for bone (Figure 5B) [6,18,19]. Besides differences in morphology between the different regions, the protein expression also differs at the different stages. Pre-hypertrophic chondrocytes are characterized by the expression of type II collagen whereas hypertrophic chondrocytes express collagen type X [20].

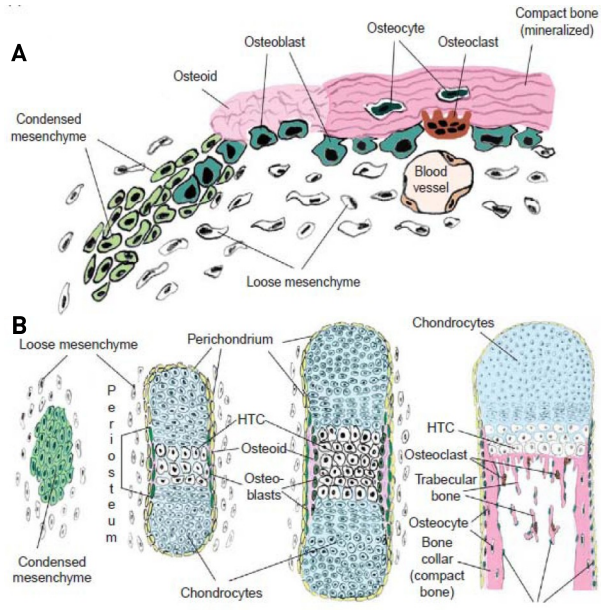

Figure 5 – Two different processes of skeleton formation: A. Intramembranous bone formation. Mesenchymal cells condense and will differentiate into functional osteoblast producing an ECM in which they will be entrapped at later stages (osteoid). Blood vessels in the surroundings will bring osteoclast to degrade the bone and new bone is deposited B. Endochondral bone formation. In this case, a condensed mesenchyme will differentiate into a cartilage template constitute of three distinct zones. One consisting of highly proliferative chondrocytes, followed by a zone of pre-hyperthrophic and a hypertrophic chondrocytes (HTC).The pre-hypertrophic chondrocytes will die and calcify and upon blood vessel infiltration osteoblast will invade this area depositing new bone. The area adjacent to the hypertrophic chondrocytes is denominated perisoteum (adapted from [42])

Communication between the different cell types that constitute bone is of paramount importance to balance anabolic and catabolic activities. For example, the main triggers of bone resorption are released by the osteoblast - receptor activator for NF-κB-ligand (RANK-L) and osteoprotegerin (OPG). RANK-L binds to its receptor on the membrane of hematopoietic cells (RANK) which in turn will activate the differentiation into a functional osteoclast. OPG is a decoy receptor that can block the RANKL/RANK interaction. Nevertheless, the availability of these two molecules can be controlled by systemic hormones such as parathyroid hormone (PTH) and estrogen [7]. Such a tightly controlled system contributes to the maintenance of bone throughout the life. Any pathology resulting in an imbalance of the anabolic or

catabolic metabolism of bone can severely affect bone homeostasis and compromise the skeleton integrity. An example of a disease resulting from such an imbalance is osteoporosis [21].

Transcriptional control of osteogenesis

Both osteoblasts and chondrocytes arise from the same precursor cells – mesenchyme-derived cells. Their distinct phenotype is the result of activation of different transcriptional regulators leading to a molecularly distinct differentiation program.

A key transcription factor regulating the differentiation of uncommitted mesenchymal cells into the osteogenic lineage is Cbfa1 or Runx2. Cbfa1/Runx2 is a member of the runt homology domain family of transcription factors. The importance of Cbfa1 for osteogenic differentiation can be observed in the phenotype of Cbfa1-deficient mice. In this mouse model, inactivation of Cbfa1 prevents the differentiation of mesenchymal cells into osteoblasts resulting in a fully cartilaginous skeleton (Figure 6) [22-24]. Moreover, several genes involved in early and late stages of osteogenic differentiation have Cbfa1 binding sites, indicating a critical role of Cbfa1 not only during commitment but also during maturation and maintenance of the osteogenic phenotype [25-27]. Furthermore, Cbfa1 is also involved in chondrogenic differentiation during the transition from non-hypertrophic to hypertrophic chondrocytes. A transgenic mouse expressing Cbfa1 only in non-hypertrophic chondrocytes (α1(II) Cbfa1) revealed endochondral ossification in places where normally it was not present [28]. The expression of Cbfa1 is prone to regulation by other elements. For example, Msx1 and Msx2, two homeobox-containing proteins, are involved in skeletal development and can interfere with the expression of Cbfa1. It has been shown that inactivation of Msx2 leads to a decrease in the expression of Cbfa1. Additionally, Msx2-deficient mice showed impaired intramembranous and endochondral ossifications indicating that Msx2 is involved in both osteogenic and chondrogenic differentiation [29,30]. Dlx5, a transcription factor belonging to the family of homeodomain proteins, is also involved in osteogenesis. It has been shown that Dlx5 is expressed at later stages of osteogenic differentiation and it can upregulate Runx2 expression [31]. Moreover, Twist transcription factors, which are basic helix-loop-helix (bHLH) – containing transcription factors, can transiently inhibit Cbfa1 function and by doing so they are able to control the onset of osteoblast differentiation [32].

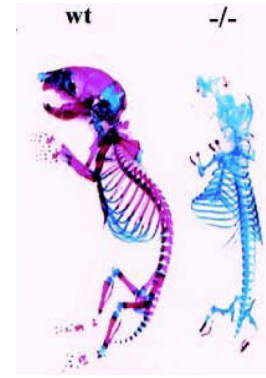

Figure 6 – Phenotype of the Cbfa1-deficient mice. Figure shows the skeleton of newborn mice stained with Alcian blue (cartilage) and Alizarin red (bone). Note that in the wild-type animal the skeleton is mainly constituted by bone whereas in the Cbfa-1-deficient mouse the skeleton is mainly cartilaginous (adapted from [28])

Several other transcription factors are known to be involved in osteogenic differentiation. For example, inactivation of Osterix (a zinc finger-containing transcription factor) in mice results in perinatal lethality due to complete absence of bone formation [33,34]. Moreover, Osterix can interact with other transcription factors such as nuclear factor of activated T cells (NFAT) and regulate bone formation [35]. ATF4, which belongs to the subfamily of cAMP-response element-binding protein/ATF basic leucine zipper proteins, has been shown to play a critical role in osteoblast differentiation and function. ATF4 is a substrate for Rsk2, a growth factor-regulated kinase. Rsk2-deficient mice

have a low bone mass phenotype due to a decrease in the bone formation rate, a marker of osteoblastic function. AFT4-deficient mice often die perinatally but a careful analysis of the embryos show a delay in skeletal development and the ones that survive show a severe low-bone-mass phenotype due to decreased bone formation. The effect in bone formation seems to be due to improper synthesis of collagen type I since ATF4-deficient mice showed a decrease in collagen type I synthesis although independent from the gene expression of α1(I) collagen [36, 37]. Tcf/Lef transcription factors are also involved in osteogenesis and are activated via the Wnt signalling pathway. Wnts are a family of secreted proteins that bind to a membrane receptor complex comprising a frizzled G-protein-coupled receptor and a low-density lipoprotein receptor-related protein (LRP). Upon binding to the receptor, an intracellular cascade is activated which leads to accumulation of β-catenin in the cytoplasm (otherwise degraded by the proteosome); its subsequent translocation into the nucleus results in activation of Tcf/Lef transcription factors [38, 39]. The involvement of Wnt signaling in osteogenic differentiation became apparent from clinical investigations showing that gain and loss-of-function in the Wnt receptor LRP5 led to high and low bone mass phenotypes, respectively [40]. Since this discovery, the role of Wnt signaling in osteogenic differentiation has been studied in great detail (Figure 7) [38, 41, 42].

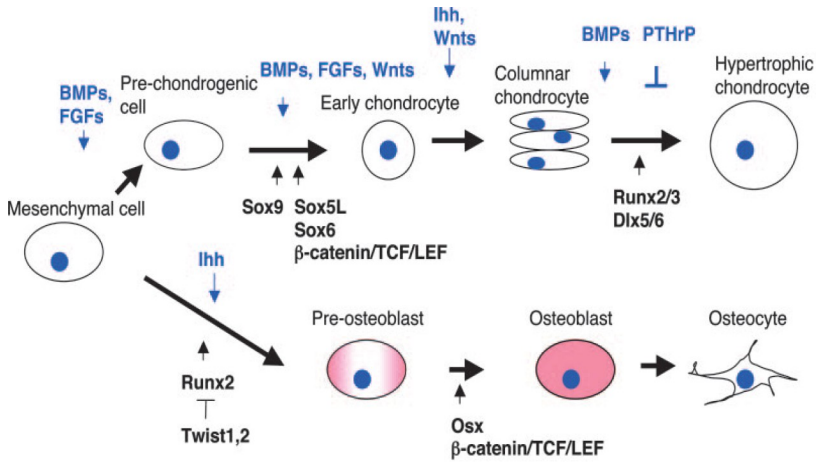

Figure 7 – Transcription control of skeletogenesis. The scheme depicts the differentiation of mesenchymal cells into the different lineages and the key transcription factors and signaling molecules involved in the differentiation cascade (adapted from [144])

According to the temporal expression of certain genes during osteogenic differentiation, three different phases occur: proliferation and synthesis of extracellular matrix (ECM), ECM maturation and finally ECM mineralization [43].

During this step-wise process, several factors such as morphogens, growth factors, cytokines, ECM proteins, among others, can lead to activation of the above mentioned transcription factors which in turn will guide the differentiation of the cells into a certain lineage. For example, factors such as bone morphogenetic proteins (BMPs), vitamin D, dexamethasone, Wnts or cyclic adenosine monophosphate (cAMP) all lead to a sequential activation of genes characteristic of differentiation towards the osteogenic lineage [12, 27]. Activation of transcription factors such as Cbfa1 and Osterix are key markers of early commitment into the osteogenic lineage but other markers such alkaline phosphatase (ALP) are typical of the early stages of differentiation. ALP is an enzyme lo-

calized in the plasma membrane of osteoblasts and, although its precise function is not known, there are studies suggesting that it is involved in mineralization, since its inhibition leads to inhibition of mineralization [44, 45]. ALP has been shown to be responsible for the cleavage of pyrophosphate - a small molecule that binds to nascent hydroxyapatite crystals and prevents further incorporation of phosphate into the crystals [46, 47]. Patients suffering from hypophosphatemia have a decrease in bone mineralization due to the absence of tissue-nonspecific alkaline phosphatase expression [48, 49]. Terminally differentiated osteoblasts will secrete an ECM mainly composed of collagen type I which can be mineralized in the presence of a phosphate source. Additionally, non-collagenous proteins such as osteocalcin and bone sialoprotein also contribute to the mineralization of the ECM and are well accepted as late markers of osteogenic differentiation [12]. Nevertheless, the mineralization of the newly deposited ECM is only possible due to the co-expression of two genes: ALP and collagen type I (Figure 8) [46].

Collagen type I has a key role in osteogenic differentiation serving as a template for mineralization. For example, in MC3T3-E1 cells, a mouse calvarial cell line commonly used in the study of osteogenic differentiation *in vitro*, the absence of collagen completely abolishes the osteogenic differentiation [50-52].

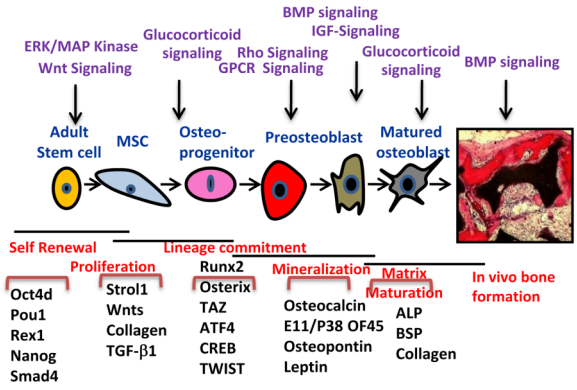

Figure 8 – Differentiation of adult stem cells into the osteogenic lineage. The scheme shows the transcription factors and signaling molecules involved on the maintenance of the stemness of adult stem cells as well as the signals that regulate the differentiation of those cells into the osteogenic lineage. The main signaling pathways involved are also show (Courtesy Dr. Siddappa)

Collagen structure and function

As mentioned above, the presence of a collagenous matrix is essential for terminal differentiation into the osteogenic lineage. Traditionally, proteins such as collagens were perceived as the scaffold of ECM, with mainly a structural role. Nowadays, however, it is believed that ECM plays an important regulatory role in cell behavior, controlling such diverse functions as proliferation, growth, cell survival, migration and differentiation (Figure 9) [53-62]. More than 20 genetically different types of collagen have been identified so far. Collagen molecules consist of three polypeptide α chains, each of them containing at least one repeating Gly-X-Y sequence, where X and Y are usually proline and hydroxyproline, respectively [63, 64]. The three chains are supercoiled around a central axis in a right-handed manner to form a triple helix. Collagen undergoes several post-translational modifications which contribute to its structural and mechanical properties. Interference with some of these modifications results in severe dysfunction of the tissue in question. For example, in the past sailors searching for new territories or doing commerce around the world, often suffered from

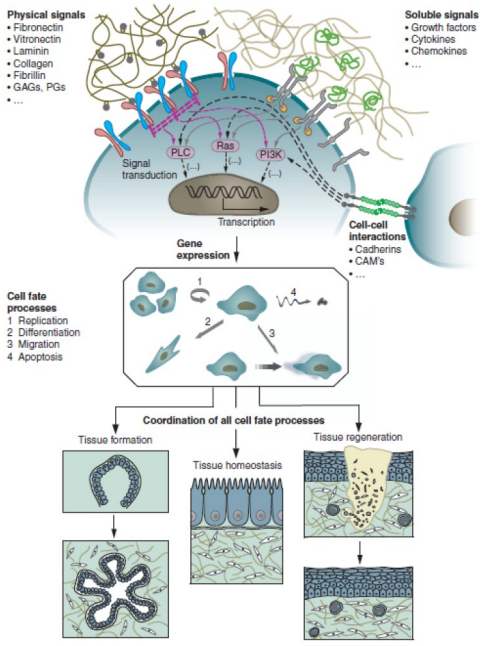

Figure 9 – ECM-cell signaling and regulation of biological processes by ECM. The scheme shows the physical and soluble signals present in ECM and the regulatory pathways that can be activated and that modulate the cell fate (adapted from [61])

by the enzyme lysyl oxidase (Figure 10) [65, 66].

The post-translational modifications of collagen in bone and other mineralizing tissues differ from those in other types of collagen matrices [67]. Proper collagen crosslinking may be important for the binding of collagen to its receptors, but it may also be important to regulate the availability of growth factors present in ECM and to enhance the mechanical properties, as well as the structure, of ECM [64, 68, 69]. Examples where the structure of collagen affects bone are patients suffering from Bruck syndrome and osteogenesis imperfecta

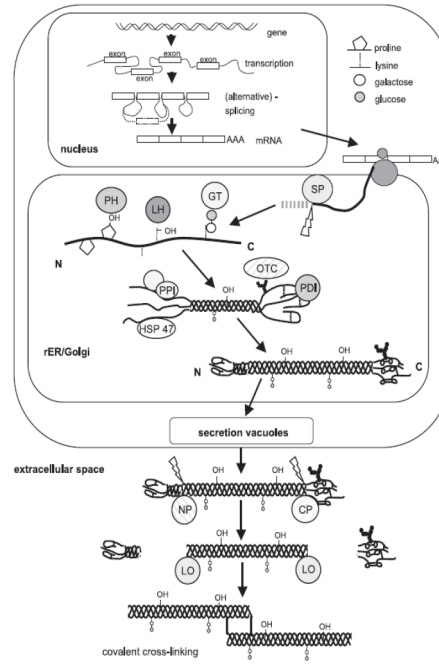

Figure 10 – Schematic representation of collagen synthesis. Depicted in the scheme are the steps involved in collagen formation, starting from transcription up to the secretion of a fully mature collagen molecule. (Figure legend: SP – signal peptide; GT – hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase; LH – lysyl hydroxylase; PH: prolyl hydroxylase; OTC – oligosaccharyl transferase complex; PDI – protein disulphide isomerase; PPI – peptidyl-prolyl cis-trans-isomerase; NP – procollagen N-preoteinase; CP – procollagen C-proteinase; LO – lysyl oxidase, HSP47 – heat shock protein 47) (adapted from [64])

scurvy. Scurvy is characterized by loss of teeth, bleeding due to the disruption of blood vessels and, in those days, could be fatal. It was later discovered that vitamin C enriched fruit would rescue the sailors. The reason behind it lays in the fact that vitamin C, also known as ascorbic acid (Asap), was a cofactor for two enzymes involved in collagen synthesis. Those enzymes – prolyl and lysyl hydroxylase – are responsible for hydroxylation of the collagen residues and, when absent, collagen is not synthesized and it is degraded inside the cell.

The last steps in the formation of the collagen molecule is the cleavage of the N and C propeptides, the spontaneous self-assembly of the resulting collagen molecules into fibrils, and the formation of covalent crosslinks [63]. Collagen crosslink formation occurs in the extracellular space and it is initiated by the conversion of specific lysine or hydroxylysine residues into the aldehydes allysine or hydroxyallysine, respectively. This crosslink reaction is catalyzed

(OI). In the first case, deficiency of bone specific telopeptide lysyl hydroxylase results in aberrant crosslinking of bone collagen leading to osteoporosis, joint contractures, fragile bones and short stature [70]. In the case of OI, mutations affecting the structure or the abundance of collagen type I can be found in the majority of the patients resulting in bone abnormalities ranging from bone fragility to high bone mineralization [71-74].

The main pathway by which collagen signals to the interior of the cell is via specific transmembrane cell surface receptors called integrins. Integrins consist of an α- and a β-subunit and have a key role in conveying information from ECM towards the interior of the cell (outside-in-signaling) as well as from the inside of the cell towards ECM (inside-out-signaling). There are 16 known α and 8 β subunits. Upon ligand binding, integrins will cluster in the cell membrane and in their intracellular part, a myriad of intracellular proteins such as vinculin, talin, paxilin and focal adhesion kinase (FAK) will be recruited leading to the formation of focal adhesion sites, initiating a cell signaling cascade that regulates cell migration, proliferation and differentiation (Figure 11) [56,75-78]. Collagen-cell interactions are mediated mainly through $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins [79-81]. For example, it has been shown that culture of rat fetal parietal bones without Asap decreased, dose-dependently, α_2 and β_1 integrins mRNA levels resulting in inhibition of calcification [82]. Moreover, it has been demonstrated that an anti-$\alpha_2\beta_1$ integrin antibody could block Asap-induced ALP activity in MC3T3-E1 cells [83]. The several steps involved in the formation of functional collagen molecules leave various possibilities to interfere with collagen formation and, given the importance of collagen in osteogenesis, several studies attempt to understand and control the process of collagen assembly hoping to improve the osteogenic differentiation of uncommitted cells.

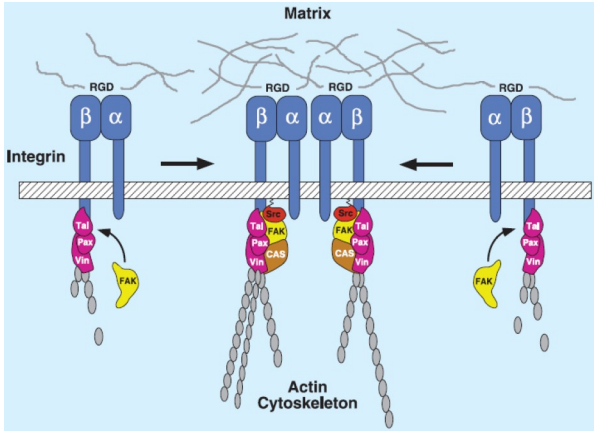

Figure 11 – Cell-matrix interaction and the assembly of focal adhesion complex. The scheme shows the interaction of the RGD motifs with the transmembrane receptors integrins and the subsequent formation of a focal adhesion complex. Upon binding to integrins there is clustering in the cell membrane and intracellular recruitment of proteins that initiate downstream signaling. (Figure legend: RGD – Arg-Gly-Asp integrin binding motif; Tal – talin; Pax – paxillin; Vin – vinculin; FAK – focal adhesion kinase; CAS – $p130^{CAS}$) (adapted from [145])

Bone tissue engineering

Decades ago diseases such as cancer and osteoporosis were not as common as these days. That does not mean they did not exist. Given the fact that such diseases usually appear later in life, and due to the short life expectancy in the beginning of the XX^{th} century, such diseases did not present a critical problem in society. Amid the increase in life expectancy and the search for better quality of life, these diseases are in the first line of research nowadays. Osteoporosis appears mainly in postmenopausal women and is characterized by the loss of bone mass which in turn is associated with

high risk of fractures in those patients [84]. Fractures in bones usually heal by themselves but in the elders or in the case of non-union fractures, that is not the case. Non-union fractures result in the loss of mechanical integrity in the skeleton.

Several nations worldwide designated the decade 2000-2010 as the Bone and Joint Decade. Some facts and figures help to illustrate the impact of bone-related diseases in US alone (from http://www.usbjd.org/about/index.cfm?pg=fast.cfm):

- Musculoskeletal conditions cost our society an estimated $254 billion every year
- One out of every 7 Americans reports a musculoskeletal impairment
- 28.6 million Americans incur a musculoskeletal injury every year
- More than half of all injuries are to the musculoskeletal system
- More than 43 million people have some form of arthritis
- It is estimated that the number of people affected by arthritis will increase to 60 million by 2020
- One out of every 2 women and 1 out of every 8 men above age 50 will have an osteoporosis-related fracture in their lifetime
- Back or spine impairments, which number 18.4 million, are the most prevalent musculoskeletal conditions for persons age 18 and older
- Sprains, dislocations and fractures account for nearly 65 percent of all musculoskeletal injuries
- More than 3 million hospitalizations are due to musculoskeletal conditions and injuries annually
- Each year, musculoskeletal conditions and injuries account for about 102.3 million visits to physicians , 10.2 million hospital outpatient visits, and 25 million emergency department visits
- Approximately 7.5 million musculoskeletal procedures are performed by physicians every year

It is clear from these facts that musculoskeletal related problems have a major impact on the health of individual patients as well as in the society.

Currently, when bone is lost or damaged, several options are available. Autologous transplantation of bone from another site is the golden standard in clinical scenarios mainly due to the absence of an immune response leading to the rejection of the graft. Nevertheless, the amount of bone that can be harvested and the donor-site morbidity associated with the collection of the tissue severely limits this option [85]. Allogeneic transplantation (bone from another human donor) is another possibility, but there is a risk of rejection in addition to the risk of disease transmission despite the stringent criteria applied by tissue banks in selecting, harvesting, and stocking bone segments.

Tissue engineering arose as an alternative to the current treatment options. Tissues are complex three-dimensional structures composed of different cell types contributing to the final function of an organ [86]. Due to its complexity, developing a biological substitute is not trivial. When doing so,

we should consider two important requirements of a tissue: mechanical and cellular integration. Mechanical integration with the host tissue is necessary as in most cases the loss of mechanical continuity needs to be addressed in first place. Cellular integration is necessary in order for the new cells to grow and to integrate in the neighbouring tissue so they can perform the biological functions necessary to restore the lost function.

Tissue engineering, as previously defined, gathers the knowledge of several distinct disciplines in order to restore the normal function of a tissue. Three key components contribute to tissue engineering applications: cells, scaffolds and signals (Figure 12). Although they can be, and sometimes are, used individually, it is believed that their combination brings additional benefits to the patient. In the next paragraphs I will briefly explain the three main components of the tissue engineering cycle.

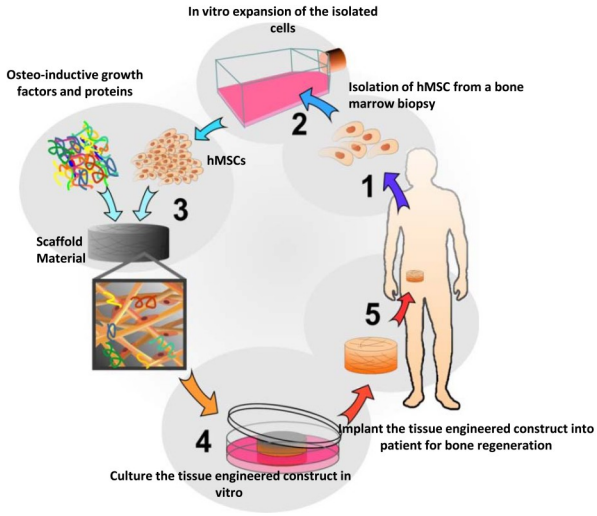

Figure 12 – The tissue engineering approach. The scheme represents the steps involved in cell-based tissue engineering approaches (adapted from [147])

Since their discovery, stem cells are an appealing cell source for tissue engineering applications due to their capacity to differentiate into different cell types of the body. Stem cells possess two basic characteristics: they are capable of self-renewal and simultaneously able to differentiate into different lineages. In the embryo, these cells appear at a specific location (the inner cell mass) early during development, and are responsible for the formation of the adult organism. These are denominated embryonic stem cells. Although pluripotent (the origin of all tissues in the body except the placenta and the umbilical cord) they are difficult to isolate and their use pose several ethical issues due to the need to destroy embryos to isolate them (Figure 13). The discovery of a population of cells in the adult organism with multilineage capacity opens a new door in the search for alternative cell sources [87].

Mesenchymal stem cells (MSCs) were isolated for the first time from bone marrow aspirates based on their ability to adhere to tissue culture plates [88]. Since then, MSCs have been isolated from many other sites, e.g. from the placenta, umbilical cord, adipose tissue and teeth, among others [89-92]. They are also referred to as colony forming unit-fibroblasts, marrow stromal fibroblasts, bone marrow derived fibroblasts, mesenchymal progenitor cells and bone marrow derived stromal cells, and they are able to differentiate into several different lineages including the chondrogenic, adipogenic, myogenic and osteogenic lineage(Figure 14) [93-98].

In the adult organism they are responsible for the maintenance of tissue homeostasis. In contrast with embryonic stem cells, adult stem cells have lower proliferation potential while maintain-

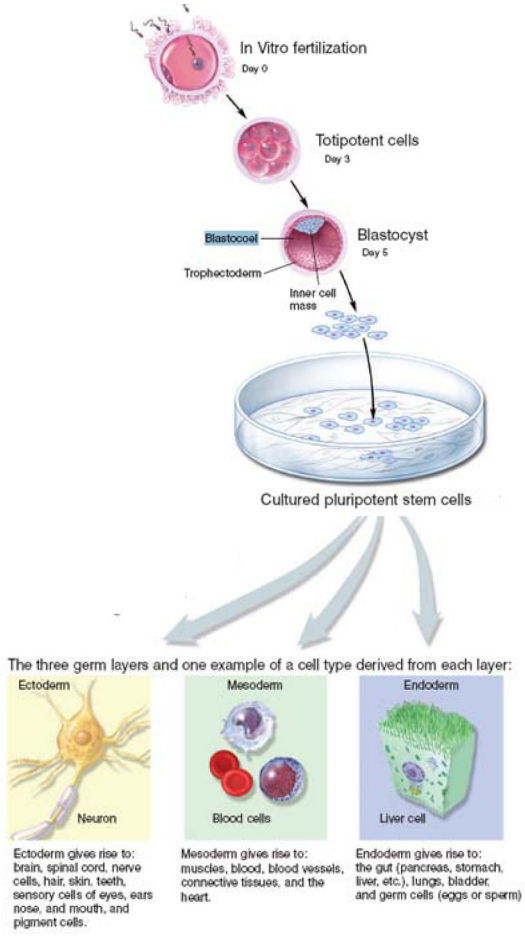

Figure 13 – Derivation of human embryonic stem cells. The scheme shows the origin of human embryonic stem cells and their pluripotency highlighted by the capacity to differentiation into cell types from the three germ layers (© 2006 Terese Winslow).

ing the stemness phenotype and, as default, they differentiate into cells found in the same germ layer from which they were isolated [99,100]. Nevertheless, there are reports showing they can also differentiate into cells from another germ layer conferring them the pluripotency characteristic of the embryonic stem cells [101-104]. More recently, an "intermediary" cell type arose: induced pluripotent stem cells (iPS cells). These cells are derived from somatic cells fully committed to a certain lineage. Using a specific cocktail of transcription factors they can be reprogrammed to an "embryonic state" overcoming the ethical problems associated with the use of embryonic stem cells [105].

Given the fact that the above mentioned cell types are capable of differentiation towards several lineages, the proper signals need to be delivered to drive the differentiation into the desired lineage. Several soluble factors like dexamethasone, cAMP, bone morphogenetic protein 2 (BMP-2) and vitamin D3 have the potential to induce MSCs into the osteogenic lineage by controlling gene activity [27]. Exposure of MSCs to these compounds is limited to the *in vitro* expansion phase because *in vivo* temporal and spatially controlled release of small molecules, although possible, is far from trivial [106-110]. One way to overcome this problem could be the genetic manipulation of the cells. By introducing a gene of interest in a certain cell type we can control the secretion of the respective protein transforming the cell into a "drug factory" [111-113]. Additionally, the discovery of RNAi allows us to control the expression of certain genes at a translational level and, as such, it represents a new tool to control the process of osteogenic differentiation [114]. Nevertheless, the discovery of new compounds is not easy and relies on a candidate approach or in the search for homology with compounds already described in the literature as osteogenic. With the advent of high-throughput screening technologies and commercially available libraries of compounds, there are increasing possibilities to find new compounds with osteogenic potential or other favourable characteristics for tissue engineering applications [115-119].

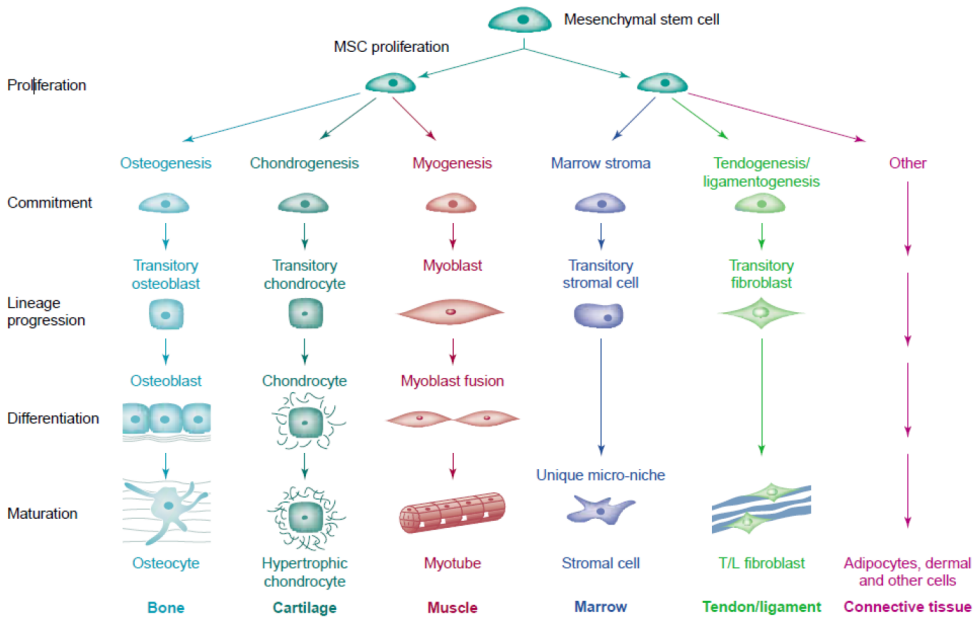

Figure 14 – Multilineage potential of mesenchymal stem cells. The scheme shows the general steps involved in the transition from a multipotent stem cell into a fully differentiated cell from different lineages (indicated below) (adapted from [99])

The final and congregating factor of the tissue engineering approach is the scaffold. Scaffolds can be divided in three groups depending on their origin: natural-derived, synthetic or hybrid scaffolds. Natural-derived scaffolds comprise scaffolds made of natural derived components. For example, decellularized matrices are a good example of this group of scaffolds. Synthetic scaffolds can be made of polymers, metals or ceramics and one of their main advantages (absent in natural scaffolds) is their reproducibility and the control over their mechanical and degradation properties. The third example is a combination of the previous two, consisting of synthetic scaffolds incorporating naturally derived components and vice-versa. To be used in tissue engineering a scaffold has to comply with some basic requisites: it should be biocompatible, meaning that upon implantation it should not trigger an inflammatory response, and it should be biodegradable, meaning that the newly formed tissue should replace the scaffold over time. The main function of a scaffold is to provide mechanical stability and to serve as a carrier either for cells or biomolecules. Moreover, the scaffold can bear instructive properties and control the differentiation by itself. A lot of research is going on nowadays in order to find the "perfect" scaffold for tissue engineering applications but most likely we are still far from "perfection" and several possibilities still need to be explored (supplementary data) [120-123].

In native tissues, the role of the scaffold is usually taken by ECM that surrounds almost every tissue in the body. ECM is secreted by the cells and not only confers mechanical stability to the tissues but also works as a growth factor reservoir due to its capacity to bind several different molecules. As such, a cell surrounded by an ECM containing growth factors can be considered the Nature's best scaffold [61, 124]. The instructive properties of ECM and the possibility to use ECM proteins

as scaffolds may be an interesting tool to conduct the differentiation of uncommitted cells into the osteogenic lineage. The use of ECM-inspired scaffolds for tissue engineering applications will be discussed elsewhere in this thesis.

Despite the fact that a combination of cells, signals and scaffolds can enhance the repair of damaged tissues, there are still concerns with this approach. Ideally, a tissue engineering solution should be efficient whilst cost-effective. However, the use of cells presents no clear increase in effectiveness and can dramatically increase the costs due to the need of clean-rooms to culture the cells and the need of a two step procedure to collect and later re-implant the cells into the patient. One way to overcome this is by designing scaffolds that, once implanted, can trigger a biological response overcoming the need to deliver bioactive molecules, or even cells, within the scaffold. For example, the differentiation of uncommitted cells into different lineages can be achieved either by controlling the elasticity of the matrix or by controlling the cell shape using fibronectin islands which limit the spreading of the cell (Figure 15) [55, 125]. Lessons from these studies can be used to design tailor-made scaffolds capable of controlling the cell fate either by spatially confining the cells or by controlling the stiffness of the scaffold.

Due to its role in the body and especially in bone, collagen has been used as a scaffold for a long time [126]. Animal-derived and recombinant collagen, decellularized scaffolds composed of collagen, electrospun fibers and hydrogels are some of the examples of collagen-based scaffolds used in tissue engineering approaches. Moreover, small peptides based on collagen sequences have been used as well as collagen motifs to drive the differentiation of cells into the osteogenic lineage [127-129]. Nevertheless, issues such as denaturation of collagen due to processing techniques, poor mechanical properties, risk of disease transmission and batch variation limit the use of collagen and highlight the need to find synthetic substitutes able to induce osteogenic differentiation.

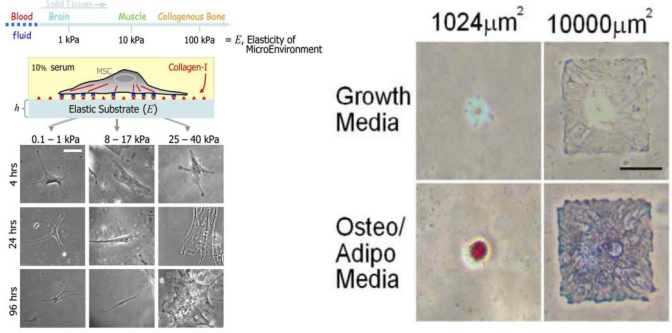

Figure 15 – Matrix stiffness and cell shape can control stem cell fate. The figure on the left shows the differentiation of mesenchymal stem cells into different lineages upon seeding on an elastic substrate with controllable stiffness. The figure on the right shows how cell shape can control the fate of an uncommitted cell. Fibronectin islands of different sizes were prepared and cells seeded on top. By exposing the cells to a mixed medium (supporting both differentiation into osteoblasts or adipocytes) it was possible to analyze the effect of cell shape into lineage commitment. Lipid stain red and ALP stain blue (adapted from [55] and [125]).

Osteoinduction

The ectopic formation of mineralized tissue upon injuries, surgeries or even in blood vessels is an undesirable phenomenon. Nevertheless, the mechanism by which bone appears in places where it usually does not exist is of extreme importance to screen functional cell-scaffold combinations for bone tissue engineering.

Urist made a seminal discovery in 1965 that changed the field of bone biology. He found that demineralised bone matrix (DBM) was able to induce bone formation ectopically upon being processed with different compounds [130]. Later, the protein responsible for this was discovered and designated bone morphogenetic protein [131]. It took 20 more years to clone BMPs, making possible their use in research [132]. Since then, no other protein has been so extensively used or studied in the field of bone biology.

BMPs belong to the transforming growth factor – β (TGF-β) superfamily of growth factors. They bind to a heterodimeric complex of transmembrane receptors termed type I and type II, which have serine-threonine kinase activity. Upon ligand binding, the type II receptor phosphorylates the type I receptor thus activating the type I kinase. This kinase phosphorylates members of the mothers against decapentaplegic (Smad) family of transcription factors, which will translocate into the nucleus to activate BMP target genes. This pathway is designated the canonical BMP signaling pathway [27, 133, 134].

To date, more than 20 different BMPs have been identified and implicated in several developmental processes and functions in the adult body such as bone remodeling, wound healing, haematopoiesis, immune response and neuronal plasticity among others [133, 135]. Because BMPs generally act as morphogens, their activity is tightly regulated at different levels in the signaling cascade. The ability of BMP binding to its receptor is controlled by several structurally different antagonists [136], such as noggin, gremlin, follistatin, sclerostin and chordin and a bone phenotype has been associated with some of these genes. For example, fibrodysplasia ossificans is a rare genetic disease characterized by congenital malformations of the big toes and by progressive heterotopic bone formation (Figure 16) [137].

BMPs are nowadays broadly used in the clinic due to their potential to induce bone formation. Nevertheless, supra-physiological doses are necessary in order to achieve bone formation. Moreover, their ability to induce bone formation combined with the high doses needed and the difficulty of releasing active BMP in a temporal and spatial way presents a big challenge for tissue engineers. Serendipity came to help us. Four years after the pioneering work of Urist describing osteoinduction by DBM, Winter reported a similar occurrence using a sponge of polyhydroxyethylmethacrylate (poly-HEMA) usually used for breast augmentation [138]. This was the first description of a material able to induce bone formation by itself. The Holy Grail of bone tissue engineered seemed to be not far: a material that was able to induce bone formation ectopically without any biological component. From those days till today several materials have shown osteoinductive potential and one particular class more often than others - calcium phosphate ceramics. Although many years passed since the first occurrence of osteoinduction by a synthetic material, the mechanism still remains unknown. It was suggested that BMPs are involved in this phenomena [139]. Nevertheless, some points against this possibility arise: while bone formation by BMPs is mainly endochondral (depending on the carrier intramembranous ossification can also occur), osteoinduction by biomaterials is mainly intramembranous as a cartilage template was never observed [140]. Moreover, bone formation occurs in the pores of material and if BMPs would be involved we would expect to see bone formation not only inside but also in the surrounding tissue. Therefore, it seems evident that the material chemistry and structure has a key role in osteoinduction. For example, it has been

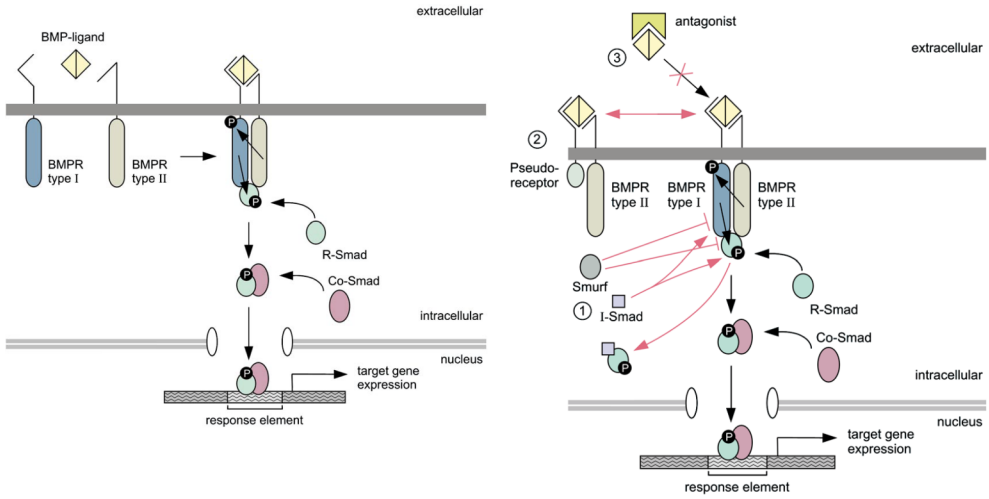

Figure 16 – Regulation of BMP signaling. The scheme shows the canonical BMP-signaling and its key players. On the left we can see the sequence of events from BMP binding to its receptors to activation of target genes which is mediated via Smad signaling. On the right we show three levels of modulation of BMP signaling: in the first case inhibitory Smads or smurf can regulate intracellular signaling, in the second case pseudo-receptors can modulate binding to the receptor and in the third case extracellular antagonists can sequester BMP, inhibiting binding to the receptor (adapted from [146])

shown that, depending on the sintering temperature, two synthetic materials with similar chemistry can have different effects, one being more osteoinductive than the other (Figure 17) [141]. Parameters such as chemistry, surface reactivity, surface area, micro and macrostructure have been shown to be involved in the process of osteoinduction.

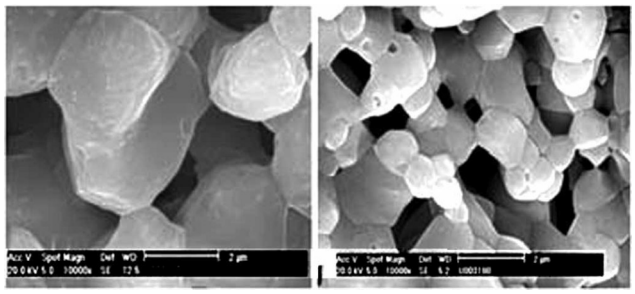

Figure 17 – Osteoinductive scaffolds. The figure shows two calcium phosphate ceramics with distinct osteoinductive potential. Both have similar chemical composition but show differences in the microporosity because of different sintering temperatures. The one on the left was sintered at 1300°C and the one on the right at 1150°C (Courtesy Dr. Habibovic)

Aims and outline of this thesis

The work presented in this thesis comprises a multidisciplinary approach to bone tissue engineering. My aim was to analyze the role of ECM in osteogenic differentiation and to use its instructive properties for cell-scaffold based tissue engineering applications. In the first part I analyze the role of ECM in the differentiation of MSCs. Moreover, the involvement of a BMP antagonist in the proliferation and differentiation of MSCs was studied. Besides studying the differentiation of cells and the role of soluble factors involved in BMP signaling, I also studied the last component of the tissue engineering cycle: scaffolds. Chapter 1 gives an overview of the field of tissue engineering focusing on bone tissue engineering and the key players. Chapter 2 highlights the role of ECM in tissue engineering with special emphasis on the lessons learned from nature and the steps taken so far to extrapolate the knowledge gathered into the development of new materials. Chapter 3 analyzes the role of collagen in osteogenic differentiation of human mesenchymal stem cells and discusses the major role it plays in this process, both *in vitro* as well as *in vivo.* Chapter 4 highlights the role of collagen crosslinking on the differentiation of a cell line commonly used in tissue engineering (MC3T3-E1 cells) and the clinically relevant human mesenchymal stem cells. Chapter 5 describes the development of macroporous scaffolds incorporating crosslinked or uncrosslinked collagen. The *in vivo* potential of these scaffolds and the response of rat and human MSCs were analyzed. Chapter 6 analyzes the effect of a BMP antagonist – chordin-like 1 - on proliferation and differentiation of human MSCs and found a new role by which a BMP antagonist can influence human MSCs biology. In chapter 7 the discovery of an osteoinductive scaffold with the potential to induce *de novo* bone formation in a critical size defect comparable to autologous bone or BMP-2 is described. Chapter 8 includes my discussion based on the results of this thesis and my final conclusions and recommendations for the future use of ECM in bone tissue engineering.

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Supplementary data - overview of materials currently used for musculoskeletal repair in Europe and US (adapted from [142])

Composition	Type	Origin	Clinical applications	Properties
Calcium phosphate, i.e. hydroxapatite; tricalcium phosphate; octacalcium phosphate	Ceramic	Synthetic	Bone regeneration, non-loading sites, bone void filler (cements, granules, coatings)	Bone bonding (bioactivity), biodegradable, tunability of degradation
Silica-based calcium phosphate	Glass ceramics	Synthetic	Bone regeneration, non-loading sites, bone void filler (granules, coatings)	Bone bonding (bioactivity), Biodegradable
Alumina	Ceramic	Synthetic	Joint replacement (knee, shoulder)	Highest tensile strength, resistance to fatigue, non-bone bonding, lubricating capacity
Titanium and alloys	Metal	Synthetic	Bone replacement, load-bearing sites, hip or dental prosthesis, spinal cages	Bone bonding (bioactivity) in some cases, non-corrosive, resistance to fatigue, high specific strength, low elasticity modulus
Stainless steel Cobalt chrome alloys	Metal	Synthetic	Bone replacement Load-bearing sites, hip or dental prosthesis, spinal cages, fixations	Corrosive to long term
Polymethylmethacrylate	Polymer	Synthetic	Bone replacement, load-bearing sites, bone void filler (cement) fixation of hip prostheses, vertebroplasty	Non-degradable
Polyesthers, i.e. poly lactide, poly glycolic acid, poly caprolactone, poly (urethane)	Polymer	Synthetic	Degradable bone fixation, soft tissue suture, bone void filler, soft tissue regeneration*, drug delivery*	Tunability by varying molecular weight of degradation and mechanical properties
Ultra high molecular weight poly ethylene	Polymer	Synthetic	Articulating component for orthopaedic prosthesis, load bearing sites	Lubricating capacity
Poly poly ethylene oxide terephtalate co butylene terephtalate	Co-Polymer	Synthetic	Cement stopper, bone void filler, soft tissue regeneration*, drug delivery*	Tunability by varying molecular weight of degradation and mechanical properties, bioactivity
Polyphosphazene	Polymer	Synthetic	Drug delivery*	Erosion degradation mechanism favorable to long term stability of the implant
Polyanhydride	Polymer	Synthetic	Drug delivery, hard and soft tissue repair*	Erosion degradation mechanism favorable to long term stability of the implant
Poly ortho esters	Polymer	Synthetic	Food additive, drug delivery*, hard and soft tissue repair*	
Poly ethylene glycol	Polymer	Synthetic	Drug and cosmetic excipient, hard and soft tissue repair*	Injectable water gel, degradable
Coral	Mineral	Natural (sea)	Bone void filler	High interconnectivity, degradable
Bone	Composite mineral/proteins	Natural (human, bovine)	Bone void filler	Similar composition as the host bone
Demineralized bone matrix	Proteins	Natural (human)	Bone void filler, cartilage regeneration*	Biodegradable, natural source of osteoinductive proteins (BMPs)
Collagen	Protein	Natural (bovine)	Hard and soft tissue repair	Biodegradable
Hyaluronic acid	Polysaccharide	Natural (human)	Soft tissues repair	Biodegradable, injectable hydrogel, naturally abundant in joint synovial fluids
Alginate	Polysaccharide	Natural (algae)	Soft tissue repair	Drug load, degradable
Agarose	Polysaccharide	Natural (algae)	Soft tissue repair*	Drug load, Degradable
Chitosan	Polysaccharide	Natural (sea crustaceans)	Soft tissue repair	Structurally similar to glycosaminoglycans (cartilage proteins)
Fibrin	Protein	Natural (human)	Soft tissue healing, bone void filler	Sealing capacity

(*) Indicates potential future clinical applications based on preclinical data.

Chapter 2

EXTRACELLULAR MATRIX AND TISSUE ENGINEERING APPLICATIONS

"Logic will get you from A to B - Imagination will take you everywhere"

Albert Einstein

Extracellular matrix and tissue engineering applications

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Abstract

Extracellular matrix is a key component during regeneration and maintenance of tissues and organs, and it therefore plays a critical role in successful tissue engineering as well. Tissue engineers should recognize that engineering technology can be deduced from natural repair processes. Due to advances in such distinct areas as biology, engineering, physics and chemistry and the possibility of using robotics to facilitate the search for new treatments, we can identify the basic principles and extrapolate them into tools to mimic the regenerative process. Ubiquitously distributed throughout the body, the extracellular matrix surrounding the cells plays a key instructive role, in addition to the previously recognized supportive role. In this review we will highlight the role of the extracellular matrix and discuss the latest technological possibilities to exploit the extracellular matrix in tissue engineering.

Extracellular matrix and its properties

"Tissue engineering is 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" [1]. These substitutes usually comprise a three dimensional (3D) scaffold providing support to cells, and growth factors to direct the differentiation of those cells.

In our body, the cell's direct environment is composed of an intricate 3D network of fibrillar proteins, proteoglycans and glycosaminoglycans (GAGs), collectively termed extracellular matrix (ECM, Figure 1A). It is the combination of cells and ECM which define the tissues in our body. For example, tissues like cartilage and bone are mainly constituted by ECM. In the first case, chondrocytes are entrapped in a highly hydrated ECM, whereas in the case of bone, ECM is highly mineralized conferring the rigidity typical of that tissue. In addition, collagen proteins secreted by cells in ligaments and tendons align along the long axis of the tissue, thus conferring resistance to load and strain. In contrast, collagen fibers in the intestine are arranged in a spiral allowing contractions that will guide the bolus through.

For a long time considered a static entity providing only support to the tissues, we now know that ECM also plays a critical role in cell signaling and tissue homeostasis, i.e., in maintaining a balance between anabolic and catabolic activities whereas the turnover of a tissue is replaced by new ECM and cellular milieu [2]. ECM acts as a sensor, conveying information from the exterior of the cell to the inside and vice-versa. Cells are exposed to a myriad of different forces and the balance between internal and external forces will elicit a cellular response (Figure 1B) [3,4]. This phenomenon is easily visualized if we think of a tennis player: the bones in the arm that the player uses the most are usually thicker than the ones in the other arm. This means, that the external stimulus (hitting the ball) triggers a change in the inside (bone growth). Somehow, ECM provides the bone forming

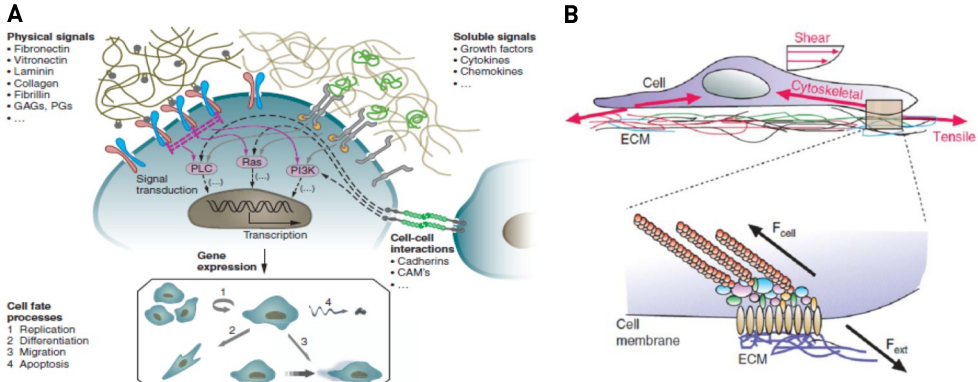

Figure 1 – A. Overview of the cell microenvironment. The cell's surrounding is composed of a highly hydrated environment containing physical and soluble signals, which can control signalling and activation of certain target genes. The activation of these genes will control the phenotype of the cell (Reprinted with permission from [17], ©2005 Nature Publishing Group). B. Schematic representation of a cell and the forces applied to it. Note that ECM plays a key role in force transmission to and from the cell (Reprinted with permission from [3]).

cells with a signal (so-called outside–in signaling) and the cell will respond accordingly by changing its gene expression profile. The external signals perceived by a cell can be as different as shear stress due to fluid flow, tensile forces via binding to ECM with different molecular composition resulting in differences in stiffness, surface topography or cytoskeletally generated forces[3,5]. The way cells sense and respond to the stimulus provided by ECM is mainly via membrane receptors called integrins and/or mechanosensitive ion channels (for a comprehensive review see references [6,7]).

The critical role of ECM in tissue formation and homeostasis is unveiled by mouse mutants for certain ECM proteins as well as some human disorders [8]. Diseases like osteogenesis imperfecta (OI), Ehlers-Danlos (ED) syndrome and epidermolysis bullosa (EB) are caused by mutations in genes encoding structural proteins such as collagen, resulting in a range of symptoms including skin blisters and erosions in the case of EB, to fatal outcomes such as disruption of blood vessels in ED syndrome [9,10].

Examples describing crosstalk between ECM and cells are reported in the literature. For example, it has been shown that during skin wound healing, a fibrin matrix is formed in the wound bed serving as a scaffold, allowing migration and proliferation of dermal fibroblasts [11] (Figure 2A). Another example of cell-ECM crosstalk is matrix elasticity, which can control the differentiation of mesenchymal stem cells (MSCs) into different lineages. Soft matrices mimicking brain tissue are neurogenic, stiffer matrices mimicking muscle are myogenic and rigid matrices mimicking bone are osteogenic, indicating that ECM properties can guide gene expression and control cell fate [12-15] (Figure 2B).

Overall, due to its 3D architecture, its mechanical properties and signaling potential, ECM is an interesting candidate for the material scientist looking for appropriate 3D scaffolds with favorable biological properties [16,17].

In this review we will first describe the composition of natural ECM and applications in tissue engineering based on natural ECM-derived materials. We will show the state-of-the-art of technologies aiming at substituting natural ECM and in the end we will identify the challenges for the future.

Natural ECM – the nature's best scaffold?

Leonardo da Vinci was one of the firsts to comprehend that understanding nature is a prerequisite to engineer solutions. Centuries later, tissue engineers striving to mimic ECM have to analyze and study natural ECM prior to designing scaffolds with similar characteristics.

The first mission is to identify the components of ECM. An obvious choice is to start with the most abundant protein: collagen. More than 20 genetically different types of collagen have been identified so far. Collagen molecules consist of three polypeptide α chains, each of them containing at least one repeating Gly-X-Y sequence, where X and Y are usually proline and hydroxyproline, respectively [18,19]. The three chains are supercoiled around a central axis in a right-handed manner to form a triple helix. Collagen molecules self-assemble into collagen fibrils, which form the collagen fibers after crosslinking. During the biosynthesis of collagen, the molecule undergoes several post-translational modifications, i.e. hydroxylation and glycosilation of particular residues. Depending on their structure and supra-molecular organization, collagens can be classified into fibrillar

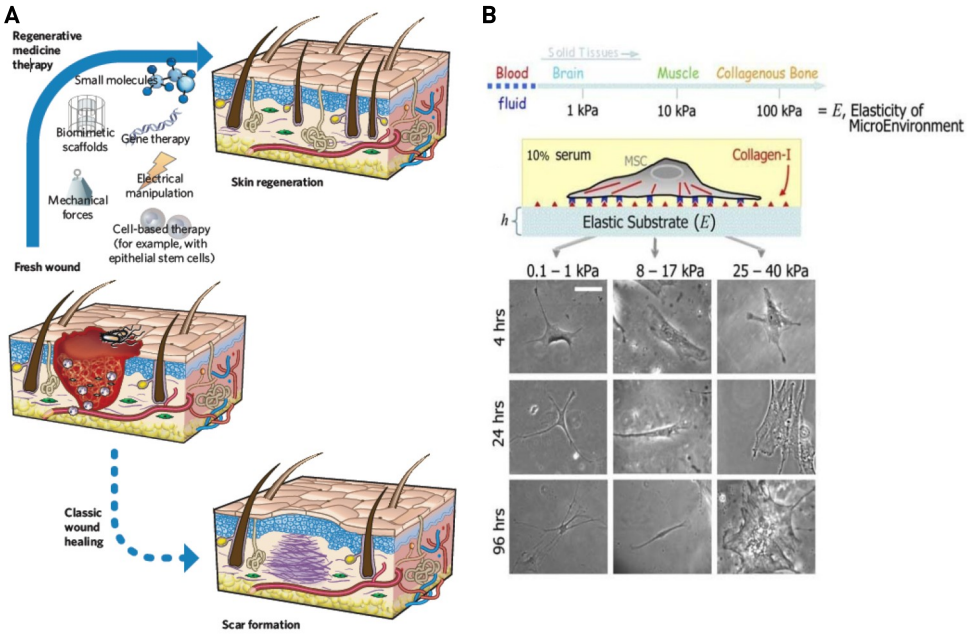

Figure 2 – A. Classical wound healing process. When skin is damaged, new tissue formation occurs followed by remodelling. Initially a scar is formed at the surface of the wound and new blood vessels appear. Fibroblasts migrate to the area and deposit a new disorganized collagen matrix which will be remodelled in a later stage. Alternatively, regenerative medicine provide us with tools to interfere with the classic healing process in order to avoid scar tissue formation and to recreate the original tissue with the same structure and function as the damaged tissue (reprinted with permission from [11], ©2008 Nature Publishing Group). B. Matrix stiffness influences the differentiation of human mesenchymal stem cells. By controlling the level of crosslinking the stiffness of the gel can be adjusted to match that of ECM of the desired tissue. Depicted in the figure are the differences in cell morphology, during time, for cells seeded on matrices with different stiffness. Note that for the stiffness typical of the brain (E. 0.1-1 kPa) cells present a branched neuron-like morphology, in the stiffness of the muscle (E. 8-17 kPa) cells showed an elongated morphology typical of myoblasts and on the stiffness of the bone (E. 25-40 kPa) cells showed a spread and cuboidal morphology typical of differentiated osteoblasts (Reprinted and adapted with permission from [13], ©2006 Elsevier).

(accounting for 90% of all collagens) and non-fibrillar collagens. For example, fibrillar collagens provide torsional stability and tensile strength and can be found in tissues such as bone, cartilage, or skin. In contrast, basement membrane collagens such as collagen type IV are more flexible giving the basement membrane its typical characteristic [18-22]. In general, collagens are mainly seen as structural proteins although they contain small sequences responsible for binding to cellular receptors.

Elastin is a protein which can be found in ECM of tissues that have the ability of transiently stretching such as skin, oesophagus, lungs or blood vessels. Tropoelastin is the soluble precursor of elastin which, upon secretion to the extracellular space, can be stabilized by covalent crosslinking between the side chains of lysine, resulting in massive macro-arrays of mature elastin. Due to the extensive crosslinking there is a decrease in the solubility. The elastic properties of elastin have been attributed to the conformational entropy between the non-polar peptide sequences and lysine sequences which are extensively crosslinked [23-26]. In short, collagen and elastin may be considered as the bricks of ECM due to their contribution to the mechanical properties of ECM.

Another mode of action can be seen with the GAGs, which contribute to the gel-like characteristics of ECM. GAGs are long unbranched carbohydrated polymers consisting of repeating disac-

charide units. These units are composed by one of two modified sugars – N-acetylgalactosamine or N-acetylglucosamine. They are responsible for growth factor sequestration and, due to their ability to retain water, they contribute to the characteristic appearance of ECM. When hydrated, GAGs are responsible for increase in tissue stiffness as they act as water pumps under mechanical loads. The reason for this can be due to water molecules binding to GAGs anionic groups as previously proposed [27]. In articular cartilage, this results in an osmotic pressure of 0.1-0.2 MPa that accounts for approximately 50% of static mechanical stiffness under compression [28]. Chondroitin sulphate A and B, heparin, heparin sulphate and hyaluronic acid are among some of the GAGs that can be found in ECM [29-31]. Chondroitin sulphate and hyaluronic acid contribute to frictional resistance against interstitial fluid flow. They are applied in cartilage tissue engineering as natural components of hydrogel -like scaffolds, because they can promote chondrocyte redifferentiation [32-35]. An extensive review of proteglycans is beyond the scope of this review and can be found elsewhere[36]. Cell attachment is another important role of ECM in many tissues. As such, some ECM proteins can be considered as the glue of ECM. Two of the most common proteins responsible for cell adhesion are fibronectin and laminin. Fibronectin (FN) is the second most abundant protein in ECM, where it is organized into a fibrillar network. It is a large glycoprotein dimer and each monomer contains three types of repeating units designated type I, II and III. In these units we can find functional domains responsible for interaction with cell surface receptors and with fibronectin itself. It can be found in two forms: soluble (in the blood plasma) or insoluble (present in ECM). Fibronectin is rich in Arg-Gly-Asp (RGD), a tripeptide important in cell adhesion via the $\alpha 5\beta 1$ integrins as well as in cell growth. Plasma fibronectin is involved in wound repair contributing to the formation of a provisory matrix whereas cellular fibronectin is incorporated into the fibrillar matrix secreted by the cell [37-42]. In addition to the RGD sequence other important sequences can be found in FN. For example, coating of materials with the FN fragment $FNIII_{7-10}$ or with the collagen mimetic peptide GFOGER enhanced osteoblastic differentiation in bone marrow stromal cells compared to uncoated materials [43, 44].

Laminin is a complex adhesion molecule especially found in the basement membrane of almost every tissue. It is composed of α, β and γ subunits arranged to form a large coiled-coil quaternary structure consisting of three short arms and one long arm [38, 45]. It has globular domains at the end of its arms which mediate the interaction with other molecules. Laminin has been involved in cell differentiation, migration and proliferation and plays a critical role in angiogenesis [46-48].

Fibrin is a fibrillar protein that plays a key role in the process of wound healing. It is formed by polymerization of fibrinogen in the presence of thrombin and subsequently undergoes crosslinking mediated via transglutaminases contributing to the clot formation during wound healing [49-51]. Fibrin matrices are currently used in the clinics and they can be used as drug delivery systems for proteins such as bone morphogenetic proteins, incorporation of bioactive peptides or as cell delivery systems [52-56].

Use of natural derived ECM proteins in regenerative medicine

All these ECM proteins have a common origin: the cell. Thus, the easiest way to obtain these proteins for tissue engineering applications is to allow cells to produce them and, after ECM deposition, to remove the cells. This is usually accomplished using detergents or other methods that will leave a decellularized matrix with most proteins in the native state [57].

The use of decellularized matrix has revealed a pivotal role of ECM on cell fate. For example, laminin is the major component of an ECM-derivative widely used in tissue engineering – Matrigel®. Matrigel® is a solubilized basement membrane preparation derived from a mouse sarcoma cell line, a tumor rich in ECM proteins. Matrigel® is able to mediate endothelial differentiation and it is also used in invasion assays to analyze tumour progression. When human umbilical vein endothelial cells (HUVECs) are seeded on Matrigel® they adopt a tube-like structure, characteristic of the first steps of vessel formation, in contrast to HUVECs seeded in normal tissue culture plates [58].

An elegant example where ECM influenced cell fate came from a study using ECM derived from embryonic stem cells. Cancerous pigment cells seeded onto ECM deposited by embryonic stem cells changed their gene expression profile from one typical of cancer cells to one resembling normal pigment cells. This exemplifies the ability of ECM to reprogram cell fate [59-61]. Similarly, bone marrow derived mesenchymal stem cells grown on murine decellularized ECM from bone marrow cells, showed increased proliferation compared to normal culture. *In vivo*, mesenchymal stem cells (MSCs) expanded on ECM displayed enhanced bone and bone marrow formation [62].

Lessons from basic science are already being applied in tissue engineering. For example, a bioartificial heart was generated by seeding cardiac and endothelial cells on ECM of a decellularized heart [63] (Figure 3). Another application has been coating of titanium with ECM prior to seeding of MSCs. Titanium meshes were coated with MSCs and decellularized after the cells had formed a

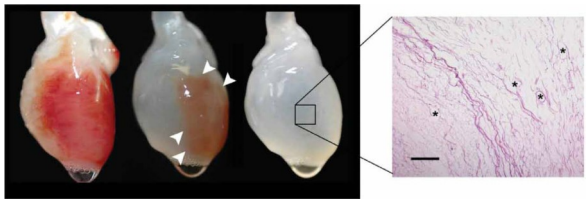

Figure 3 – Decellularization of whole rat heart. The figure shows the sequential process of decellularization upon perfusion of the heart with a detergent. The inset shows that no intact cells or nuclei are present after the process and that vasculature conduits were preserved during the decellularization (asterisks) (Reprinted with permission from [63], ©2008 Nature Publishing Group).

confluent layer and had deposited ECM on the metal. Next, the material was re-seeded with MSCs and ECM-functionalized scaffolds showed enhanced calcium deposition compared to uncoated titanium [64].

There is increasing evidence that ECM proteins also play an important role in stem cell niches. Stem cell niches are anatomical structures, including cellular and acellular components, which integrate local and systemic factors to regulate stem cell proliferation, differentiation, survival and localization. They are responsible to maintain the self-renewal potential of stem cells and, at the same time, replenish the body with differentiated cells when necessary [65]. A critical role of ECM proteins in the stem cell niche has been demonstrated in the case of tendon tissue. Depletion of biglycan and fibromodulin, two ECM proteins, affected the differentiation of tendon stem/progenitor cells, thus impairing tendon formation *in vivo* [66].

Some ECM-derivatives have found their way to the clinic. Acellular human dermal matrices generated by freeze-drying are used to treat skin wounds [67]. Another product commonly used in the clinic as a bone graft substitute is demineralized bone matrix (DBM). DBM is obtained by acid extraction of the mineralized component of bone, while maintaining the organic component comprised of collagen and non-collagenous proteins. Among these proteins, an important role in DBM osteoinductivity is played by the presence of bone morphogenetic proteins (BMPs), which contribute to the differentiation of local MSCs into the osteogenic lineage [68-70].

Plagiarism allowed. Scientists attempt to copy nature.

An important strategy of tissue engineering is to enhance the natural regenerative capacity of the adult human body. It has been a long way from the stage of a single cell to the multicellular organism we are today. Tissue engineers cannot expect to mimic the complexity of a multicellular organism using a single technique. Rational combination of techniques will allow us to walk on a road that nature followed for thousands of years and, by analyzing ECM that constitute this multicellular organism, we can try to copy nature's best scaffold.

While designing ECM-inspired tissue engineering grafts one needs to take in consideration the advantages and limitations of the available techniques, such as decellularization of natural tissues, hydrogel polymerization and electrospinning. Such techniques will influence many properties such as mechanical, adhesion/signalling, architecture and remodeling. Implications on the above mentioned techniques and on their bias on the resulting ECM-inspired scaffolds are discussed hereafter.

Mechanical properties

To mimic the mechanical properties of ECM, three biomaterials/techniques are commonly used: hydrogels, electrospinning and rapid prototyping. Due to their capacity to swell and entrap water, hydrogels are considered as a logical choice to mimic hydrated ECM. In the presence of a crosslinking agent, these biomaterials form a crosslinked fibrillar network, at micro and nano scale dimensions, when dissolved in an aqueous medium. Cells can be encapsulated in the liquid form of the biomaterial and entrapped in the 3D fibrillar network during the crosslinking reaction (Figure 4A and 4B). Increasing the percentage of the material dissolved results in hydrogels characterized by a network with increased stiffness but decreased pore size and nutrient diffusion properties [71, 72]. Therefore, when designing hydrogel scaffolds for tissue engineering it is critical to maintain a balance between mechanical and diffusion properties to allow scaffold integrity and cell viability during tissue development.

Hydrogels that found applications in tissue engineering comprise natural and synthetic polymers, or combinations thereof forming semi-interpenetrating networks. Among natural polymers, fibrin glue is successfully used in the clinics as a wound repair scaffold [73, 74]. Synthetic hydrogels are also considered, because their non-toxicity (biocompatibility) and bulk properties can be controlled during synthesis. Photopolymerizable hydrogels like poly(ethylene glycol-diacrylate) (PEGDA) have found promising applications in cartilage regeneration (Figure 4C) [75, 76]. Thermosensitive hydrogels such as poly(N-isopropylacrylamide) (PNIPAAm) and Pluronic® (polyethylene oxide –

polypropylene oxide – polyethylene oxide block copolymers) are also interesting biomaterials for connective tissues, myocardic and bone tissue engineering [77-79].

Electrospinning of polymers is gaining considerable attention because it allows the deposition of fibers with a nano to micrometer resolution, which resembles ECM fibrillar composition [80, 81]. These fibers can be produced using natural ECM proteins or synthetic polymers [82-88]. By varying the processing parameters, it is possible to achieve fibers with variable architecture and surface topology, which are known to influence cell adhesion [89-91]. Interestingly, when scaffolds are comprised of micro (∅ ~ 2-15 μm) and nanofibers (∅ ~ 100-900 nm), cell-material interactions are enhanced and synthetic polymers gain further instructive properties for tissue regeneration compared to the same polymeric scaffolds made of larger pore diameter (> 100 μm) [91-93]. Thus, micro- and nano-scaled fibers may acquire some of the natural ECM functions.

Hydrogels and electrospun micro and nano fibrous scaffolds can mimic the physicochemical properties and the topographical and structural features of ECM, but unfortunately do not match in most cases the mechanical properties of the tissues to be regenerated. To do so, rapid prototyping fabrication technologies might be a good alternative. It has been shown that with these techniques it is possible to generate scaffolds with modulable physicochemical and mechanical properties while maintaining a completely interconnected pore network for cell migration and nutrient diffusion [91, 94-98]. These 3D structures can be optimized to match the mechanical properties of a number of soft and hard tissues like meniscal and articular cartilage [96]. However, rapid prototyping techniques are limited by the resolution of the main struts composing 3D scaffolds, which is confined to the order of hundreds of micrometers. As ECM has physical features well below this limit, ranging from the micron to the nano scale, perhaps the best solution to fabricate ECM-inspired scaffolds passes through the combination of the above mentioned biomaterials and techniques to create a structure with matching physicochemical and mechanical properties. Theoretical modeling elegantly disclosed properties of collagen molecules which can be crucial for the design of new materials. When the complexity of a biological system can be introduced in such models, scientist can gain insights on the mechanical properties which, for the time being, are only partially understood [22].

Adhesion/signaling properties

When designing new materials, tissue engineers should achieve biofunctionalization of those materials in order to improve cell adhesion and, at the same time, provide biological cues able to recruit cells and control their activity. For example, peptides derived from two ECM proteins (laminin and N-cadherin) were incorporated in fibrin and tested for their potential application in nerve regeneration. Mixing these peptides with fibrin resulted in an 85% increase in the number of regenerated axons when compared with unmodified fibrin [52]. Similarly, laminin-derived recognition sequences such as IKLLI or IKVAV preserved viability, reduced apoptosis and increased insulin secretion of β-cells [99]. Modification of the PEGDA backbone with RGD peptides, the peptide motif responsible for binding to the integrin family of adhesion receptors, was essential to achieve embryonic stem cell adhesion and their subsequent differentiation into the chondrogenic lineage [100]. In another example, a collagen mimetic peptide was used to endow biomaterials with properties

to attract growth factors, such as vascular endothelial growth factor (VEGF). This resulted in improved morphological features of endothelial cells, indicative of tubulogenesis [101].

A key issue in improving the biological properties of biomaterials is the density at which the biological moieties are presented to the cells. An important advance in this area has been achieved by using molecular self-assembly. For instance, functionalized synthetic amphiphilic peptides have been used to support differentiation of neurons, mineralization of bone hydroxyapatite, enamel formation and regeneration and adhesion of bladder smooth muscle cells to branch-peptide-amphiphile functionalized scaffolds, which open a new window of possibilities for tissue engineering [102-110]. Amphiphilic peptides possess a hydrophobic and hydrophilic region. Functional groups such as the RGD peptide can be incorporated in the molecule to provide control over biological functions (Figure 4D) [102, 104, 105, 111-115]. It has been shown that tethered small molecules could directly influence the cell fate of human mesenchymal stem cells (hMSCs), with charged phosphate groups leading to osteogenesis and hydrophobic *t*-butyl groups inducing adipogenesis [116]. Another impor-

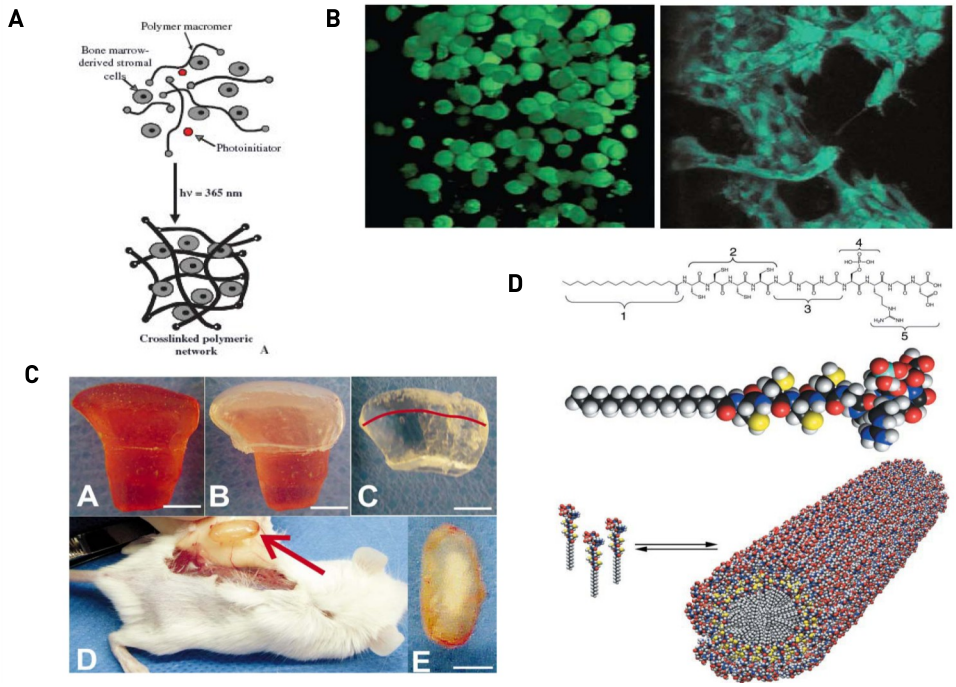

Figure 4 – A. Schematic diagram of the photoencapsulation process of cells in a hydrogel. Cells, a polymer macromer and a photoinitiator are combined and exposed to light in order to form a crosslinked polymeric network containing cells surrounded by a highly hydrated network (Reproduced with permission from [136], ©2005 Wiley-VCH). B. Changes in the morphology of human mesenchymal stem cells encapsulated in a functionalized degradable PEG gel. First, cells present a round morphology which will change to a more spread and tissue-like structure during time due to interactions between the cells and the hydrogel (From [137]. Reprinted with permission from AAAS). C. Example of a tissue engineered hydrogel with a pre-defined shape and spatial separation of cell types. The hydrogel was designed to mimic the shape of the articular condyle and, in a two step process, mesenchymal stem cells committed either into the chondrogenic or in the osteogenic lineage were encapsulated. Upon implantation in an immunodeficient mouse the construct was recovered and the presence of bone and cartilage could be observed (reproduced from [76] with kind permission of Springer Science and Business Media). D. Chemical structure of a peptide amphiphile with key structural features (1 – long alkyl tail (hydrophobic), 2 – four cysteine residues, 3 – flexible linker consisting of three glycine residues, 4 – phosphorilated serine to drive hydroxyapatite mineralization, 5 – RDG adhesion ligand), the molecular model and finally the self-assembly scheme of the peptide amphiphile into a cylindrical micelle. (From [102]. Reprinted with permission from AAAS).

tant aspect of biomaterial functionalization is the difficulty to attach functional groups to the materials. Each moiety has to be combined with a material of interest in a separate process of chemical synthesis. Considering the enormous number of possible variables in material functionalization, i.e. using peptide sequences or antibodies, streamlining this process is an important step towards biofunctionalization and high throughput screening of biomaterials. An elegant approach to achieve this uses ureido-pyrimidinone (UPy) moieties, which form non-covalent hydrogen bonds strong enough to create mechanically viable biomaterials. In addition, a UPy-functionalized biomaterial can be functionalized with libraries of UPy-functionalized peptides by simple mixing. It has been shown that by using UPy-functionalized polymers in combination with UPy-modified biomolecules cell adhesive materials can be created [117]. Libraries of functionalized biomaterials can be screened on material arrays. For instance, robotic spotting technology has been applied to analyze combinations of ECM domains immobilized on a hydrogel surface. Thirty two different combinations of five different ECM molecules were tested for their capacity to maintain the phenotype of rat primary hepatocytes or to induce differentiation of mouse embryonic stem cells [118, 119].

Spatial organization in ECM

In the previous paragraphs we have seen that it is possible to control the mechanical properties and molecular cues of biomaterials such that they mimic those of ECM of the tissue of interest. So far, both properties and also cell seeding have been homogenous throughout the material, whereas body tissues are complex structures in which multiple cell types and their respective ECM are spatially highly organized. For instance, an endothelial cell layer covers the inside of a blood vessel, smooth muscle cells surround the tube to support it, and an ECM separates the two cell types [120]. Evidently, engineering the correct spatial control of cells and ECM molecules within tissue engineered constructs is a challenge in which chemical science can create valuable tools. For instance, dielectrophoretic forces have been applied to control the spatial deposition of articular chondrocytes within photopolymerizable PEG hydrogels. Clusters of 3 to 18 cells each were created in a hydrogel and their cluster size, dose-dependently, determined deposition of a cartilage matrix by the cells. This shows that micro-organization (a previously uncontrolled variable) influences cell behavior (Figure 5A) [121]. Another technique which can be used to introduce heterogeneity in scaffold architecture is electrospinning. By changing the parameters during the deposition process, one can fabricate multi-layered scaffolds, each with unique properties as seen in tissues with a layered hierarchical architecture such as cartilage [122]. Increased complexity can be added by electrospraying different cell types at the same time as the electrospun fibers are deposited. This allowed, for example, the creation of tubular scaffolds seeded with smooth muscle cells, which maintained viability through a thickness of 300-500 μm, supporting successful vascular regeneration [123].

Spatial organization occurs not only at the tissue level, as demonstrated by the examples above, but also at the single cell level. For example, a muscle cell is elongated because this represents the most optimal mechanical properties, whereas lipid storage is most optimal in a spherically shaped adipocyte [124, 125]. In these examples, form follows function and technology to control cell shape will benefit cell function. However, biologists are increasingly aware of the fact that function can also follow shape. Several manuscripts describe that geometric presentation of ECM proteins to

a cell is a powerful tool to influence cell function. A classical example of this is a study in which a poly(dimethyl siloxane) (PDMS) stamp was used to create patches of fibronectin of variable sizes on a glass surface (Figure 5B). Subsequently, MSCs were allowed to adhere to the surfaces and it was found that depending on the patch size, the MSCs either differentiated into fat cells or bone cells (Figure 5C and 5D) [125-128]. Using similar technology, the choice between life and death of a cell was influenced by the shape the cell was forced into [129]. Even more delicate examples show that it is possible to deposit, with well-defined geometries, different ECM components at a subcellular resolution using automated printing techniques based on atomic force microscopy. Sub-cellular feature sizes of 6-9 µm were achieved using two components: fibronectin and a commercially available mixture of laminin/collagen type IV. The ratio between these components varied and affected cell alignment [130].

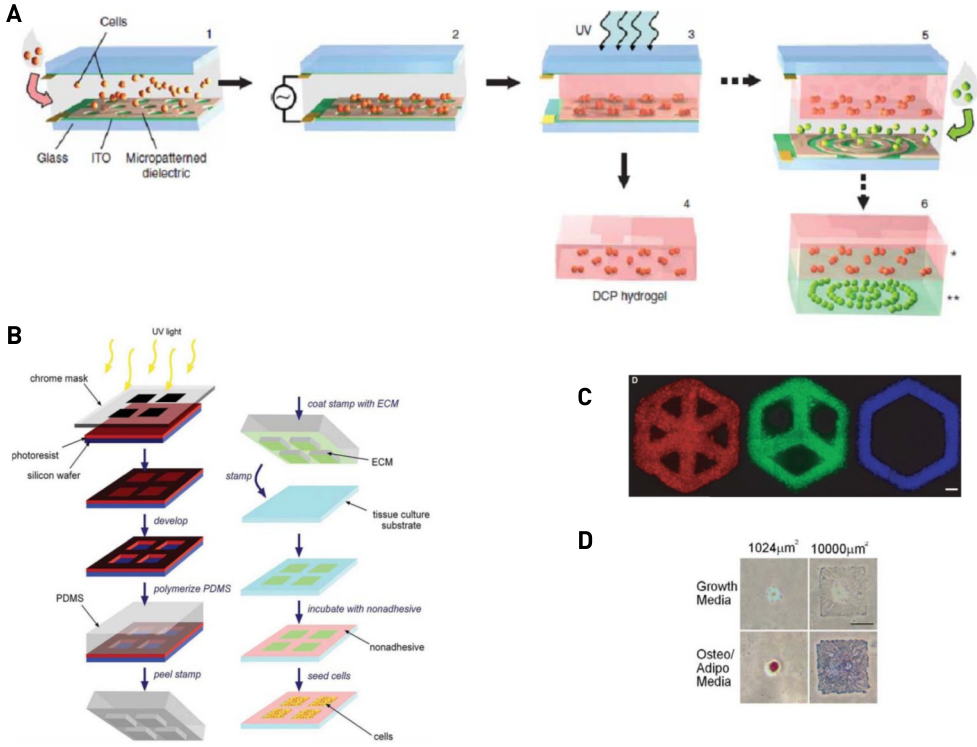

Figure 5 – A. Production of hydrogels with controllable incorporation of cells. Cells are localized using dielectrophoretic forces in micropatterned gaps of the dielectric layer. Exposure to UV light polymerizes the hydrogel thus keeping the cells embedding in a defined position. Once polymerized the gel can be removed and used for further applications. This process can be repeated using every time different cell organization schemes, cell types or hydrogel formulations. (**Legend:** DCP – dielectrophoretic cell patterning; ITO – indium tin oxide) (Reprinted with permission from [121], ©2006 Nature Publishing Group). B. Scheme of ECM protein deposition onto a surface using microcontact printing. A PDMS stamp is produced using photolithography and ECM proteins are coated on the stamp and transferred to the substrate prior to treatment with a non-adhesive compound (Reprinted and adapted with permission from [126], ©2005 Elsevier). C. Examples of microscale patterns created using photopatterning. Viability of the cells in the different patterns is indicated by fluorescent dyes. Scale bar is 500 µm (Reproduced from [128] with permission of FASEB; permission conveyed through Copyright Clearance Center, Inc.). D. Controlling cell fate by cell shape. A fibronectin spot with a defined size was deposited and cells seeded on the protein were cultured in a mixed medium allowing differentiation either into adipocytes or osteoblasts. Cells that grew on the smallest fibronectin spot (1024 µm²) became adipocytes whereas the cells grown in the larger spots (10000 µm²) became osteoblasts (Reprinted and adapted with permission from [125], ©2004 Elsevier).

Remodeling

As if mechanical and cell signaling properties and spatial organization do not already provide sufficient engineering challenges, time is another variable in tissue function. Tissues are not fixed structures but undergo constant remodeling and a bio-mimetic approach for ECM engineering needs to address this aspect as well. Many ECMs are degraded by cell-secreted proteases, such as matrix metalloproteinases (MMP) and serine proteases [131, 132]. ECM remodelling is not only important for ECM maintenance but also to allow cells to migrate. Furthermore, during ECM remodelling, growth factors entrapped in the matrix will be released, which can act as morphogens controlling tissue formation. Proteases are highly specific and degrade ECM at defined sequences. This has been used to design materials with specific proteolytic sites which allow ECM remodelling and new tissue ingrowth in the implant [80, 133, 134]. For instance, protease-functionalized hydrogels loaded with the bone inducing growth factor BMP-2 showed that the extent of bone formation depended on the proteolytic activity of the matrix [134, 135].

Challenges for the future

In this review we have highlighted the biological complexity of the ECM and discussed engineering solutions to design ECM-mimicking scaffolds for tissue regeneration. Scaffolds from the next generation will not merely function as mechanical support, but act as instructive matrices that guide cells to correct tissue regeneration, growth, and development. So far, efforts have focused on chemical modifications of the biomaterial backbone by inserting peptide sequences or on physical processing by downscaling the characteristic dimensions of the base elements (for example, fibers, struts and pore walls) forming the 3D scaffolds. As nature has provided us with multidimensional and multifunctional tissues, we should aim at mimicking this complexity more closely. A possibility is to combine different scaffold fabrication technologies at different scales, to recreate scaffolds with physical and mechanical properties resembling those of ECM. This would also mean incorporating different biomolecules in different regions of the scaffold to hierarchically replicate the biological signals that govern tissue development and homeostasis. In a further effort to bring the worlds of biology and material chemistry together, it could be even envisioned creating chemically modified biomaterials that allow recruitment of cells and biological molecules in situ, thus preventing cell culture techniques *in vitro* before implantation. Possibilities are seemingly unlimited, as more and more biomaterials are synthesized every day and technologies are being developed. Since investigating each single combination would require extensive costs and resources using standard biological evaluation, the search for the most optimal ECM-like scaffold should pass through initial high-throughput screening. High content screening of cell/material interaction allows studying the influence of thousands of biomaterials and ECM proteins on cellular activity. Nevertheless, the integration of mechanical and biological cues is still not optimal. When designing ECM-inspired scaffolds we need to overcome a major hurdle limiting their use for tissue engineering applications: nutrient diffusion limitations. A compromise between mechanical properties and diffusion capacity as to be achieved so the scaffold can have desirable mechanical properties without impairing diffusion of nutrients or by-products of the metabolism. Additionally, spatial control of ligands is a challenge for

both biologists as well as material scientists as the decision on which biological cues and their spatial distribution can dictate cell fate. In that respect, degradation properties and the concomitant release of degradation products need to be carefully controlled in order to retain the properties of the scaffold, to avoid inflammatory responses as well as an increase in the local concentration of certain compounds capable of hindering cellular functions. Integration of different technologies can also be hampered by the need of combining different processing techniques which can result in incompatibility of building blocks or creation of fracture/degradation zones within the constructs. When considering the use of biological systems in an attempt to produce recombinant ECM proteins for incorporation in scaffolds one should be aware that most available systems are not capable of mimicking the complex post-translational modifications necessary to have a functional protein. Most likely, new systems to produce and isolate these proteins need to be develop.

We have witnessed the eras of genomics and proteomics but we envisage the era of materiomics to bridge the gap between natural and engineered tissue development.

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Chapter 3

COLLAGEN INFLUENCES DIFFERENTIATION OF HUMAN MULTIPOTENT MESENCHYMAL STROMAL CELLS

"Success is going from failure to failure without loosing enthusiasm"

Winston Churchill

Collagen influences differentiation of human multipotent mesenchymal stromal cells

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Abstract

Human multipotent mesenchymal stromal cells (hMSCs) are multipotent cells, which in the presence of appropriate stimuli can differentiate into different lineages such as the osteogenic, chondrogenic and adipogenic lineage. In the presence of ascorbic acid (Asap), multipotent mesenchymal stromal cells (MSCs) secrete an extracellular matrix (ECM) mainly composed of collagen type I and we assessed a potential role of collagen in hMSC differentiation and stem cell maintenance. We observed a sharp reduction in proliferation rate of hMSCs in the absence of collagen type I, concomitant with a reduction in osteogenesis *in vitro* and bone formation *in vivo*. In line with a positive role for collagen type I in osteogenesis, gene expression profiling of hMSCs in the absence of Asap demonstrated increased expression of genes involved in adipogenesis and chondrogenesis and a reduction in the expression of osteogenic genes. We also observed that matrix remodelling and anti-osteoclastogenic signals were high in the presence of Asap. The presence of collagen type I during the expansion phase of hMSCs did not affect their osteogenic and adipogenic differentiation potential. In conclusion, the collagenous matrix supports both proliferation and differentiation of osteogenic hMSCs but on the other hand presents signals stimulating matrix remodelling inhibiting osteoclastogenesis.

Introduction

Tissue engineering focuses on the restoration, maintenance or improvement of diseased or damaged tissues [1-3]. Currently, when a tissue is damaged or lost, autografts or synthetic prostheses are used in an attempt to restore its function. Nevertheless, problems such as multiple surgeries, insufficient material availability, risk of infection, failure of graft materials or rejection by the host, are commonly associated with these treatments. Presently, research is focused on the use of autologous cells for tissue engineering applications in an attempt to increase integration of grafts as well as to enhance the biological efficacy of cell-based therapies [4, 5]. Over the past decade, there has been much interest in the use of human multipotent mesenchymal stromal cells (hMSCs) for tissue engineering. hMSCs are multipotent cells, able to differentiate, depending on the stimulus, into several lineages including the osteogenic, chondrogenic and adipogenic lineage *in vitro* [6, 7]. Because these cells can be easily isolated from bone marrow aspirates and expanded *in vitro*, they can be used for various cell-based therapeutic approaches such as tissue-engineering, cell therapy, and as cytokine and growth factor factories [8]. In stem cell biology, the growth factor milieu is acknowledged as very important for maintenance of stemness. For example, propagation of undifferentiated pluripotent mouse embryonic stem cells is dependent on the cytokine leukemia inhibitory factor (LIF) [9]. Similarly, during *in vitro* culture of hMSCs, basic fibroblast growth factor is used to enhance their growth rate and to select for a sub-population of hMSCs with a more extensive expansion potential [10]. Growth factors and other diffusible molecules, such as dexamethasone, cyclic adenosine 3', 5' – monophosphate (cAMP), 1,25-dihydroxy-vitaminD (vitD3) and bone morphogenetic protein 2 (BMP-2) are also used to drive osteogenic differentiation of hMSCs [11-13]. On the other hand, the presence of extracellular matrix (ECM) proteins can be crucial for maintenance of stemness and differentiation. For instance, the tendon stem cell niche is dependent on the presence of biglycan and fibromodulin, which highlights the instructive potential of these molecules in addition to the well known structural role of ECM proteins [14, 15].

hMSCs are typically expanded in the presence of ascorbic acid (Asap), which plays a key role in the synthesis of ECM [16]. Asap is an essential cofactor for the enzymes prolyl and lysyl hydroxylase, which hydroxylates proline and lysine residues, respectively, in collagen, during post-translational modifications of this protein [17]. As such, hMSCs are expanded in the presence of a matrix which is mainly composed of collagen type I. Collagen type I is also the most abundant protein in ECM of bone and no collagen is produced in the absence of Asap [18]. It has been reported that addition of Asap to the culture medium greatly enhanced the growth rate and extended the lifespan of hMSCs [19, 20]. In addition, Asap has been shown to promote differentiation and mineralization of MC3T3-E1 preosteoblast cells by enhancing the accumulation of mature collagen in ECM [21-23]. Based on the known instructive role of ECM in some contexts, it can be expected that the matrix deposited by culture-expanded hMSCs has an effect on the stemness and differentiation potential of the cells growing on it.

Cells attach to ECM proteins mainly via specific transmembrane receptors called integrins. They consist of an α- and a β-subunit and play an important role in relaying information from ECM towards the cell ("outside-in-signaling") and from the cell towards ECM ("inside-out-signaling") during cellular adhesion and migration [24, 25]. The interaction of integrins with intracellu-

lar proteins such as focal adhesion kinase (FAK) initiates a cell signaling cascade which regulates cell proliferation and differentiation and controls adhesive properties of the cell. For instance, binding of FAK to the α_1 integrin cytoplasmic tail leads to the formation of focal adhesion sites [26, 27].

ECM also plays an important role during chondrogenic differentiation. During this process, cells change from the characteristic fibroblast-like morphology to large round cells surrounded by abundant ECM, due to a dynamic reorganization of the cytoskeletal network [28]. This ECM is a dense connective tissue, consisting of a highly organized network of collagen (mostly type II) and large aggregating proteoglycans [28]. In hMSCs, SOX9 regulates expression of the gene encoding type II collagen during chondrogenic differentiation [29]. Collagen type II is not expressed by hMSCs cultured in proliferation or osteogenic medium [6].

In this study we investigated the effect of the collagenous matrix deposited during expansion of hMSCs. We confirmed the positive effect of collagen type I on osteogenic differentiation of hMSCs and we investigated whether the presence of collagen type I during the expansion phase has a consequence for subsequent adipogenic, osteogenic and chondrogenic differentiation.

Materials and Methods

Cell culturing

Bone marrow aspirates (5-15 mL) were obtained from patients who had given written informed consent. hMSCs were isolated and proliferated as described previously, unless stated otherwise [30]. Briefly, aspirates were resuspended using a 20-gauge needle, plated at a density of $5x10^5$ cells/cm^2 and cultured in MSC proliferation medium, which contains minimal essential medium (α-MEM; Life Technologies, Gaithersburg, MD), 10% foetal bovine serum (FBS; Life Technologies), 0.2 mM L-Ascorbic acid 2-phosphate magnesium salt (Asap; Life Technologies), 2 mM L-glutamine (L-glut; Life Technologies), 100 U/mL penicillin (Life Technologies), 100 µg/mL streptomycin (Life Technologies) and 1 ng/mL basic fibroblast growth factor (bFGF; Instruchemie, Delfzijl, The Netherlands). Cells were grown at 37ºC in a humid atmosphere with 5% CO_2. Medium was refreshed twice per week and cells were used for further subculturing or cryopreservation on reaching near confluence. hMSC basic medium was composed of hMSC proliferation medium without bFGF, hMSCs osteogenic medium was composed of hMSC basic medium supplemented with 10^{-8} M dexamethasone (dex; Sigma) and hMSC mineralization medium was composed of basic medium supplemented with 10^{-8} M dex and 0.01 M β-glycerophosphate (BGP; Sigma).

Cell proliferation assay

Cell proliferation was assessed using an Alamar blue assay according to the manufacturer's protocol. Briefly, culture medium was replaced with medium containing 10% Alamar blue solution (Biosource, Camarillo, CA, USA) and cells were incubated at 37ºC for 4h. Fluorescence was measured at 590 nm on a Perkin Elmer LS50B plate reader.

ALP flow cytometry

To analyze the role of Asap in ALP expression of hMSCs, cells were grown in the presence or absence of Asap either in basic or in osteogenic medium. Different concentrations of Asap were used and its effect on ALP expression was analyzed.

After 7 days of culture, cells were trypsinized and incubated for 30 minutes in block buffer (PBS with 5% bovine serum albumin (BSA; Sigma) and 0.05% NaN_2) and in PBS with 1% BSA plus primary antibody (anti-ALP B4-78; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) for 30 minutes or with isotype control antibody (mouse anti-human IgG_1; BD Biosciences). Cells were then washed three times with washing buffer (PBS with 1% BSA and 0.025% NaN_2) and incubated with secondary antibody (rat anti-mouse IgG_1 PE; BD Biosciences) for 30 minutes. Cells were washed two times and resuspended in 300 µL of washing buffer plus 10 µL of Viaprobe (BD Biosciences) for live/dead cell staining and only living cells were used for further analysis. ALP expression levels were analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Mineralization and calcium assay

For mineralization experiments, hMSCs were seeded in triplicate at 5000 cells/cm² in T25 culture flasks in basic or osteogenic medium, either in the presence or absence of Asap. In each experiment, hMSC mineralization medium was used as a positive control and hMSC basic medium containing BGP as a negative control. The total calcium deposition was assayed using a calcium assay kit (Sigma Diagnostics) according to manufacturer's protocol. Briefly, the culture medium was aspirated, cells were washed twice with calcium- and magnesium-free PBS (Life Technologies) and incubated overnight with 0.5 N HCl on an orbital shaker at room temperature. The supernatant was collected and the calcium content was measured at 575 nm (Perkin Elmer, Lambda 40).

Adipogenic assay

hMSCs were seeded in triplicate at 25 000 cells/well in 24-wells plates and grown in adipogenic medium containing Dulbecco's modified Eagle's medium (DMEM, Gibco), 10% FBS, 100 U/mL penicillin with 100 µg/mL streptomycin, 0.2 mM Indomethacin (Sigma), 0.5 mM isobutylmethylxanthine (IBMX; Sigma), 10^{-6} M dexamethasone and 10 mg/mL human insulin (Sigma). As a negative control, DMEM with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin was used. After 21 days, cells were fixed for at least 4h in 0.5 g $CaCl_2.2H_2O$ (Merck) in 45 mL MilliQ and 5 mL of 4% formaldehyde (Sigma), rinsed with water, incubated for 5 minutes in 60% isopropanol (Sigma) and stained for 5 minutes in freshly filtered (0.22 µm) Oil Red O solution (3 mg/mL in 60% isopropanol, Sigma). After rinsing with demineralized water, pictures of the stained cells were made using a Nikon digital sight DS-Fi1 camera.

Chondrogenic assay

To test the chondrogenic potential of hMSCs, 200 000 cells per well were seeded in round-bottom 96-wells plates and pelleted by centrifugation. Chondrogenic medium consisted of DMEM supplemented with 40 µg/mL proline (Sigma), 50 µg/mL insulin transferrin selenium (ITS-premix;

Sigma), 100 U/mL penicillin, 100 µg/ml streptomycin, 50 µg/mL Asap, 100 µg/ml sodium pyruvate (Gibco), 10 ng/mL TGF-β3 (R&D systems), 10^{-7} M dexamethasone and 500 ng/mL BMP-6 (kindly provided by Genera Incorporated). After 21 days, pellets were fixed with cold formalin for histological analysis, dehydrated, embedded into paraffin and stained with alcian blue 8GX (Sigma). For analysis of glycosaminoglycans (GAG), pellets were digested with 1 mg/mL proteinase K (Sigma), lysed and absorbance was measured after adding 1,9-dimethylmethylene blue chloride (DMMB) solution in PBE buffer (14.2 g/L Na_2HPO4 and 3.72 g/L Na_2EDTA, pH 6.5; Sigma). To correct for cell number, Cyquant cell proliferation assay kit (Invitrogen) was used according to the manufacturer's instructions.

Collagen biochemical analysis

We determined the collagen content and the ratio hydroxyproline/proline (Hyp/Pro) as a measure of collageneous to non-collageneous proteins. To determine the collagen contents, hMSCs were cultured for 21 days in the presence or absence of Asap, either in basic or in osteogenic medium. After 21 days, samples were hydrolyzed (110ºC, 20-24h) with 750 µL 6 M HCl, dried and redissolved in 800 µL of water containing 10 µM pyridoxine (internal standard for hydroxylysylpyridinoline (HP) and lysylpyridinoline(LP)) and 2.4 mM homoarginine (internal standard for amino acids; Sigma). Samples were diluted 5-fold with 1% (vol/vol) heptafluorobutyric acid (HFBA; Fluka) in 10% (vol/vol) acetonitrile for crosslinking analysis; aliquots of the 5-fold diluted sample were diluted 20-fold with 0.1 M sodium borate buffer (pH 8.0) for amino acid analysis. Derivation and subsequent chromatography of the amino acids, as well as chromatography of the crosslinks were performed as described [31, 32]. Collagen content was calculated from the total amount of Hyp in each sample, assuming 300 Hyp residues per collagen molecule and a molecular weight of collagen of 300 kD.

Microarray analysis

To analyze the role of Asap in the gene expression profile of hMSCs we seeded 5000 cells/cm² in T25 flasks either in basic or in osteogenic media, in the presence or absence of Asap, for 10 days.

Total RNA was isolated using an Rneasy mini kit (Qiagen) and on-column DNase treated with 10U RNase free DNase I (Gibco) at 37ºC for 30 minutes. DNase was inactivated at 72ºC for 15 minutes. The quality and quantity of RNA was analyzed by gel electrophoresis and spectrophotometry.

Affymetrix 900649 GeneChip® Human Exon 1.0 ST Arrays were hybridized and the fluorescence intensity was normalized by dividing the fluorescence intensity of each sample by the medium intensity of the array. The fold induction reflects the ratio between the fluorescence intensity in the first condition versus the fluorescence intensity of the second condition. We generated four different lists (genes upregulated and downregulated in basic medium versus basic medium without Asap and genes upregulated and downregulated in osteogenic medium versus osteogenic medium without Asap). Only genes co-expressed in the two media were considered for further analysis. DAVID, an online annotation tool, was used for identification of enriched functional groups within gene lists [33].

In vivo bone formation

To evaluate the effect of Asap on *in vivo* bone formation by hMSCs, we seeded 2x10^5 cells onto three porous biphasic calcium phosphate (BCP) ceramic scaffolds of approximately 2-3 mm, containing 20% β-tricalcium phosphate and 80% hydroxyapatite sintered at 1300ºC, prepared in house. Cells were cultured *in vitro* for 14 days in the presence of osteogenic medium or osteogenic medium without Asap.

In order to analyze if the absence of Asap had an effect on proliferation when cells were grown on the scaffolds for 14 days, a DNA assay was performed. Briefly, cells were seeded as described above and lysed at day 14 using a 0.1% Triton X-100 solution in PBS. DNA was quantified using the cyQuantGR dye by measuring the fluorescence at 520 nm.

Prior to implantation, the tissue-engineered constructs were washed with PBS. Six immunodeficient mice (HsdCpb:NRI-nu, Harlan, Horst, The Netherlands) were anesthetized using isoflurane. The surgical sites were cleaned with ethanol, subcutaneous pockets were created and three particles were implanted. After 6 weeks the implants were retrieved and fixed in 0.14 M cacodylic acid buffer pH 7.3 containing 1.5% glutaraldehyde. The fixed samples were dehydrated in ethanol series and embedded in methyl methacrylate. Sections were processed on a histological diamond saw (Leica SP1600, Wetzlar, Germany) and stained with 1% methylene blue (Sigma) and 0.3% basic fuchsin solution (Sigma). At least 5 sections per scaffold were imaged and the percentage of bone per scaffold area calculated using a PC-based system with KS400 software (version 3, Zeiss, Germany). All experiments were approved by the local animal care and use committee.

Statistical analysis

The data was analyzed using Student's paired t-test ($P<0.05$).

Results

Effect of collagen on in vitro proliferation of human multipotent mesenchymal stromal cells

We quantified the amount of collagen deposited by hMSCs after 21 days of expansion in the presence or absence of Asap, either in basic or in osteogenic medium. In the presence of Asap, collagen could be detected and no statistically significant difference could be observed in the amount of collagen deposited by cells grown in either basic or osteogenic medium (209 ± 71 versus 212 ± 4 µg of collagen per sample, respectively). In the absence of Asap, almost no collagen could be detected in the samples in both conditions (less than 1 µg of collagen per sample), which means a more than 1000-fold reduction and 200-fold reduction in collagen content in basic and osteogenic medium respectively (Figure 1A). We further analyzed the ratio of collagenous to non-collagenous proteins (expressed as the hydroxyproline/proline ratio) in the presence or absence of Asap in both basic and osteogenic medium. In the absence of Asap, there was a significant reduction in this ratio, independently of the medium (20-fold reduction in basic medium and 44-fold reduction in osteogenic medium; Figure 1A), demonstrating a decrease in the amount of collagenous proteins synthesized in the absence of Asap. Interestingly, we also observed a significant difference in the Hyp/Pro ratio between basic and osteogenic medium, which suggests a shift in protein synthesis upon osteogenic differentiation (Figure 1A).

We analyzed the effect of collagen deposition during expansion of hMSCs. There was a marked difference in cell morphology between cells grown in the presence or absence of Asap (Figure 1B). The most striking difference between hMSCs grown in the two conditions was the appearance of stress fibers in the absence of Asap, which we even observed when cells were allowed to grow in the presence of Asap for the first 3 days before depleting the medium of Asap. Similarly, there was a decrease on ALP expression as well as on calcium deposition when cells were grown for the first 3 days in Asap and further culture in its absence (data not shown). To analyze the effect of collagen on the efficiency of hMSC isolation, we plated bone marrow aspirates either in the presence or absence of Asap. Cells grew until one of the conditions reached near confluence, upon which they were trypsinized and the number of cells was calculated. We observed a marked decrease in the number of cells in the absence of Asap for three out of four donors tested (Figure 1C). Because the number of CFU-Fs/mL of bone marrow was not determined, we cannot exclude that the increase in cell number in the presence of Asap is due to higher cloning efficiency. To investigate a potential effect on proliferation, we analyzed the effect of a range of concentrations of Asap on proliferation of hMSCs. Figure 1D clearly shows that addition of Asap, independent of the medium or concentration tested, resulted in enhanced proliferation of hMSCs. The effect was dose-dependent and reached a plateau at a concentration of 0.2 mM, which is the concentration typically used in hMSC expansion medium.

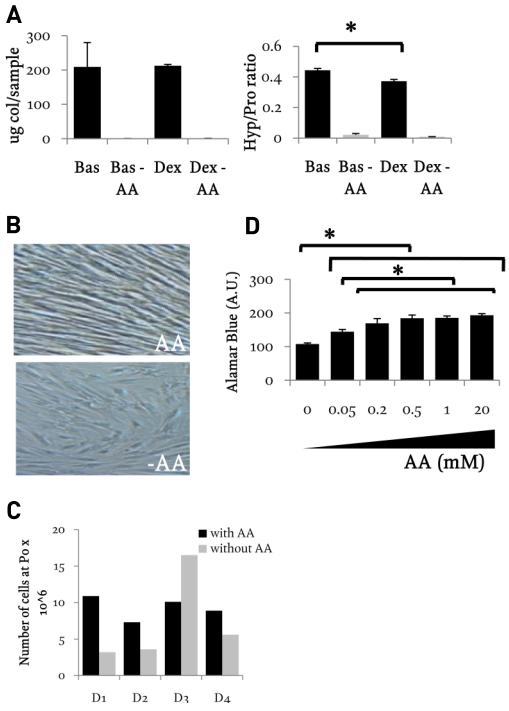

Figure 1 – Absence of Asap inhibited collagen deposition and altered cell morphology and proliferation A. Collagen content and Hyp/pro ratio (ratio between collagen/non-collagenous proteins) in hMSCs grown for 21 days in basic or osteogenic medium in the presence or absence of Asap. Note that in the absence of Asap hardly any collagen could be detected in the samples. The low hyp/pro ratio in the absence of Asap was the result of absence of collagen B. hMSCs were cultured for 21 days in basic or osteogenic medium in the presence or absence of Asap. Note that cells grown in the absence of Asap showed marked differences in cell morphology when compared to the respective control with Asap C. Bone marrow cells were obtained from various donors and the hMSC yield after passage 0 was calculated. Note that in the absence of Asap there was a reduction in the number of hMSCs for all the donors except one (donor 3). D. Proliferation of hMSCs cultured in osteogenic medium for 7 days in the presence of increasing concentrations of Asap. Note that there was an increase in proliferation of hMSCs when Asap was present at any given concentration.

Ectopic bone formation by hMSCs in the absence of Asap

In ectopic bone formation models, hMSCs are cultured *in vitro* prior to implantation in the presence of Asap but the beneficial effect of its presence for the *in vivo* bone forming capacity is not known. Here, we investigated the *in vivo* bone forming capacity of hMSCs grown on ceramic scaffolds for 14 days, in the presence or absence of Asap. First, we analyzed the number of cells on the scaffolds after 14 days of *in vitro* culture. Figure 2A shows a 35 % reduction in the number of cells on the scaffolds when cultured in the absence of Asap. Secondly, we analyzed the *in vivo* bone forming potential of these cells upon subcutaneous implantation in immunodeficient mice. Six weeks after implantation, 4 ± 1.5 % of bone tissue per scaffold area was seen in the case of hMSCs grown in the presence of Asap whereas only 2 ± 2.5 % of bone per scaffold area was observed in cells grown in the absence of Asap (Figure 2B), clearly demonstrating the positive effect of Asap on ectopic bone formation by hMSCs.

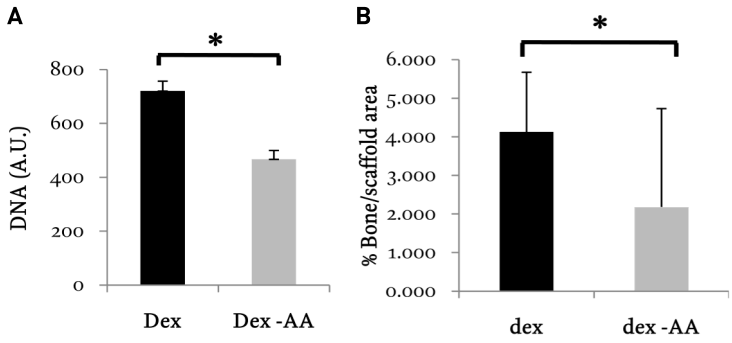

Figure 2 – Effect of Asap on in vivo bone formation by hMSCs A. hMSCs were cultured for 14 days on scaffolds in the presence or absence of Asap and the amount of DNA was quantified. Note the significant decrease in the amount of DNA when Asap was absent from the culture medium B. hMSCs were cultured in vitro for 14 days on BCP 1300 in the presence of osteogenic medium with or without Asap. The constructs were implanted subcutaneously in immune-deficient mice for 6 weeks and after explantation they were stained for newly formed bone with basic Fuchsin. At least 5 sections per scaffold were imaged and the percentage of bone per scaffold area calculated. Note the decrease in the percentage of bone per scaffold area when Asap was absent from the culture medium.

Effect of Asap in the osteogenic program of hMSCs

Based on the previous results on changes in morphology and a decrease in *in vivo* bone formation, we analysed the effect of collagen on the osteogenic program of hMSCs. Quantification of calcium accumulation by hMSCs, a phenotypical late marker of osteogenic differentiation, showed an almost complete inhibition of calcium deposition in the absence of Asap (Figure 3A). More importantly, dex-induced ALP expression of hMSCs, an early marker of osteogenic differentiation, was significantly reduced in the absence of Asap (40 % reduction in the percentage of ALP positive cells between cells grown in osteogenic medium versus osteogenic medium without Asap) indicating that the osteogenic program was impaired early in the differentiation cascade (Figure 3B).

Effect of Asap on the gene expression profile of hMSCs

Next, we analyzed genome-wide gene expression in the presence or absence of collagen. We performed a microarray study on hMSCs cultured for 10 days either in basic or in osteogenic medium, in the presence or absence of Asap. Some of the changes in gene expression reflected our earlier observations. For instance, we observed that cells grown in the presence of Asap were enriched for genes related to the cell cycle and mitosis (see supplementary data). Furthermore, genes involved in osteogenesis such as ALP where highest expressed in the presence of Asap. In contrast, noggin, a BMP antagonist, was upregulated in the absence of Asap. Interestingly, the proteases cathepsin K and cathepsin S were expressed higher in the presence of Asap, independent of the medium (5.9-fold in basic versus basic –AA and 6.5-fold in osteogenic versus osteogenic –AA for cathepsin K, 2-fold for cathepsin S). In addition, several interferon-induced genes were upregulated upon exposure to Asap, suggesting that interferon-signalling is enhanced in the presence of a collagenous matrix. The expression of some genes was lower in the presence of Asap, which represents a group of genes suppressed by the presence of collagen. For instance, we observed lower levels of expression of transforming growth factor beta 2, aggrecan, hyaluronan synthase 1 and cartilage oligomeric matrix protein, all known to be involved in chondrogenic differentiation. CCAAT/enhancer binding protein (C/EBP) gamma expression, a gene involved in adipogenic differentiation, was also lower in the presence of Asap. The expression of genes with an endothelial signature, such as endothelial differentiation lysophosphatidic acid G-protein-coupled receptor 7 and vascular endothelial growth factor A were highest in the absence of Asap (a list of all the genes regulated can be found in supplementary data).

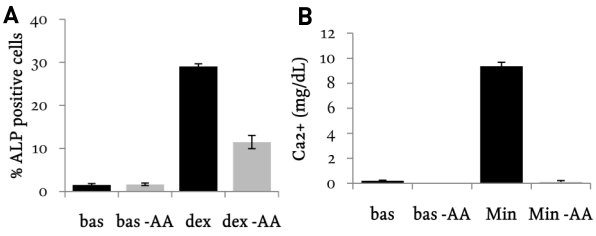

Figure 3 – Absence of Asap impairs the osteogenic differentiation of hMSCs. A. ALP expression by hMSCs cultured in basic or osteogenic medium, either in the presence or absence of Asap, for 7 days. Note the induction of ALP when cells were exposed to dexamethasone. Additionally, there was a significant decrease in the percentage of ALP positive cells in the absence of Asap. B. Calcium accumulation by hMSCs cultured in basic (with BGP) or mineralization medium, in the presence or absence of Asap, for 21 days. Note that in the absence of Asap hardly any calcium could be detected in mineralization medium.

Absence of Asap and its effects on cell fate decision

Our results show that by adding Asap to the culture medium we enhance the proliferation of hMSCs and differentiation into the osteogenic lineage but, by doing so, we may compromise differentiation into other lineages. To confirm this, we expanded hMSCs, in the presence or absence of Asap and differentiated them into the osteogenic, adipogenic and chondrogenic lineages. When cells where expanded in the absence of Asap their osteogenic potential was not significantly affected in two of the four donors tested whereas a positive effect was seen in two other donors (Figure 4A). Upon exposure to an adipogenic differentiation protocol, we observed an increase in the number of Oil Red O-positive cells when hMSCs from three donors were expanded in the absence of Asap (Figure 4B). In the case of chondrogenic differentiation of hMSCs we observed that

two out of four donors did not undergo chondrogenic differentiation irrespective of the presence or absence of Asap (data not shown). Moreover, the two donors that did differentiate into the chondrogenic lineage gave opposite results. In one case the expansion in the absence of Asap resulted in an increase in the amount of GAG per DNA whereas in the other case there was a significant reduction (Figure 4C).

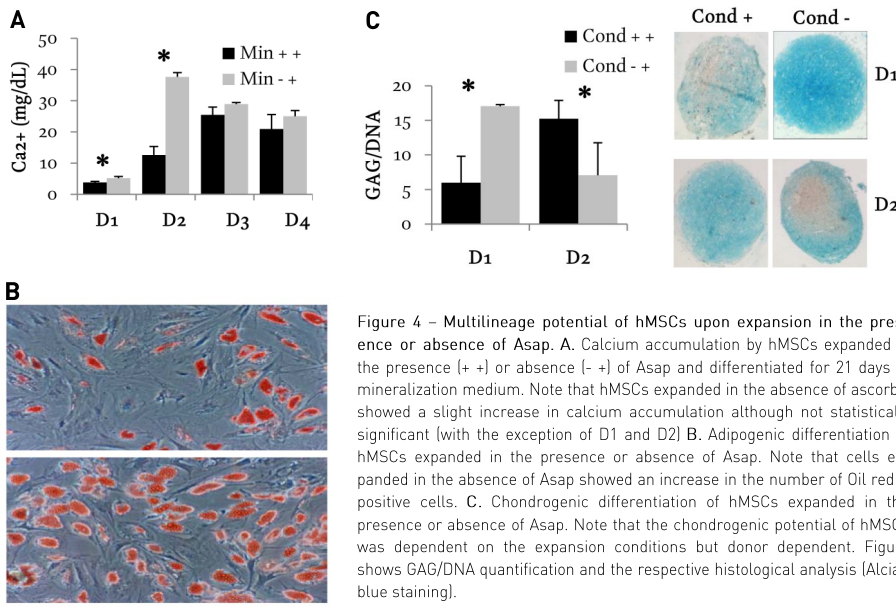

Figure 4 – Multilineage potential of hMSCs upon expansion in the presence or absence of Asap. A. Calcium accumulation by hMSCs expanded in the presence (+ +) or absence (- +) of Asap and differentiated for 21 days in mineralization medium. Note that hMSCs expanded in the absence of ascorbic showed a slight increase in calcium accumulation although not statistically significant (with the exception of D1 and D2) B. Adipogenic differentiation of hMSCs expanded in the presence or absence of Asap. Note that cells expanded in the absence of Asap showed an increase in the number of Oil red O positive cells. C. Chondrogenic differentiation of hMSCs expanded in the presence or absence of Asap. Note that the chondrogenic potential of hMSCs was dependent on the expansion conditions but donor dependent. Figure shows GAG/DNA quantification and the respective histological analysis (Alcian blue staining).

Discussion

Soluble factors are typically used in the culture medium to drive differentiation of hMSCs. For example, dexamethasone, Asap and BGP are used for osteogenic differentiation, TGF-β for chondrogenic differentiation and indomethacin, IBMX and insulin for adipogenic differentiation [6].

During osteogenic differentiation, hMSCs undergo a stepwise process leading to the deposition of an ECM, mainly composed of collagen type I, which will be mineralized in a later stage [34]. Asap is an essential co-factor for the activity of a key enzyme involved in collagen synthesis – prolyl hydroxylase [35]. In the absence of Asap, collagen deposition by the cells was drastically reduced resulting in impaired osteogenic differentiation and proliferation. For example, MC3T3-E1 cells, a murine pre-osteoblastic cell line, did not differentiate into the osteogenic lineage in the absence of Asap [36]. These observations are in line with our data showing that in the absence of Asap, hMSCs produce minimal amounts of collagen leading to an inhibition of proliferation. In most tissue engineering applications, the number of cells is critical and it is therefore recommended to expand cells in the presence of Asap because the yield after the first passage is higher in its presence. The mitogenic effect observed in the presence of Asap can be ascribed either to the role of ECM as a growth factor reservoir or by directly activating a mitogenic pathway. In the former, the growth factors secreted by the cells are soluble in the medium in the absence of collagen, and lost during medium refreshment. In the latter case, integrin signalling may be impaired and the downstream signals that lead

to activation of mitogen-activated protein kinase activation are absent in the absence of collagen [37, 38]. In addition to the mitogenic role of ECM we also observed an effect on osteogenesis. We observed that ALP expression and calcium deposition were both decreased in the absence of Asap pointing towards a role of collagen in osteogenic differentiation [39]. Interestingly, the effects on ALP expression and calcium accumulation were similar irrespective of the initial presence of Asap and further culture without Asap for the remaining period of the assay. In that case, the effect of ECM deposited while cells were exposed to Asap most likely disappeared upon removal because ECM was remodelled and degraded by matrix degrading enzymes without new deposition due to the absence of Asap. In this respect, it is interesting to observe that cathepsin K, an osteoclast-related gene also expressed in osteoblasts, is one of the genes highly expressed in the presence of Asap indicating that concomitant with ECM deposition there is a signal to remodel it, which may lead to release of growth factors from ECM [40]. Interestingly, in the presence of Asap, several interferon-induced proteins were upregulated. Recently, it has been shown that interferon-gamma is a strong inhibitor of osteoclast activity and furthermore, its exogenous addition accelerates osteogenic differentiation of hMSCs [41]. This poses the interesting possibility that collagen represents an anti-osteoclastic and pro-osteogenic signal by activating interferon-mediated signalling.

Further mining of the microarray data suggested that in the absence of Asap, differentiation into other lineages was favoured since genes involved in differentiation towards those lineages were upregulated. This would indicate that Asap not only has an effect on proliferation of the cells but also plays a role in cell fate decision by controlling the switch between different lineages. Despite the positive effect of collagen on osteogenesis, our gene expression data suggest that in its presence the multilineage potential of hMSCs is maintained, which is similar to the effect of dexamethasone. Despite the fact that dexamethasone is generally used as an osteogenic factor *in vitro*, expansion in its presence does not impair multilineage differentiation. To favour osteogenic differentiation, not only dexamethasone is necessary but also the presence of an ECM that serves as a template for mineralization and as a reservoir of growth factors. Likewise, expansion of hMSCs in the absence of Asap did not hamper the osteogenic potential as seen by calcium deposition.

Conclusion

In conclusion, we show that collagen plays a positive role in proliferation and osteogenic differentiation of hMSCs and addition of Asap to the culture medium favours *in vivo* bone formation by hMSCs. Additionally, our genome-wide expression data shows that both in the presence and absence of collagen, hMSCs keep their multipotency and we disclose an interesting cross-talk between collagen and matrix remodelling.

Acknowledgements

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Note

Supplementary data available upon request

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Chapter 4

THE ROLE OF COLLAGEN CROSSLINKING IN DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS AND MC3T3-E1 CELLS

"It is not because things are difficult that we do not dare; it is because we do not dare that they are difficult"

Seneca

The role of collagen crosslinking in differentiation of human mesenchymal stem cells and MC3T3-E1 cells

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Abstract

Collagen is the main component of the extracellular matrix of bone and it has both structural and instructive properties. Collagen undergoes many post-translational modifications, including extensive crosslinking. Although defective crosslinking has been implicated in human syndromes, e.g. osteogenesis imperfecta or Ehlers-Danlos syndrome, it is not clear to which extent crosslinking is necessary for collagen's instructive properties during bone formation. Here we report that inhibition of collagen crosslinking in the mouse pre-osteoblast cell line MC3T3-E1 impairs the osteogenic program. Genome-wide expression profiling of BAPN-treated and control cells revealed that matrix deposition by MC3T3-E1 cells provides a feedback signal driving cells through the differentiation process, which is strongly impaired when crosslinking is inhibited. Interestingly, inhibition of crosslinking did not affect osteogenic differentiation of human mesenchymal stem cells (hMSCs), shown by the expression of alkaline phosphatase and genome-wide gene expression analysis. Surprisingly, it does enhance matrix mineralization. In conclusion, collagen crosslinking harbours instructive properties in MC3T3-E1 differentiation, but plays a more passive role in differentiation of bone marrow-derived hMSCs.

Introduction

Extracellular matrix (ECM) is a complex and dynamic component of many tissues in the body. Traditionally, ECM proteins such as collagens were perceived as the scaffold of ECM, with mainly a structural role. Nowadays, however, it is believed that ECM plays an important regulatory role in cell behavior, controlling such diverse functions as proliferation, growth, cell survival, migration and differentiation [1-9]. For example, ECM proteins biglycan and fibromodulin were identified as critical components controlling the fate of stem cells in the tendon stem cell niche. Depletion of these two components affects proliferation and differentiation, leading to ossification rather than tendon cell differentiation. Furthermore, Tenascin C is an ECM protein that has the ability to block RhoA activity. Thus, it can control the cell shape and actin stress fiber organization of chondrocytes thereby inhibiting the dedifferentiation process that these cells undergo during culturing [10, 11].

ECM also plays a critical role in osteogenic differentiation. MC3T3-E1 cells is a murine pre-osteoblastic cell line in which the osteogenic program is regarded as a matrix-driven process. Proliferation and differentiation of MC3T3-E1 cells into osteoblasts is influenced by matrix stiffness and it is critically dependent on the presence of ECM [12, 13]. For instance, in the absence of ascorbic acid, collagen type I protein production is impaired in MC3T3-E1 cells, resulting in inhibition of osteogenic differentiation. Moreover, treatment of the cells with an inhibitor of collagen synthesis or with antibodies against $\alpha 2\beta 1$ integrins, the transmembrane receptors mediating collagen/cell interaction, results in an abolishment of the osteogenic program [13, 14].

The instructive property of ECM may be an interesting tool to direct the osteogenic differentiation program of human mesenchymal stem cells (hMSCs) for bone tissue engineering applications. hMSCs are bone marrow derived cells which were isolated for the first time due to their ability to adhere to tissue culture plates [15]. hMSCs, also referred to as colony forming unit-fibroblasts, marrow stromal fibroblasts, bone marrow derived fibroblasts, mesenchymal progenitor cells and bone marrow derived stromal cells, are able to differentiate into several different lineages including the chondrogenic, adipogenic, myogenic and osteogenic lineage [16-21].

During osteogenic differentiation of hMSCs, the uncommitted multipotent cell differentiates into a functional mature osteoblast, which secrets a matrix that will later be mineralized. The differentiation of hMSCs into the osteogenic lineage is a stepwise process in which different signaling pathways (e.g. transforming growth factor beta and Wnt signaling) and nuclear receptors (e.g. glucocorticoid and vitamin D3) play a complementary role [22]. Several soluble factors like dexamethasone, cyclic adenosine monophosphate (cAMP), bone morphogenetic protein 2 (BMP-2) and vitamin D3 have the potential to induce hMSCs into the osteogenic lineage [22]. Exposure of hMSCs to these compounds is limited to the *in vitro* expansion phase because *in vivo* controlled release of small molecules is far from trivial. An alternative strategy relies on the use of natural or synthetic ECM proteins to induce differentiation into the desired lineage. Proteins can be combined with scaffold materials and literature has shown proof of principle for this approach. For example, when cells were cultured in a collagen type I matrix with a controlled stiffness they irreversibly differentiated into the osteogenic lineage [3]. In addition, hMSC differentiation into the osteogenic lineage was enhanced when the cells were exposed to ECM proteins such as vitronectin, laminin-5 and collagen type I [6, 23, 24]. This effect might be mediated through ECM - focal adhesion kinase (FAK) –

Extracellular signal-related kinase 1/2 (ERK) activation [25, 26]. Thus, ECM proteins represent a potential pro-osteogenic stimulus for bone tissue engineering.

Interestingly, it is not only the sheer presence of collagen but also the type and structure that control the function of ECM. Examples where the structure of collagen affects bone are patients suffering from Bruck syndrome and osteogenesis imperfecta (OI). In the first case, deficiency of bone specific telopeptide lysyl hydroxylase results in aberrant crosslinking of bone collagen leading to osteoporosis, joint contractures, fragile bones and short stature [27]. In the case of OI, mutations affecting the structure or the abundance of collagen type I can be found in the majority of the patients resulting in bone abnormalities ranging from bone fragility to high bone mineralization [28-31]. Moreover, when hMSCs were proliferated on denatured collagen, they showed an increased retention of osteogenic potential compared to expansion on tissue culture plates [32].

Structural changes in the molecules that are the constituents of the matrix will likely result in structural changes in the matrix itself and, concomitantly, in changes in cell signaling [33].

Collagen undergoes several post-translational modifications which contribute to its structural and mechanical properties. Interference with some of these modifications results in severe dysfunction of the tissue in question. One of the last steps in the formation of the collagen molecule is the cleavage of the N and C pro-peptides, spontaneous self-assembly of the resulting collagen molecules into fibrils and formation of covalent crosslinks [34]. Collagen crosslink formation occurs in the extracellular space and it is initiated by the conversion of specific lysine or hydroxylysine residues into the aldehydes allysine or hydroxyallysine, respectively. This crosslink reaction is catalyzed by the enzyme lysyl oxidase [35, 36].

The post-translational modifications of collagen in bone and other mineralizing tissues differ from those in other types of collagen matrices [37]. Proper collagen crosslinking may be important for the binding of collagen to its receptors, but it may also be important to regulate the availability of growth factors present in ECM and for the mechanical properties of ECM [38-40].

In this manuscript we describe the effect of collagen crosslinking on the differentiation of MC3T3-E1 cells and hMSCs cells into the osteogenic lineage.

Materials and Methods

Cell culturing

Bone marrow aspirates (5-15 mL) were obtained from patients who had given written informed consent. hMSCs were isolated and proliferated as described previously [41]. hMSC proliferation medium was composed of minimal essential medium (α-MEM; Gibco), 10% foetal bovine serum (FBS; Lonza), 0.2 mM ascorbic acid (Asap; Sigma Aldrich), 2 mM L-glutamine (L-glut; Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco) and 1 ng/mL basic fibroblast growth factor (bFGF; Instruchemie, Delfzijl, The Netherlands). Basic medium was composed of hMSC proliferation medium without bFGF, hMSC osteogenic medium was composed of hMSC basic medium supplemented with 10^{-8} M dexamethasone (dex; Sigma) and hMSC mineralization medium was composed of basic medium supplemented with 10^{-8} M dex and 0.01 M β-glycerophosphate (BGP; Sigma).

The MC3T3-E1 cell line was purchased from Riken Cell bank (RCB 1126) and cultured in MC3T3-E1 basic medium consisting of α-MEM (Biowhittaker), 10% foetal calf serum (FCS; Hyclone), 2 mM L-glutamine (Invitrogen) and 100 U/mL penicillin (Life Technologies), 10 µg/mL streptomycin (Life Technologies). MC3T3-E1 control medium was composed of basic medium supplemented with 50 µg/mL Asap (Sigma) and 10 mM BGP. MC3T3-E1 mineralization medium was composed of control medium supplemented with 100 ng/mL rhBMP-2 (R&D systems).

Assessment of cell proliferation was performed using an Alamar blue assay according to the manufacturer's protocol. Briefly, culture medium was replaced with medium containing 10% (vol/vol) Alamar blue solution (Biosource, Camarillo, CA, USA) and cells were incubated at 37ºC for 4h. Fluorescence was measured at 590 nm on a Perkin Elmer LS50B plate reader.

Alkaline phosphatase flow cytometry and biochemical analysis

The effect of BAPN on alkaline phosphatase (ALP) expression of hMSCs was studied by flow cytometry on cells seeded at 1000 cells/cm² and grown under various conditions. Each experiment included a negative control (cells grown in hMSCs basic medium), a positive control (cells grown in hMSCs osteogenic medium) and one or more experimental conditions (0.04 to 0.5 mM BAPN). After 7 days of culture, cells were trypsinized and ALP expression was analyzed as described previously [42].

For MC3T3-E1 cells we analyzed ALP activity biochemically. To this end, cells were lysed in 0.2% Triton X-100 buffered with 0.1 M potassium-phosphate at pH 7.8. Subsequently, 40 µL of CDP-star reagent (Roche) was added to a 10 µL aliquot of cell lysate and incubated for 30 minutes in the dark. Chemoluminescence was measured in a Victor plate reader (Perkin Elmer, Wellesley, MA, USA). Acidic phosphatase (ACP) activity was used to correct for the cell number. To this end, 5 µL of cell lysate was combined with 100 µL of buffered substrate solution (2.7 mM 4-nitrophenyl phosphate/0.1 M NaAc, pH 5.5). After 90 minutes, the reaction was terminated by addition of 10 µL of 1M NaOH and the absorbance at 405 nm was determined.

Mineralization and calcium assay

For mineralization experiments, MC3T3-E1 cells were seeded at 5x10⁴cells/cm², in control and mineralization medium, in the presence or absence of 1.6 mM BAPN for 10 days. After this period the cells were lysed and matrix-associated calcium was solubilized in 150 µL of 0.5 M HCl at 37ºC overnight. A 2 µL aliquot of the solubilized calcium was combined with a solution of 0.25 M 2-amino-2-methyl-1,3-propandiol (AMPD), 8.5 mM 8-hydroxyquinoline and 190 µM o-cresolphthalein complexone (Sigma-Aldrich, Zwijndrecht, The Netherlands). Absorption was measured at 600 nm and compared to that of a standard curve of hydroxyapatite dissolved in 0.5 M HCl.

hMSCs were seeded in triplicate at 5000 cells/cm² in T25 culture flasks and incubated with 0.5 mM BAPN for 21 days. In each experiment, hMSC mineralization medium was used as a positive control and hMSC basic medium containing BGP as a negative control. The total calcium deposition was assayed using a calcium assay kit (Sigma Diagnostics) according to manufacturer's protocol. Briefly, the culture medium was aspirated, cells were washed twice with calcium- and magne-

sium-free PBS (Life Technologies) and incubated overnight with 0.5 M HCl on an orbital shaker at room temperature. The supernatant was collected and the calcium content was measured at 575nm (Perkin Elmer, Lambda 40). To visualize the calcium deposits, hMSCs were grown as mentioned above and fixed in formalin after 21 days of culture. One gram of alizarin red (Sigma) was added to 50 ml of demiwater and the pH adjust to 4.1 - 4.3. The solution was added for 3 minutes to the hMSCs previously fixed with formalin.

Collagen biochemical analysis

MC3T3-E1 cells were seeded onto 10 cm dishes in the presence of mineralization medium and cultured in the presence or absence of 1.6 mM BAPN. Collagen analysis was performed has previously described [35]. Briefly, MC3T3-E1 cells were grown for 16 days. Cultures were rinsed three times with PBS and extracted with 500 µL/10 cm dish of 1 M NaCl/ 0.05 M Tris/ 1 mM EDTA/ 10 mM N-ethylmaleimide (NEM)/ protease inhibitor (Roche) pH 7.5. After 5 minutes of incubation, cells were detached using a cell scraper, homogenized and centrifuged at $5x10^4$ g for 2h at 4ºC. The resulting supernatant was designated the neutral salt extract. Fifty micrograms of neutral salt extract was loaded on a 4-12% BIS/TRIS SDS-PAGE gel and stained with Coomassie blue.

To determine the level of collagen crosslinking and the collagen secreted by hMSCs we cultured the cells in basic or in mineralization medium, in the presence or absence of 0.5 mM BAPN. After 21 days, samples were washed three times with PBS and hydrolyzed (110ºC, 20-24h) with 750 µL 6 M HCl, dried and redissolved in 800 µL of water containing 10 µM pyridoxine (internal standard for hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP)) and 2.4 mM homoarginine (internal standard for amino acids; Sigma). Samples were diluted 5-fold with 1% (vol/vol) heptafluorobutyric acid (HFBA; Fluka) in 10% (vol/vol) acetonitrile for crosslinking analysis; aliquots of the 5-fold diluted samples were diluted 20-fold with 0.1 M sodium borate buffer (pH 8.0) for amino acid analysis. Derivation and subsequent chromatography of the amino acids, as well as chromatography of the crosslinks was performed as described [43, 44]. Collagen content was calculated from the total amount of Hyp in each sample, assuming 300 Hyp residues per collagen molecule and a molecular weight of collagen of 300,000 D.

RNA isolation and quantitative PCR

The effect of inhibition of lysyl oxidase on expression of osteogenic marker genes was analyzed by seeding hMSCs at 5000 cells/cm^2 in T25 flasks in osteogenic medium, with or without BAPN for 10 days. Total RNA was isolated using an Rneasy mini kit (Qiagen) and on column DNase treated with 10 U RNase free DNase I (Gibco) at 37ºC for 30 minutes. DNase was inactivated at 72ºC for 15 minutes. The quality and quantity of RNA was analyzed by gel electrophoresis and spectrophotometrically. 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 collagen type I (COLI) and 18s rRNA amplification and 1 µL of undiluted cDNA was used for other genes. PCR was performed on a Light Cycler real time PCR machine (Roche) using a SYBR green I master mix (Invitrogen). Data was analyzed using Light Cycler software version 3.5.3 using the fit point method by setting the noise band to the exponential phase of the reaction to exclude back-

ground fluorescence. Expression of osteogenic marker genes genes was calculated relative to 18s rRNA levels by the comparative ΔCT method [45].

Microarray analysis and statistical analysis

In order to analyze the effects of inhibition of lysyl oxidase on the gene expression profile of MC3T3-E1 cells and hMSCs undergoing osteogenic differentiation we performed a whole genome analysis.

A time course analysis was performed on MC3T3-E1 cells cultured in mineralization medium in the presence or absence of BAPN. At least two microarrays were hybridized per time point (0, 24, 36, 48, 60 and 72h). RNA was isolated using the Trizol reagent according to the protocol supplied by the manufacturer (Invitrogen, Breda, The Netherlands). cRNA labeling, hybridization of Affymetrix Mouse Genome 430A Genechips and data processing was performed as described previously [46].

Normalization and statistical analysis of the data was performed using the error model developed for Affymetrix GeneChips carried out using Rosetta Resolver Version 4.0 [47]. In the case of hMSCs, 5000 cells/cm^2 were seeded in T25 flasks in mineralization medium in the presence or absence of 0.5 mM BAPN. Total RNA was isolated using an Rneasy mini kit (Qiagen) and on-column DNase treated with 10 U RNase free DNase I (Gibco) at 37ºC for 30 minutes. DNase was inactivated at 72ºC for 15 minutes. The quality and quantity of RNA was analyzed by gel electrophoresis and spectrophotometrically. Affymetrix 900649 GeneChip® Human Exon 1.0 ST Arrays were hybridized and the fluorescence intensity was normalized by dividing the fluorescence intensity of each sample by the median intensity of the array. Statistical analysis was performed by application of a 2-way ANOVA model on each gene's profile using treatment and time as factors, resulting in p-values for the effect of treatment, time and for the interaction between treatment and time. These p-values were corrected for multiple testing using Benjamin-Hochberg (BH) resulting in estimated false discovery rates (FDR). Genes were ordered based on minimal FDR for the treatment effect or the interaction effect. Genes were defined as significantly differentially expressed if this minimal estimated FDR<=1%.

Focal adhesion assembly

To analyze the assembly of focal adhesions by hMSCs we cultured cells on glass coverslips in basic or osteogenic medium in the presence or absence of 0.5 mM BAPN. After 7 days, cells were fixed with 70% ethanol, permeabilized with 0.2% Triton X-100 in PBS for 20 minutes at room temperature and washed with PBS. Subsequently, samples were blocked with 10% FBS in PBS for 1h at room temperature. A mouse anti-human vinculin monoclonal antibody (clone V284, Santa Cruz Biotechnology) was used as primary antibody. Samples were incubated with 1:100 diluted primary antibody for 2h at 37ºC, washed with 0.05% Tween in PBS and incubated with 1:200 diluted secondary antibody (goat anti-mouse conjugated with AlexaFluor 488) and 1:40 phalloidin conjugated with rhodamine (Invitrogen) for 1h at 37ºC. Afterwards, samples were washed with 0.05% Tween in PBS, dried and fixed with mounting medium containing DAPI (Vectashield). Cells were imaged using a BD Pathway™ Bioimager.

Results

Inhibition of lysyl oxidase inhibits osteogenic differentiation of MC3T3-E1 cells

In order to analyze the role of collagen crosslinking in osteogenic differentiation of MC3T3-E1 cells we used BAPN, an irreversible inhibitor of lysyl oxidase, and analyzed its effects on collagen crosslinking, proliferation, ALP expression (an early marker of osteogenic differentiation) and calcium accumulation (late marker of osteogenic differentiation). The time points selected to analyze each of these parameters were based on previous studies for BMP-induced osteogenic differentiation of MC3T3-E1 cells [48].

Firstly, to confirm that BAPN was able to inhibit collagen crosslinking, we analyzed the form of collagen extracted from MC3T3-E1 cells cultured for 16 days in the presence or absence of 1.6 mM BAPN. In the presence of BAPN we observed an accumulation of non-crosslinked α1 and α2 tropocollagen chains (Figure 1A). This indicates that BAPN leads to a decrease in the level of collagen crosslinking, causing the accumulation of tropocollagen molecules in the neutral salt extract.

Secondly, to examine whether collagen crosslinking influences proliferation of MC3T3-E1 cells, we exposed the cells to increasing concentrations of BAPN, either in control or in mineralization medium. We observed that, independent of the medium, proliferation of MC3T3-E1 cells was not affected by BAPN (Figure 1B).

Thirdly, we analyzed ALP expression of MC3T3-E1 cells upon exposure to different concentrations of BAPN, both in control and mineralization medium. As expected, a robust induction of ALP levels was evident for the MC3T3-E1 cells in mineralization medium compared to control medium, due to the exposure to BMP-2 (Figure 1C). There was, however, no significant change in ALP expression in MC3T3-E1 cells cultured in control medium in the presence of increasing concentrations of BAPN. In contrast, when cultured in mineralization medium in the presence of increasing concentrations of BAPN, we observed a dose dependent decrease in ALP expression (Figure 1C).

Finally, in order to analyze the ability to deposit calcium, we cultured MC3T3-E1 cells in control or in mineralization medium, either in the presence or absence of 0.4 mM BAPN, for 10 days. Exposure of MC3T3-E1 cells to mineralization medium for 10 days resulted in a 3-fold increase in calcium accumulation relative to the control. Interestingly, when MC3T3-E1 cells were cultured in mineralization medium in the presence of BAPN, we observed a 2-fold decrease in calcium accumulation relative to MC3T3-E1 cells cultured in mineralization medium alone (Figure 1D).

In conclusion, BMP-2-induced ALP expression and calcium accumulation in MC3T3-E1 cells were repressed when the level of collagen crosslinking was reduced.

Lysyl oxidase activity is required for ECM gene expression of MC3T3 cells

To unravel the molecular mechanisms by which BAPN inhibits osteogenic differentiation of MC3T3-E1 cells we analyzed the gene expression profile of MC3T3-E1 cells undergoing osteogenic differentiation in the presence or absence of 1.6 mM BAPN. Figure 2A shows the expression intensities of several relevant transcription factors and ECM-related genes in BAPN-treated and control cells as a function of time. In accordance with the effect of BAPN on alkaline phosphatase enzymatic activity, expression of the cognate alkaline phosphatase gene (*AKP2*) was repressed in BAPN-treated MC3T3-E1 cells (Figure 2A). Moreover, we observed that many ECM-related genes and

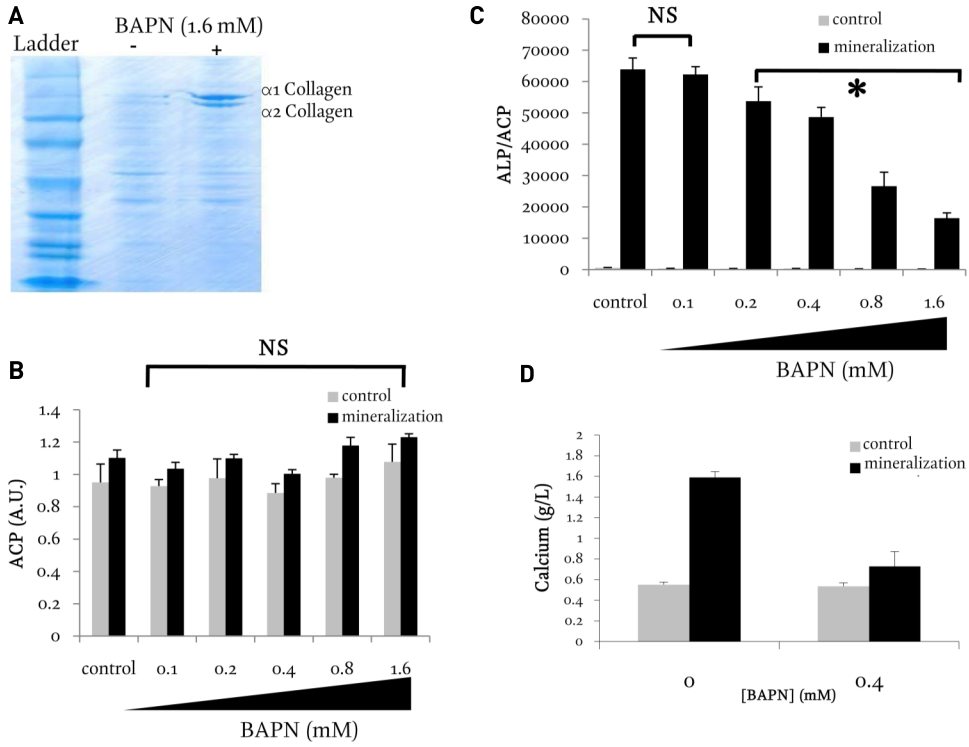
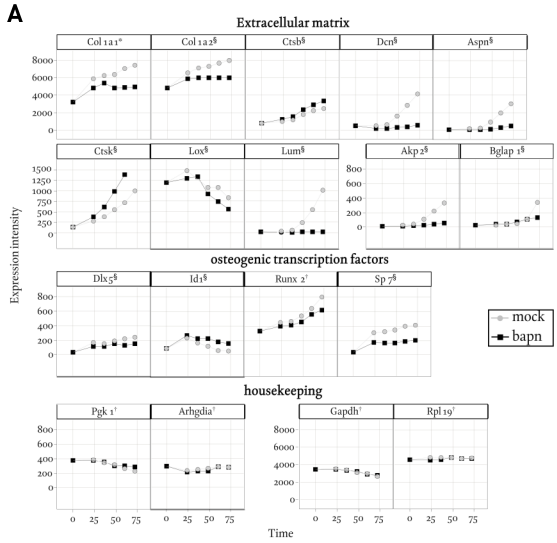


Figure 1. Inhibition of lysyl oxidase impairs *in vitro* osteogenesis of MC3T3-E1 cells. A. Collagen solubility of MC3T3-E1 cells grown for 16 days in the presence or absence of 1.6 mM BAPN. B. Proliferation of MC3T3-E1 cells in the presence of increasing concentrations of BAPN either in control or in mineralization medium. C. ALP expression by MC3T3-E1 treated with control or mineralization medium in the presence of different concentrations of BAPN. D. Calcium accumulation by MC3T3-E1 cells upon exposure to 0.4 mM BAPN for 10 days either in control or in mineralization medium.

osteogenic transcription factors were expressed at a lower level in MC3T3-E1 cells exposed to BAPN when compared to the control. The expression of the collagen type I α1 chain and the collagen type I α2 chain occur as early as 24h in mineralization medium and was lower when MC3T3-E1 cells were exposed to BAPN. The steep rise in collagen expression in control medium coincides with an increase in the expression of the enzyme responsible for the crosslinking - lysyl oxidase (Lox). Treatment with BAPN led to a lower level of Lox expression. Additionally, genes such as decorin, asporin and lumican, which are known to be involved in matrix assembly and are highly expressed in later stages of differentiation, were not induced when MC3T3-E1 cells were exposed to BAPN. The osteogenic marker BGLAP1 was also expressed at a low level upon exposure to BAPN in comparison with control cells. The lack of induction of the *BGLAP1* and *ALPL* genes coincides with the lack of induction of other ECM related genes, pointing at a relation between the expression of bone markers and ECM genes in osteogenic differentiation of MC3T3-E1 cells. Interestingly, transcription factors that control the osteogenic differentiation program (Runx2, Sp7 and Dlx5) showed a low expression upon exposure to BAPN in comparison with control cells. The lack of induction of the early transcription factors may explain the effects of BAPN on *LOX*, *COL1A1* and other ECM-

related genes, as these are genetically downstream of the Runx2-Sp7 pathway [49]. Although our data indicates that the majority of genes involved in osteogenic differentiation failed to be induced upon exposure of MC3T3-E1 to BAPN, some genes were upregulated. Among those, we observed an upregulation of cathepsins and some metalloproteinases (Figure 2A and supplementary data). Interestingly, a well known BMP-2 target gene – *ID1* – was upregulated upon exposure of MC3T3-E1 cells to BAPN. BAPN did not affect the expression of housekeeping genes indicating that the effect observed, either in ECM genes as well as in genes encoding transcription factors, was not the result of an a-specific effect of BAPN on the cellular processes.

Together, these results show that BAPN inhibits collagen crosslinking, and thereby affect the transcriptional program underlying osteogenic differentiation of MC3T3-E1 cells.



B

Gene	Control	BAPN	
COL1A1	7962	7647	Extracellular matrix
COL1A2	6900	7141	
DCN	4502	4470	
ASPN	244	157	
CTSB	5323	5693	
CTSK	1087	1507	
LOX	3260	3270	
LUM	1800	2319	
ALPL	661	621	
BGLAP1	197	181	
RUNX2	280	238	Osteogenic transcription factors
SP7	26	25	
DLX5	129	113	
ID1	256	184	
ARHGDIA	241	298	Housekeeping
PGK1	1973	2075	
GAPDH	867	796	
RPL19	51	33	

C

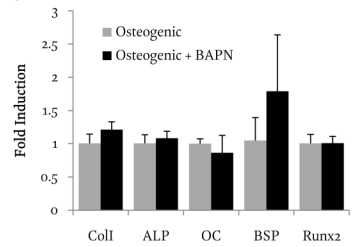


Figure 2. Gene expression profile of MC3T3-E1 and hMSCs upon treatment with a lysyl oxidase inhibitor. A. Panel of osteogenic related genes and their respective fluorescence intensity during osteogenic differentiation of MC3T3-E1 cells in the presence (squares) or absence of BAPN (circles). B. Gene expression profile of hMSCs in control and in BAPN treated cells for 10 days. The table shows the fluorescence intensity of the same genes as in panel A C. Gene expression profile of hMSCs culture for 10 days in control and in medium containing BAPN. Expression of a panel of osteogenic markers is indicated as fold induction compared to the control medium. Legend: *COL1A2* – procollagen, type I, alpha 2; *COL1A1* – procollagen, type I, alpha 1; *DCN* – decorin; *ASPN* – asporin; *LOX* – lysyl oxidase; *LUM* – lumican; *ALPL* – alkaline phosphatase 2, liver; *BGLAP 1* – bone gamma-carboxyglutamate protein, related sequence 1; *RUNX2* – runt related transcription factor 2; *SP7* – trans-acting transcription factor 7; *DLX5* – distal-less homeobox 5; *ARHGDIA* – RIKEN cDNA 6720463E02 gene; *PGK 1* – phosphoglycerate kinase 1; *GAPDH* – glyceraldehydes-3-phosphate dehydrogenase; *RPL 19* – ribosomal protein L19; *ID1* – inhibitor of DNA binding 1; *CTSB* – cathepsin B; *CTSK* – cathepsin K. (* FDR≤ 5%; § FDR≤ 0.1%, † FDR≥ 10%)

Inhibition of lysyl oxidase influences in vitro osteogenic differentiation of hMSCs

Next, we examined whether inhibition of lysyl oxidase has similar effects on our cell model for tissue engineering applications – hMSCs. When hMSCs were exposed to 0.5 mM BAPN, crosslinks were absent as indicated by the undetectable levels of the pyridinoline crosslinks HP and LP of the collagen present in the deposited matrix (in the absence of lysyl oxidase activity the telopeptide hydroxylysine residues can not be modified into aldehydes and as so crosslinking is absent), demonstrating that BAPN, as in the case of MC3T3-E1 cells, was able to inhibit the activity of lysyl oxidase in hMSCs (Figure 3A). This concentration of BAPN can be considered equipotent to the one used in the case of MC3T3-E1 cells since in both cases there was inhibition of lysyl oxidase. Moreover, upon exposure of hMSCs to BAPN, the collagen content was reduced when compared to the respective controls (a decrease of 54% in the case of basic versus basic + BAPN and 93% in the case of mineralization versus mineralization + BAPN) (Figure 3B). Additionally, there was a decrease in the Hyp/Pro ratio which represents the ratio of collagen versus non-collagenous proteins. When hMSCs were exposed to BAPN we observed a 32% decrease in the case of basic versus basic + BAPN and 57% in the case of mineralization versus mineralization + BAPN for the Hyp/Pro ratio (Figure 3C). Taken together, these results showed that BAPN was able to inhibit lysyl oxidase activity and impair collagen crosslinking of hMSCs.

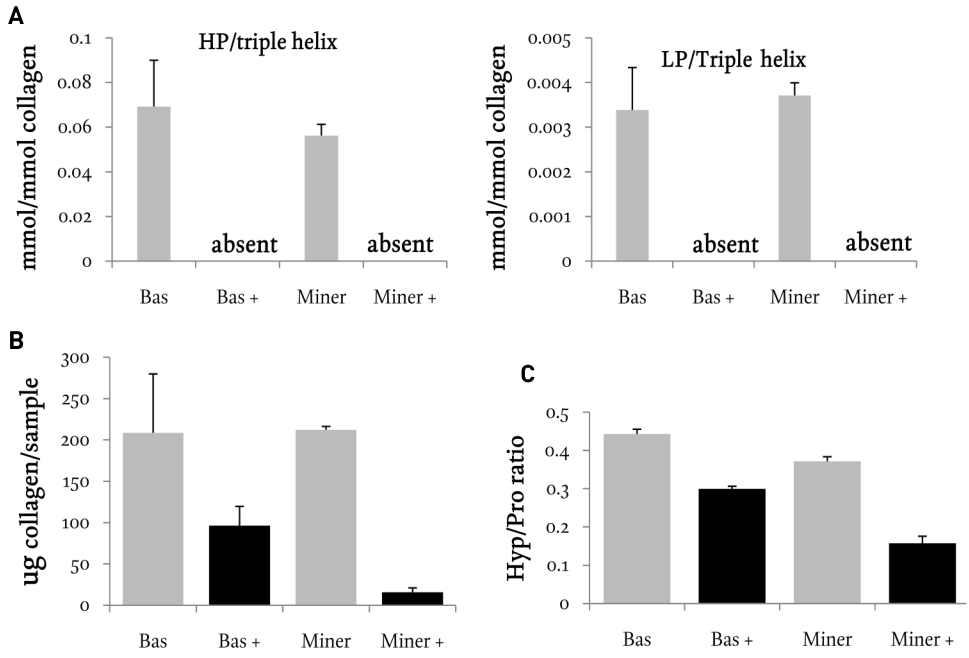


Figure 3. Collagen biochemical analysis in the presence of an inhibitor of collagen crosslinking. A Analysis of collagen crosslinking upon treatment with BAPN for 21 days. B. Collagen content upon exposure of hMSCs for 21 days to BAPN.. C. Effect of inhibition of crosslinking on Hyp/Pro ratio. Legend: Bas = basic medium, Bas + = basic medium plus BAPN, Miner = mineralization medium, Miner + = mineralization plus BAPN.

Given the fact that upon exposure to BAPN, hMSCs showed changes in collagen structure and synthesis, we analyzed if those changes would have an effect on integrin signalling. In order to analyze this we analyzed focal adhesion assembly in the presence or absence of BAPN. Our analysis showed that BAPN did not affect focal adhesion assembly (Figure 4A).

Subsequently, we analyzed the effect of lysyl oxidase inhibition on proliferation and ALP expression. We cultured hMSCs in basic (data not shown) and in osteogenic medium in the presence of increasing concentrations of BAPN for 7 days. BAPN did not affect proliferation of hMSCs during the period of culture considered (Figure 4B). Surprisingly, dex-induced ALP expression was not affected by exposing hMSCs to increasing concentrations of BAPN (Figure 4C).

Next, we performed a whole genome analysis on hMSCs exposed to BAPN. Exposure of hMSCs to BAPN for 10 days had very little or no effect on gene expression. In contrast with MC3T3 -E1 cells, we saw that upon exposure of hMSCs to BAPN, no ECM genes were downregulated at the time point considered. Additionally, there were no drastic changes in the expression of osteogenic transcription factors, as well as in the housekeeping genes, when hMSCs were exposed to BAPN (Figure 2B). To validate the results from the microarray, we performed qPCR for a panel of osteogenic marker genes in hMSCs cultured for 10 days in osteogenic medium in the presence or absence of 0.5 mM BAPN. Similarly to the results of the microarray, no statistical significant changes in the expression of these osteogenic genes was observed (Figure 2C). These results demonstrate a good correlation between the microarray measurements and qPCR results, as previously shown [46]. Because lysyl oxidase is involved in the crosslinking of collagen, which is the main component of the matrix deposited by terminally differentiated osteoblasts, we analyzed the effect of BAPN on matrix mineralization. We cultured hMSCs in basic or mineralization medium in the presence or absence of 0.5 mM BAPN for 21 days and measured calcium accumulation. Surprisingly, there was an up to 6-fold increase in calcium deposition by hMSCs treated with BAPN when compared to hMSCs treated with mineralization medium alone for all six donors tested. The increase in calcium deposition was observed only for hMSCs cultured in mineralization medium (Figure 4D).

Discussion

In vitro osteogenic differentiation of MC3T3-E1 cells and effects of lysyl oxidase inhibition

Differentiation of MC3T3-E1 cells into the osteogenic lineage is regarded as a matrix driven process. In the presence of ascorbic acid the cells deposit a collagen matrix which later will be mineralized in the presence of inorganic phosphate [50]. The synthesis and deposition of this collagen matrix provides a positive feedback signal to activate and maintain the osteogenic program. In the absence of that signal the cells do not undergo osteogenic differentiation [13]. The positive feedback signal provided by ECM can be affected by the structure and abundance of collagen. It has been shown that proliferation, as well as calcium deposition by MC3T3-E1 cells grown on denatured collagen, can be enhanced, dose-dependently, by adding increasing concentrations of micro fibrillar collagen to the culture system [51]. This shows that it is possible to interfere with the signal provided by the collagen matrix in order to enhance the osteogenic potential of MC3T3-E1 cells.

As previously shown, inhibition of lysyl oxidase inhibits collagen crosslinking and affects collagen structure resulting in the accumulation of soluble tropocollagen. Similar to the results showing

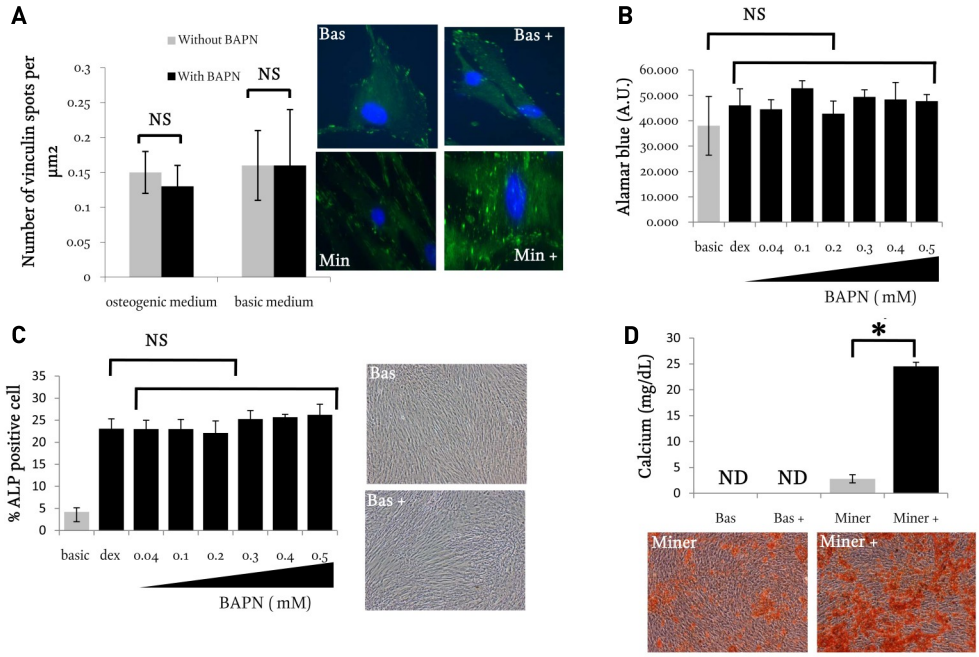


Figure 4. Inhibition of lysyl oxidase influences *in vitro* osteogenesis of hMSCs. A. hMSCs were cultured for 7 days in basic or osteogenic medium in the presence or absence of 0.5 mM BAPN. Nuclear staining was used to analyze the number of cells, actin staining was used to analyze the morphology and size of the cell and vinculin staining was used to analyze assembly of focal adhesion B. Proliferation of hMSCs cultured in osteogenic medium for 7 days in the presence of different concentrations of BAPN. C. ALP expression by hMSCs treated with different concentrations of BAPN in osteogenic medium for 7 days. D. Calcium accumulation by hMSCs cultured in basic and osteogenic medium in the presence or absence of 0.5 mM BAPN for 21 days. The pictures show the calcium distribution in the sample (alizarin red staining). Legend: Bas = basic medium, Bas + = basic medium plus BAPN, Miner = mineralization medium, Miner + = mineralization plus BAPN and ND = not detectable.

a decrease on BMP-induced ALP expression upon blocking of integrins, we observed a dose dependent decrease in ALP expression upon exposure of MC3T3-E1 cells to BAPN [52]. Moreover, our results showed that calcium deposition by MC3T3-E1 was also decreased when collagen crosslinking was inhibited. In the absence of collagen crosslinking the collagen is weakly associated with the cell membrane and it is more prone to proteolytic degradation [35]. As such, we can not rule out that the effects we observed are the result of lower abundance of collagen.

It has been shown that proliferation of MC3T3-E1 cells was impaired in the absence of ascorbic acid [53]. In our case, we did not observe effects on proliferation upon exposure to BAPN which indicates that sufficient levels of collagen was still present in our culture. Interestingly, treatment of MC3T3-E1 cells with BAPN led to a lack of induction of expression of the transcription factors Dlx5, Runx2 and Sp7. Since these factors are known to initiate osteogenic differentiation, their downregulation suggests a feed back mechanism in which ECM supports maintenance of expression of these early genes (Figure 5). The positive feedback signal provided by collagen upon stimulation with BMP-2 was abrogated by BAPN leading to a lack of induction of ECM-related genes. The decrease in the expression of ECM genes supports the observed decrease in mineralization as these proteins may act as mineralization nucleators [54]. Impairment of differentiation was not a result of inhibition

of BMP signaling because Id1, a direct target of BMP2-induced Smad signaling, was not negatively affected but was actually slightly upregulated upon exposure of MC3T3-E1 cells to BAPN. This suggests that the collagen matrix acts downstream of Smad signaling to enhance Dlx5, Runx2 and Sp7. The exact nature of the signal is currently unknown.

Effects of lysyl oxidase inhibition on in vitro differentiation of hMSCs

Because collagen plays an essential role in osteogenic differentiation of MC3T3-E1 cells, we were interested in analysing its role in hMSCs, which are widely used in bone tissue engineering. So far, mainly soluble factors have been used to enhance osteogenic differentiation of hMSCs, but it is technically not trivial to present these factors to the cells after implantation [22]. Exposure of implanted cells to ECM-coated scaffolds could be an interesting alternative. Unveiling the role of the collagen matrix on osteogenic differentiation of hMSCs is therefore required [9]. In that respect, work done by others showed that ECM deposited by MC3T3-E1 cells can affect the differentiation into the osteogenic lineage [55]. The immense potential of ECM has been demonstrated by using decellularization methods in order to remove the cellular components and seeding new cells onto the matrix. ECM produced in this way was able to influence the differentiation of the newly seeded cells and at the same time it provided an appropriate structure to support cell grow [2, 56-58].

We made two interesting observations. First, osteogenic differentiation of hMSCs is not sensitive to the level of crosslinking of the matrix, in contrast to MC3T3-E1 cells. Not only was ALP activity in hMSCs not influenced by inhibition of collagen crosslinking but also the global gene expression profile of hMSCs was virtually unchanged after exposure to BAPN for 10 days. Only 20 genes where regulated two-fold or more between BAPN-treated and untreated cells. Among those, no ECM genes were present. This indicates that the positive feedback loop provided by the crosslinked collagen matrix observed in MC3T3-E1 cells is absent in hMSCs. The decrease in collagen abundance observed in hMSCs exposed to BAPN could be attributed to an increase in proteolytic degradation of collagen due to a reduction in the number of crosslinks or due to the fact that most of the collagen was soluble and, as such, was not accounted for in the analysis.

The second interesting observation is the increase in calcium deposition upon exposure of hMSCs to BAPN. Normally, calcium deposition starts at specific residues in the collagen molecule, in particular in the gap junctions between adjacent collagen molecules. Inhibition of crosslinking may expose cryptic nucleation sites thus leading to enhance mineralization [59-61]. Gene expression data rule out an increase in matrix components as an explanation for enhanced mineralization. As such, we hypothesize that the absence of crosslinks in the collagen matrix provides more space for crystal growth in the collagen fiber. The observed increase in mineralization might be similar to the enhanced mineralization in osteogenesis imperfecta (OI) patients which can be due to an increase in the density of nucleation centers in the bone matrix of the patients [29]. Although collagen provides the structural framework for mineral deposition, non-collageneous proteins are responsible for the initiation and regulation of biomineralization [54]. Another hypothesis for the increase in calcium accumulation is that improper deposition of collagen results in the release of some non-collageneous proteins which will act as mineralization nucleators.

The results presented herein highlight the fact that the results gathered in one cell type cannot be directly extrapolated to another cell type without taking in consideration the species difference or even the origin and differentiation state of the cells [62]. In this particular case we analyse the role of collagen crosslinking in a commonly used cell line in bone cell biology – MC3T3-E1 cells and compare it to the more clinical relevant hMSCs. These cells are from different origins and show a different differentiation potential. Whereas hMSCs can differentiate into several lineages depending on the initial trigger, MC3T3-E1 are pre-committed into the osteogenic lineage. Furthermore, MC3T3-E1 cells originate from murine calvaria whereas hMSCs originate from human bone marrow. As such, we cannot exclude that the differences observed herein are due to the different origins of these two cells types.

Conclusion

In conclusion, we show that the differentiation of MC3T3-E1 cells into the osteogenic lineage is impaired as a result of inhibition of collagen crosslinking. Exposure of MC3T3-E1 cells to BMP-2 was sufficient to drive the osteogenic differentiation program leading to collagen production, which in turn fed back to osteogenic transcription factors. The inhibition of collagen crosslinking abolishes this positive feedback loop resulting in impaired osteogenic differentiation (Figure 5). In contrast, the osteogenic differentiation process of hMSCs was not affected by the levels of collagen crosslinking. We did however observe an interesting effect of crosslinking on mineralization.

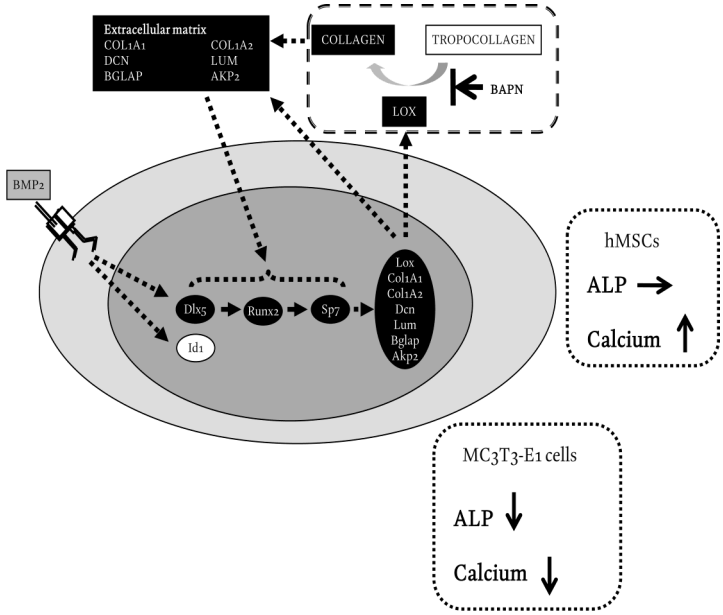


Figure 5. Proposed mechanism of action of BAPN on MC3T3-E1 cells. The figure depicts the osteogenic differentiation of MC3T3-E1 cells upon exposure to BMP-2 and ECM. In black are the genes and proteins negatively affected by BAPN whereas in white are the genes and proteins that were upregulated. Proteins are represented by square boxes whereas RNAs are in circles. Rounded dot squares indicate the phenotype on both hMSCs and MC3T3-E1 cells upon exposure to BAPN, whereas rounded dashed square represents a mechanism conserved in both cell types.

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Note

Supplementary data available upon request.

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Chapter 5

IN VIVO EVALUATION OF HIGHLY MACROPOROUS SCAFFOLDS FOR BONE TISSUE ENGINEERING

"We are all born ignorant, but one must work hard to remain stupid"

Benjamin Franklin

In vivo evaluation of highly macroporous scaffolds for bone tissue engineering

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Abstract

During the last decades different materials of both natural and synthetic origin have been developed with the aim of inducing and controlling osteogenic differentiation of mesenchymal stem cells. In order for that to happen it is necessary that the material to be implanted obey a series of requirements, namely: osteoconduction, biocompatibility and biodegradability. Additionally, they must be low-priced, easy to produce, shape and store.

Hydroxyapatite is a well known ceramic with composition similar to the mineral component of bone and is highly biocompatible and easy to obtain and/or process. On the other hand, collagen is the main structural protein present in the human body and bone. In this study, a polymer replication method was applied and a highly porous hydroxyapatite scaffold was produced. Collagen was later incorporated in order to improve the biological properties of the scaffold while resembling the bone composition. The scaffolds were characterized by means of scanning electron microscopy, fourier transform infrared spectroscopy and energy dispersive spectroscopy. *In vitro* and *in vivo* testing was performed in all scaffolds produced. The goal of this study was to evaluate the *in vivo* osteogenic potential of mesenchymal stem cells (MSCs) from two different species seeded on the different hydroxyapatite based porous scaffolds with collagen type I. The results indicate that all scaffolds exhibit relevant bone formation, being more prominent in the case of the HA scaffolds.

Introduction

During the last years, the tissue engineering field has emerged as one of the most promising areas for treatment of patients that have lost or damaged organs or tissues. Its aim has been the development of bone inducing therapies using a combination of materials and cells. However, a scaffold for bone tissue engineering has to comply to a series of requirements, namely: porosity, osteoconduction, biocompatibility, biodegradability and others [1]. Additionally, they should be easy to process, produce, store and more importantly, cost effective.

Ceramic scaffolds have been developed for many years for bone tissue engineering as key alternative to allografts or xenografts. One of the most widely used ceramic in this field is hydroxyapatite. This ceramic, with a composition similar to bone, has received attention for its excellent biocompatibility, biodegradability, easy processing and osteoconductivity properties [2, 3].

Additionally, the scaffold architecture has an important function when designing an implant for bone regeneration, since it may determine the rate and degree of bone ingrowth into the construct [2, 3].

Parameters such as macroporosity (which facilitates cell migration and enhances nutrient and ion transport); microporosity (to improve bone ingrowth due to its high specific area and provides attachment locations for osteoblasts) and pore interconnectivity (facilitates bone ingrowth, provides volume area necessary for vascularization to occur as well as removal of waste products) are crucial. On the other hand, collagen is the main structural protein present in the human body. The good biological properties of collagen, as well as its biocompatibility, biodegradability and easy processing have resulted in a frequented use of this protein for biomedical applications [4-9].

Hydroxyapatite is a biocompatible, osteoconductive calcium phosphate and can be easily combined with other materials in order to improve its properties. The most common examples are hydroxyapatite reinforced with synthetic polymers (e.g. Polyglycolic-Lactic Acid PGLA) or hydroxyapatite combined with natural polymers (e.g. chitosan and collagen) [10-22]. However, there is the need to improve collagen mechanical properties and rate of enzymatic degradation, thus leading to the employment of crosslinking procedures [4, 23, 24]. There are several methods to crosslink collagen, but the chemical methods are the most efficient. Agents such as glutaraldehyde, formaldehyde and diisocyanates introduce crosslinks between two e-amino groups of lysine and/or hydroxylysine residues and characterize the chemical crosslinking. Crosslinking of collagen using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) was shown to be non-cytotoxic *in vitro* and its biocompatibility was previously demonstrated [4, 23-26].

In order to obtain a scaffold that meets the criteria described previously, a highly porous hydroxyapatite scaffold was obtained using the polymer replication method [27]. To improve its biological properties and mimic the bone composition, collagen type I was coated onto the surface of the ceramic scaffold. In this way, the ceramic scaffold can act as a support template whereas the collagen can serve as a promoter of cell adhesion and proliferation [8].

The goal of this study was to evaluate the *in vivo* osteogenic potential of mesenchymal stem cells (MSCs) from two different species seeded on the different hydroxyapatite based porous scaffolds with collagen type I.

Materials and Methods

Scaffold preparation

The scaffolds were prepared using polyurethane (PU) sponges kindly provided by Recticel (Belgium). These PU sponges were impregnated with a ceramic slurry as reported previously [27]. Briefly, a ceramic slurry was prepared using several Hydroxyapatite (HA):Water:tensioactive ratios, with the best ratio being 6:4:0.2 [27]. The PU sponge was squeezed to remove the excess slurry and submitted to the following sintering cycle: heating at 1ºC/min followed by a 1h stage at 600ºC; heating at 4ºC/min followed by another stage of 1h at 1350ºC and then the samples were cooled inside the oven. Afterwards, the sponges were cut into 5 mm cubes.

Type I insoluble collagen (Sigma) was swollen overnight in 0.01 M HCl (Sigma) at 4ºC. The dispersion was later homogenized (Ultra Turrax T25, IKA) and filtered. HA scaffolds were impregnated with uncrosslinked collagen (HA COL) by applying vacuum as a driving force. To increase the mechanical properties of collagen, collagen the composites were crosslinked using the EDC/NHS conjugation method (HA COL XL) [4, 25, 26, 28, 29]. After the modification procedure, the composites were frozen in liquid nitrogen and lyophilized. The latter were sterilized by gamma irradiation by exposure to a Co source of 900,000 Ci (Gammaster, Ede, The Netherlands) until a total dose of 25 KGy was reached [60].

As a well established scaffold for bone tissue engineering, biphasic calcium phosphate (BCP) particles were used as reference material, which were prepared using the hydrogen peroxide foaming method as previously described [30, 31]. For the preparation of the ceramic, in-house made BCP powder was used. Porous green bodies were produced by mixing this powder with 2% hydrogen peroxide solution (1.0 g powder/1.2 ± 0.05 mL solution) and naphthalene particles (710-1400 m; 100 g powder/30 g particles) at 60°C. The naphthalene was then evaporated at 80°C and the green porous bodies were dried. Afterwards, the porous bodies were sintered at 1300°C for 8 hours.

The HA scaffolds and the BCP particles were sterilized by autoclaving (120ºC during 20 minutes).

Scaffold characterization

The porous structure of the scaffolds was analyzed by scanning electron microscopy (SEM), on which prior to observation, its surface was gold sputtered (Carringdon) and analyzed on a Philips XL 30 ESEM- FEG.

Cell culture

Six week-old male wistar rats were euthanized using CO_2. Under sterile conditions, femurs were removed and placed in culture medium (CM) consisting of α—minimal essential medium (α-MEM, Life Technologies, Baltimore, MD), 15% fetal bovine serum (FBS, Cambrex, Walkersville, MD), 100 U/mL penicillin (Life Technologies), 10 µg/mL streptomycin (Life Technologies), 0.2 mM ascorbic acid (Asap, Life Technologies), 2 mM L-glutamine (Life Technologies) and 10^{-8} M dexamethasone (Sigma, St. Louis, MO). The epiphyses were cut and the diaphyses flushed using a 21-gauge needle connected to a 10 mL syringe filled with the culture medium. The cells from two rats were pooled, resuspended and plated in four 75 cm^2 tissue culture flasks and grown at 37ºC in a humid atmos-

phere with 5% CO_2. Rat mesenchymal stem cells (rMSCs) were isolated by their adherence to tissue culture plastic. After two days, medium was changed and non adherent cells removed. Medium was refreshed twice a week until cells reached confluence. Upon confluence, cells were trypsinized with 0.25% trypsin - 1 mM EDTA for five minutes at 37ºC and counted.

Bone marrow aspirates (5-20 mL) were obtained from donors with written informed consent. Human mesenchymal stem cells (hMSCs) were isolated and proliferated as described previously [32]. Briefly, aspirates were resuspended using 20 G needles, plated at a density of $5x10^5$ cells/cm^2 and cultured in hMSCs proliferation medium (PM) consisting of á-MEM, 10% FBS, 0.2 mM Asap, 2 mM L-glutamine, 100 U/mL penicillin, 10 µg/mL streptomycin and 1 ng/mL basic fibroblast growth factor (bFGF, Instruchemie, The Netherlands). Cells were grown at 37ºC in a humidified atmosphere with 5% CO_2. After two days medium was changed and non adherent cells removed. Medium was refreshed twice a week until cells reach confluence. Upon confluence, cells were trypsinized with 0.25% trypsin - 1 mM EDTA for five minutes at 37ºC and counted.

hMSCs osteogenic medium (OM) was composed of hMSCs proliferation medium with 10^{-8}M dexamethasone but without bFGF.

Cell proliferation assay

To quantify the number of cells on the scaffolds prior to implantation we performed a CyQuant DNA assay (Invitrogen). Briefly, the scaffolds loaded with cells were gently washed with PBS and lysed using a 0.1% Triton X-100 solution in PBS. After sonication the samples were centrifuged for 10 minutes at 3500 rpm and 4ºC and the pellet was discarded. DNA was quantified using the CyQuantGR dye by measuring the fluorescence at 520 nm.

Scanning electron microscopy analysis

To analyze the distribution of the cells immediately after seeding and prior to implantation, the samples were analyzed by SEM. Briefly, the culture medium was removed, the samples were washed twice with PBS and fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylic acid buffer pH 7.3 for 30 minutes at room temperature.

Dehydration was performed with a series of ethanol solutions of increasing concentration beginning with 70% and progressing through 80%, 90%, 96% and 100% absolute ethanol. The samples were kept in absolute ethanol and transferred to the critical point dryer and dried using CO_2. The samples were golden sputtered (Carringdon) and analyzed on a Philips XL 30 ESEM- FEG.

Ectopic bone formation

To evaluate the *in vivo* bone forming potential of rat and human cells seeded on the different scaffolds, $1.5x10^5$ and $2x10^5$ cells in 150 µL of medium were seeded onto 5 mm HA scaffolds or 2-3 mm BCP particles, respectively. Three particles were used per condition and cells were left standing for 4h in 150 µL of medium. After 4h 2 mL of osteogenic medium was added and the cells were grown for 7 days prior to implantation. During this period the medium was refreshed once.

Six immune-deficient male mice (Hsd-cpb:NMRI-nu, Harlan) were anesthetized with isoflurane. Four subcutaneous pockets were made and on each pocket three particles loaded with cells

were implanted. The incisions were closed using a vicryl 5-0 suture. After 6 weeks the mice were sacrificed using CO_2 and samples were explanted, fixed in 1.5% glutaraldehyde (Merck, Rahway, NJ) in 0.14 M cacodylic acid (Fluka, Haupphage, NY) buffer pH 7.3, dehydrated and embedded in methylmethacrylate (Sigma) for sectioning. Approximately 10 µm-thick sections were processed on a histological diamond saw (Leica saw microtome cutting system). The sections were stained with basic fuchsin (stains newly formed bone pink) and methylene blue (stains the remaining fibrous tissue blue). The HA based and the BCP scaffolds remained unstained and appeared black in the sections. Quantitative histomorphometry was performed as previously described [33]. Briefly, five randomly selected sections were made from each sample and high resolution (5400 dpi) micrographs were made of these sections. Micrographs where pseudocolored (bone in red color and material in green color) and image analysis was carried out with a computer-based system equipped with KS400 version 3.0 software (Carl Zeiss Vision, Oberkochen, Germany). Prior to measurements, the system was geometrically calibrated with an image of a disk of known dimensions. A custom made macro was developed in KS400 to measure bone/ceramic surface ratios.

All experiments with animals were approved by the local Animal Experimental Committee.

Results

Scaffold characterization

Scaffold architecture was observed by SEM and displayed in figure 1. The HA scaffolds (Figure 1A) used in this study were characterized by a homogeneous macroporosity with a diameter larger than 300 µm and micropores of 1-10 µm with established interconnectivity [34]. Upon incorporation of collagen no significant changes were observed in terms of porosity (Figure 1B).

The mechanical properties of the HA scaffolds were evaluated (manuscript in press) [35]. The samples were evaluated under compressive mechanical testing, with a maximum compressive stress value of 0,07 ± 0,03 N/mm². These values are in accordance with the literature [36]. In addition, the results have indicated that the fracture is initiated at the scaffolds inner core and rupture was due to the reduced pore strut thickness [35].

In order to observe the presence of collagen on the HA scaffolds, energy dispersive spectroscopy (EDS) characterization was performed (Figure 2). The collagen coating was heterogeneous and characterized by a thin and dispersed mesh of collagen fibers deposited on the HA surface. The Fourier transform infrared (FTIR) spectrum of the HA scaffold with and without collagen type I was obtained and the characteristic peaks can be observed in Figure 3. There is a clear OH^- peak at 3571cm^{-1}, followed by some broad peaks between 2920 and 2002 cm^{-1} that may correspond to HPO_4^{2-} groups as previously reported in literature [37]. Phosphate u_3 bands were identified by two peaks at 1089 and 1037 cm^{-1}, whereas the u_1 band is present at 960 cm^{-1}. Furthermore, the phosphate u_2 band was also observed at 473 cm^{-1} being followed by phosphate u_4 bands at peaks 602 and 504 cm^{-1}, respectively. With the incorporation of collagen, typical amide bands appear at 3426, 1738, 1638 and 1539 cm^{-1}.

The BCP scaffolds used in this study had been previously characterized and are fully crystalline with a composition of 80±3% HA and 20±3% β-TCP (beta tricalcium phosphate) [30]. The scaffolds present highly interconnected macropores with an average pore size of around 400 µm. Using im-

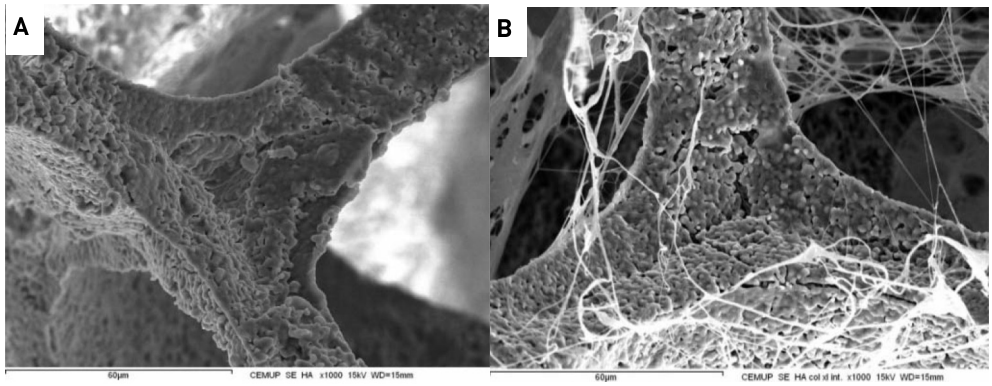

Figure 1 – SEM micrographs of hydroxyapatite (HA) based scaffolds used in the study. Figures represent the surface and the microstruture of HA scaffolds without collagen (A), and with crosslinked collagen (B). The HA based scaffolds present macroporosity (100-400 µm), microporosity (1 to 10 µm) and interconnected porosity. The incorporation of collagen did not affect the porosity of the materials, being the surface characterized by fine collagen coating and spread fibers (B). Scale bar: 60 µm.

age analysis of ESEM photographs and mercury porosimetry measurements, the total porosity and macroporosity were determined at 68±2% and 55±5%, respectively. Microporosity was also observed (3%), exhibiting micropores o. 7 µm in diameter.

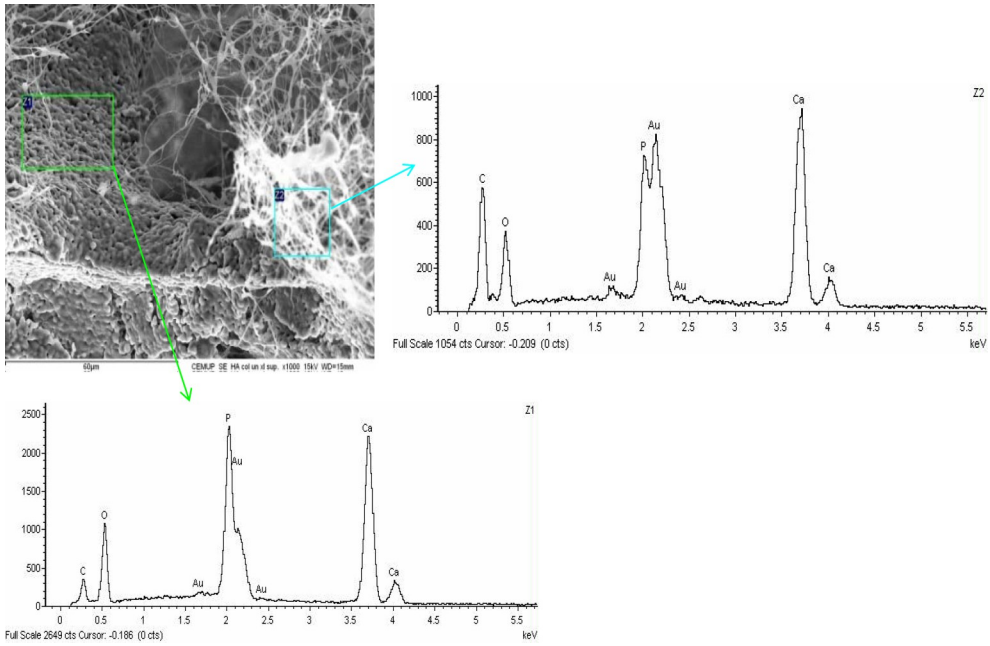

Figure 2 – SEM micrographs and EDS of the scaffold with collagen (HA COL). The figure shows the heterogeneous composition of the scaffold, showing HA (green box) and collagen (blue box) and their respective EDS graphs. Note the presence of the calcium and phosphorous peaks, as expected. After the collagen incorporation, the coating is characterized by the appearance of the carbon peak.

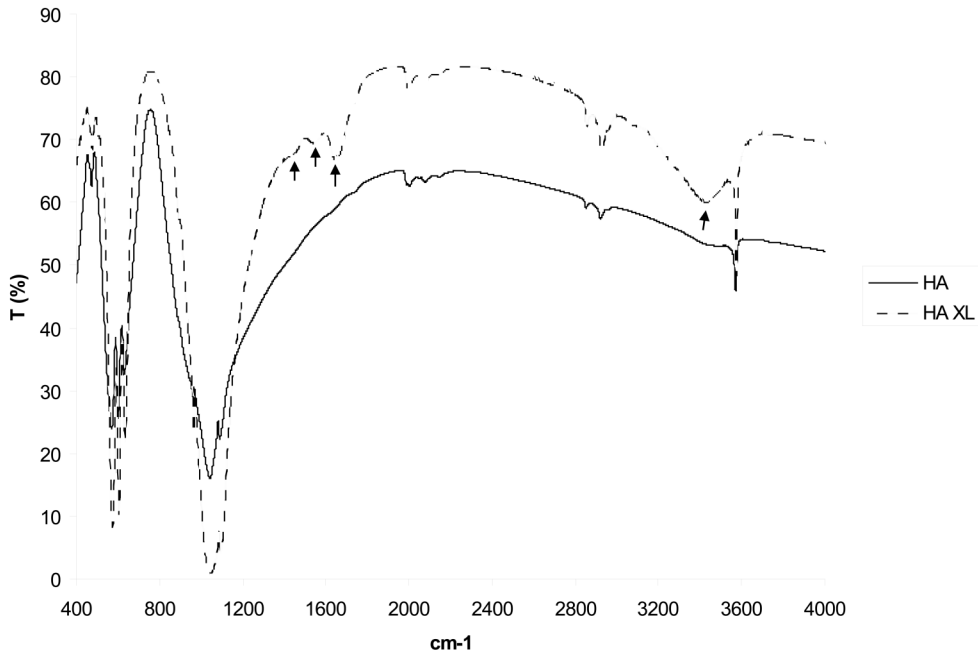

Figure 3 – FTIR spectra of the HA scaffold (HA) and HA with collagen (HA COL). The figure shows the presence of the characteristic peaks associated to both hydroxyapatite and collagen. Note the appearance of the typical amide bands upon the incorporation of collagen at 3426, 1738, 1638 and 1539 cm^{-1}. There is a clear OH^- peak at 3571 cm^{-1} and characteristic phosphate peaks at 1089, 1037 cm^{-1} and 960 cm^{-1}.

Cell number and distribution on the scaffolds

To analyze the distribution and quantify the cells in the scaffolds, SEM analysis and a DNA assay, respectively, were performed on the scaffolds loaded with hMSC and rMSC cells at day 1 (after seeding) and at day 7 (prior to implantation). In the case of HA scaffolds, the cells were well spread and present on several areas of the scaffolds whereas on the collagen coated scaffolds, the presence of a dense mesh of collagen fibers seemed to obstruct the cell migration into the pores. The control samples (BCP particles) showed good cell adhesion and proliferation even inside the pores during the 7 days of *in vitro* culture, as previously reported in literature (Figure 4) [30, 31].

The results of the DNA assay (Figure 5) at day 1 showed that there are more hMSCs than rMSCs on the scaffolds (as expected due to the different seeding densities). This showed that there were no differences in cell attachment between the two cell types since the higher values of hMSCs correspond to higher seeding density. Moreover, it was observed that the incorporation of collagen on the HA scaffolds had a negative effect on the number of cells in the case of rMSCs. Additionally, it was observed that collagen crosslinking resulted in a further reduction in cell numbers at day 1. In the case of hMSCs at day 1, it was seen that the incorporation of uncrosslinked collagen seems to have a positive effect on adhesion/proliferation when compared to HA alone. This effect disappears upon crosslinking of the collagen resulting in lower amount of DNA when compared to the control (HA scaffolds).

The results from the DNA assay (Figure 5) showed that there were no differences in cell attachment between rMSCs and hMSCs as can be seen at day 1. Since more hMSCs were implanted than rMSCs that explains the higher values for the DNA assay in the case of hMSCs. Moreover, the incorporation of collagen seems to have a negative effect on cell adhesion/proliferation in the case of rMSCs. The results at day 7 indicate that cell proliferation occurred during this period in all the scaffolds tested, as indicated by the higher values of DNA at day 7 when compared to day 1.

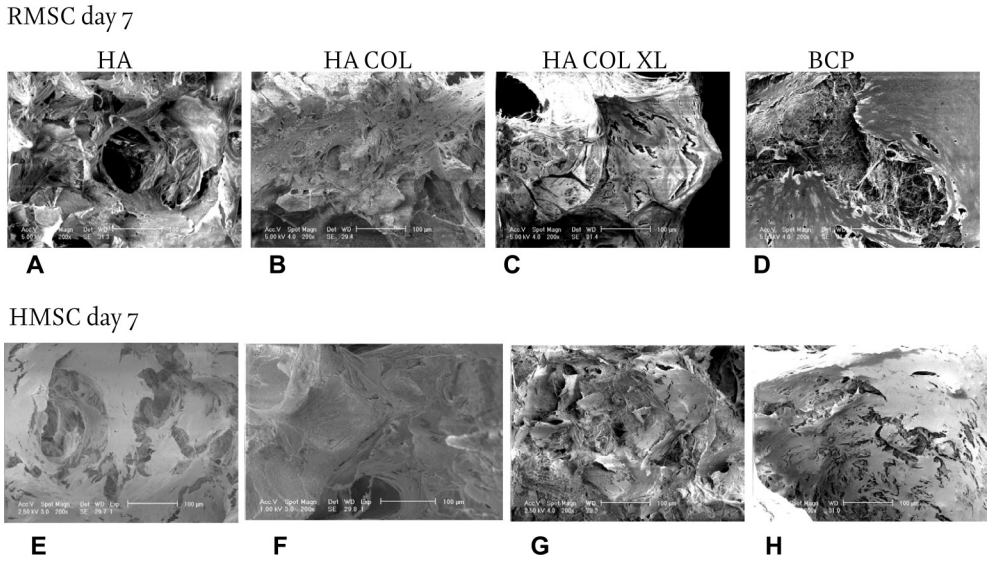

Figure 4 – ESEM micrographs of rat MSCs (A-D) and human MSC (E-H) seeded on HA scaffolds (A, E), HA COL (B, F), HA COL XL (C, G) and BCP (D, H) at day 7 respectively. The cells were cultured for 7 days in the presence of osteogenic medium. Note that the cells attached and proliferated onto the scaffold pores surface. Scale bar: 100 µm.

A difference was noticed between the number of rMSCs and hMSCs at day 1. This can indicate that rMSCs and hMSCs have different proliferation rates during the time period considered, with rMSCs proliferating slightly faster than hMSCs. Additionally, it was observed that the number of cells between HA and HA with uncrosslinked collagen were comparable whereas for the case of HA with crosslinked collagen there was a decrease in the number of cells. This indicates that the negative effect observed at day 1 for rMSCs on HA with uncrosslinked collagen was overcome whereas the positive effect seen on uncrosslinked collagen in the case of hMSCs at day 1 was absent at day 7. In both cells types, the uncrosslinked collagen resulted in a significant decrease on cell number both at day 1 and day 7 when compared with HA scaffold alone. It should be noted that the differences in cell numbers may be statistically significant, but the differences are relatively small.

Due to differences in the chemical composition and structure, the number of cells between HA scaffolds and BCP were not compared, since the later was included in the experiment only as a control.

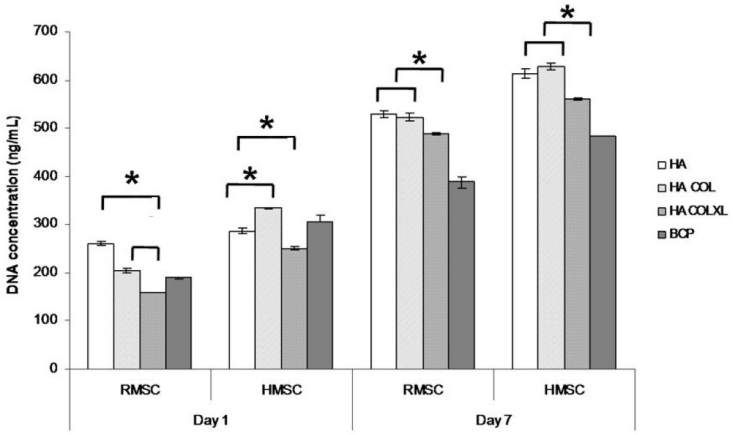

Figure 5 - DNA assay for rat and human MSCs seeded on the different ceramics. Rat and human MSCs were grown during 7 days in vitro in the presence of osteogenic medium. A quantitative DNA assay was performed at day 1 and 7 after seeding. The difference between rat and human can be explained by the different initial cell seeding densities ($1.5x10^5$ and $2x10^5$, respectively).

In vivo bone formation

To analyze the in vivo bone forming potential of rMSCs and hMSCs on HA scaffolds and HA scaffolds with collagen crosslinked or uncrosslinked, the cells were seeded onto the different scaffolds and implanted in immuno-deficient mice for 6 weeks.

Our results showed a significant difference in newly formed bone between rMSCs and hMSCs. We clearly saw that rMSCs surpass hMSCs regarding the bone forming potential, even though, initially, more hMSCs were seeded on the constructs (Figure 6). Moreover, comparing the effects of the different scaffolds it was observed that in the case of rMSCs, the presence of collagen does not have an beneficial effect when compared to the HA scaffold alone.

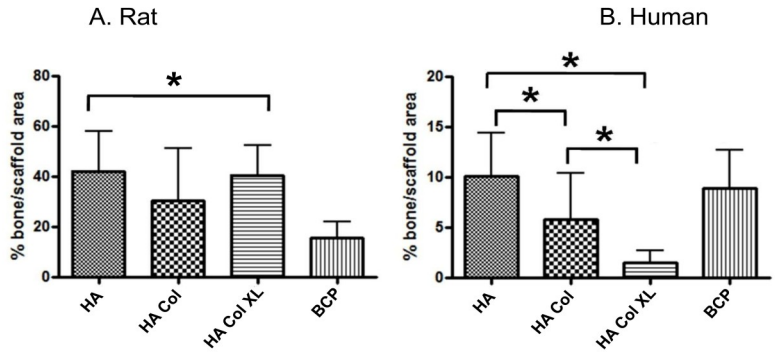

Figure 6 – In vivo bone formation by rat and human MSCs. Ceramics seeded with rat (A) or human (B) MSCs were implanted during 6 weeks in nude mice. The constructs were recovered after 6 weeks and stained for newly formed bone with basic fuchsin. At least 5 sections per scaffold were imaged and the percentage of bone per scaffold area was calculated. Note that in the case of rat MSCs the percentage of bone per scaffold area on the HA scaffolds was significantly different from the BCP whereas in the case of human MSCs there was a significant reduction on the percentage of bone per scaffold area for HA with collagen.

In contrast with rMSCs, it was observed that the incorporation of collagen in the HA scaffolds had a negative effect on the in vivo bone forming potential of hMSCs. Additionally, when the collagen was crosslinked, that difference was even more evident.

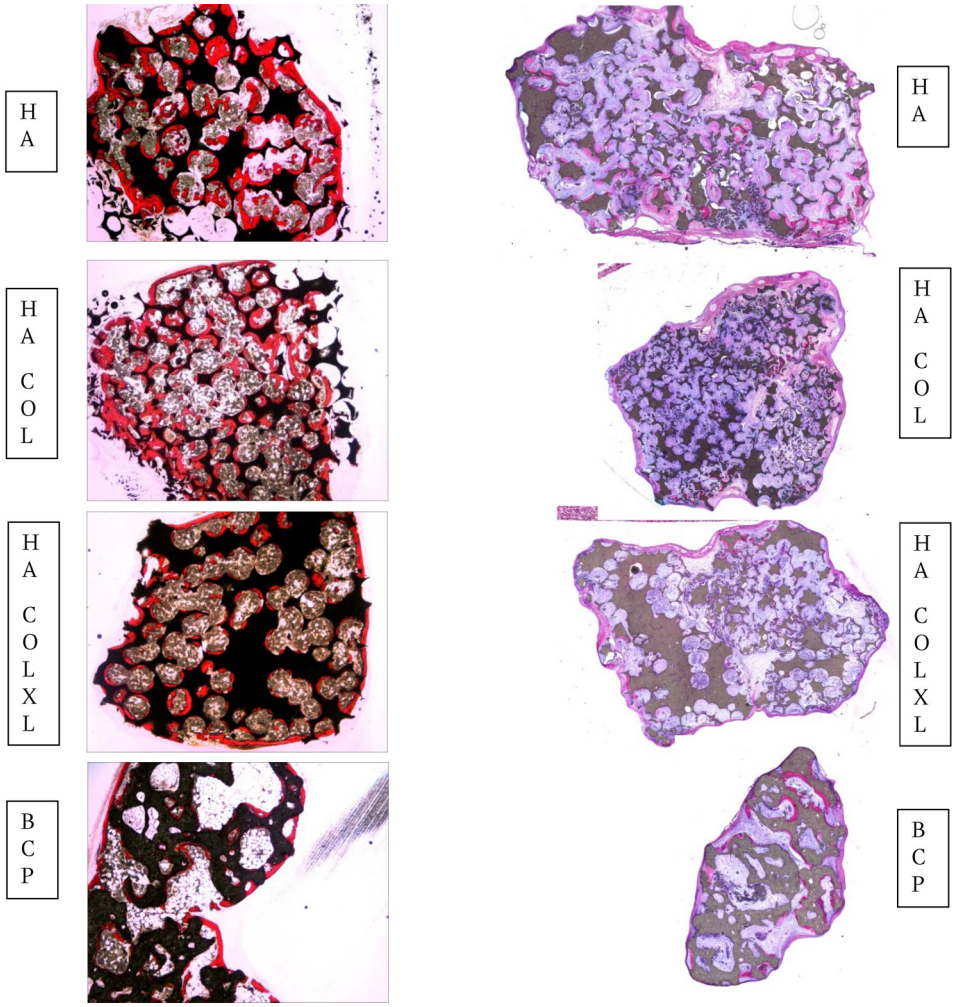

Figure 7 – Representative histological sections of the HA scaffold , HA COL, HA COL XL and BCP. New bone was formed and bone marrow by rat (left panel) and human (right panel) MSCs, respectively after 6 weeks implanted in nude mice. The newly formed bone is present in the periphery of the scaffold as well as on the pores interior Histological sections were stained with basic fuchsin and methylene blue. Magnification: 40X.

Discussion

When bone is damaged or lost it is necessary to treat the patients either by using autologous bone transplants or by using tissue engineered constructs with the potential to form bone *in vivo*. A relevant amount of bone graft is necessary to treat these patients. In this study highly porous HA scaffolds were developed. In order to improve their biological performance, collagen type I was successfully incorporated on the HA scaffolds. The results showed that the incorporation of collagen did not hinder the cell attachment and proliferation, as well as the *in vivo* bone forming potential, when compared to HA scaffolds alone.

The data illustrated the presence of a fiber mesh upon incorporation of collagen on the HA scaffolds. Moreover, on the crosslinked collagen samples, this fine mesh seems denser, which can explain, on its turn, the lower number of cells on the scaffolds for this condition on both cell types. A denser mesh of collagen may delay the migration of the cells onto the scaffold.

In this study, different initial cell seeding densities were used for rMSCs and hMSCs because rat MSCs typically proliferate faster than human MSCs. The data presented in this study shows that after 7 days of *in vitro* proliferation on the scaffold, the number of rMSCs was still lower than the number of hMSCs, which indicates that the initial difference in the seeding density was maintained. Nevertheless, that difference was slightly reduced, after 7 days (the ratio rMSCs vs hMSCs for each of the scaffolds changed between day 1 and day 7). A significant difference in the amount of newly formed bone was observed between rat and human MSCs. This difference could not be attributed to the scaffolds used since the same scaffolds for both cell types were used.

One hypothesis is related with a species difference on the differentiation potential between the two cell types [38]. Additionally, Olivo *et al* showed that proliferating cells can be necessary in order to have *in vivo* bone formation [39]. This fact raises the possibility that in the case of rMSCs, proliferation advances upon implantation, whereas in the case of hMSCs cells stop proliferating upon implantation hence, less bone is formed. Moreover, since rMSCs were exposed to dexamethasone (an osteogenic stimulus) immediately after isolation, that might have triggered the cells towards the osteogenic lineage, whereas in the case of hMSCs the cells were only exposed to an osteogenic stimulus 7 days prior to implantation which might not be sufficient to differentiate all the cells in the osteogenic lineage [38].

Interestingly, the incorporation of collagen on the HA scaffolds did not affect the *in vivo* bone formation in the case of rMSCs, in sharp contrast with the case of hMSCs. Despite the fact that for both cell types, the crosslinking of collagen resulted in a reduction of proliferation of the cells on the scaffold prior to implantation, that reduction was not reflected in the amount of bone formed *in vivo* by rMSCs. This indicates that for rMSCs, other factors rather than the initial cell numbers, influence the *in vivo* bone forming potential. As mentioned above, it is believed that the potential of the cells to proliferate *in vivo* is a crucial factor that dictates the final outcome. The incorporation of collagen on the HA scaffolds does not enhance the proliferation of rMSCs onto the scaffolds more than in the HA alone. This is in line with the DNA assay at day 7 where the presence of collagen does not result in higher values of DNA.

Moreover, the incorporation of collagen may affect signals affecting the proliferation and differentiation of hMSCs and those signals can be different depending on the level of crosslinking of the

collagen. It is not possible to exclude a toxic effect due to the crosslinking of the collagen, on the *in vivo* bone formation. The stabilization of the scaffold by the two different cell types can also influence the *in vivo* bone formation potential. The proliferation of rMSCs but not of hMSCs stabilizes the collagen fibers whereas in the case of hMSCs the lack of stabilization may result in degradation of the fibers resulting in loss of cells from the construct since, as seen on SEM, the presence of collagen fibers works as a sieve where most of the cells are entrapped.

Another parameter affecting bone formation is the degradation of the scaffold with a concomitant release of calcium and phosphate ions. It has been shown that BCP and HA scaffolds implanted during one year in the femoral cortical bone of dogs did not show signs of degradation [40]. Moreover, the scaffolds we implanted were sintered at higher temperature than the ones used in the above mentioned study resulting in decrease on the degradation rate [41, 42].

Conclusion

In this study, different types of scaffolds for bone tissue engineering were evaluated. The scaffolds produced met key properties that bone tissue engineering scaffolds should exhibit, namely: macroporosity, microporosity and pore interconnectivity. Macropores with diameter greater than 100 μm were achieved as well as a highly defined microporosity with pores ranging from 1 to 10 μm. Interconnected porosity was also achieve as observed by SEM and confirmed by other techniques such as μ-CT [34].

Analyzing the bone forming ability of cells seeded on the different scaffolds it was observed that relevant amounts of bone were formed, regardless the presence of collagen, for rMSCs. In the case of hMSCs, it was found that the HA scaffolds performed as good as the control scaffold (BCP). Nevertheless, the presence of collagen had a negative effect on *in vivo* bone formation, independently of the crosslinking process.

Acknowledgments

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Chapter 6

EFFECT OF CHORDIN-LIKE 1 ON MC3T3-E1 AND HUMAN MESENCHYMAL STEM CELLS

"There is only one good, knowledge, and one evil, ignorance"

Socrates

Effect of chordin-like 1 on MC3T3-E1 and human mesenchymal stem cells

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Abstract

Since the discovery that bone morphogenetic proteins (BMPs) are able to induce ectopic bone formation, considerable effort has been devoted to apply it for bone regeneration. BMP activity needs to be temporally and spatially controlled and the organism has devised ways to achieve it. Here we show that the BMP inhibitor chordin-like 1 can interfere with BMP-2 signalling thereby affecting the osteogenic differentiation of MC3T3-E1 cells. Besides its function as a BMP antagonist, we observed that chordin-like 1 enhances the proliferation of human mesenchymal stem cells (hMSCs) in a BMP-2-independent manner. When MC3T3-E1 cells were exposed to recombinant chordin-like 1 there was an inhibition of ALP expression whereas in the case of hMSCs no effect was observed. However, chordin-like 1 enhanced dose-dependently the proliferation of hMSCs. This effect is probably BMP-2-independent because the chordin-like 1 concentration that stimulates proliferation does not interfere with BMP signalling monitored by a Smad-dependent reporter gene. Our data point towards a novel BMP-independent role of chordin-like 1 in hMSC proliferation.

Introduction

The seminal discovery of bone morphogenetic proteins (BMPs) spurred by the research of Urist on osteoinductivity opened a new era in the field of bone biology [1]. Urist showed that decalcified bone has the potential to induce de novo bone formation when implanted in extraskeletal sites. The factor responsible for this phenomenon was later isolated and designated BMP. The intrinsic potential of BMPs to induce bone formation in ectopic locations has been explored ever since.

BMPs belong to the transforming growth factor – β (TGF-β) superfamily of growth factors. They bind to a heterodimeric complex of transmembrane receptors termed type I and type II which have serine-threonine kinase activity. Upon ligand binding, the type II receptor phosphorylates the type I receptor thus activating the type I kinase. This kinase will phosphorylate members of the mothers against decapentaplegic (Smad) family of transcription factors which will translocate into the nucleus to activate BMP target genes. This pathway is designated the canonical BMP signalling pathway [2-4].

To date, more than 20 different BMPs have been identified and implicated in several developmental processes and functions in the adult body such as bone remodelling, wound healing, haematopoiesis, immune response and neuronal plasticity among others [5]. For example, during embryonic development, a BMP-4 gradient is responsible for the ventralization process. When BMP-4 is blocked, the ventral mesoderm develops as dorsal mesoderm resulting in impaired embryonic development [6]. Because BMPs generally act as morphogens, their activity is tightly regulated at different levels in the signalling cascade. The ability of BMP binding to its receptor is controlled by several structurally different extracellular antagonists, such as noggin, gremlin, follistatin and chordin. Moreover, pseudo-receptors such as BAMBI can modulate BMP signalling at the receptor levels whereas inhibitory Smads and Smurfs can control it at the intracellular level [7,8]. Failure to modulate BMP signalling can severely affect skeletal homeostasis. For example, fibrodysplasia ossificans progressiva (FOP) is a rare genetic disease characterized by congenital malformations of the great toes and by progressive heterotopic bone formation. FOP patients have a constitutively active BMP type I receptor which can activate downstream signalling even in the absence of ligand binding [9-13]. Moreover it has been shown that, in contrast with control cells lines, cell lines derived from FOP patients did not show an increase in the expression of BMP antagonists such as noggin or gremlin upon stimulation with BMP-4, indicating an impairment in the auto-regulatory feedback loop that controls the magnitude and boundaries of BMP signalling. Furthermore, FOP cells showed a dramatically attenuated response to BMP-4 stimulation compared with control cells. This phenotype highlights the importance of BMP modulation in the regulation of bone formation [14].

Chordin-like 1, also known as chordin-like, ventroptin or neuralin is a secreted glycoprotein containing three characteristic cysteine-rich repeats (CRs) also known as von Willebrand factor C domains (vWF-C) [15]. The CR1 and CR3 regions of chordin-like 1 are most homologous to the CR3 of chordin. In the case of chordin, the CR1 and CR3 possess the BMP-binding capacity although the activity of individual CRs is lower than the full length chordin [16-18]. These CRs are conserved in many other proteins such as von Willebrand factor, connective tissue growth factor (CTGF), procollagens, amnionless, cysteine-rich motor neuron protein (CRIM-1), Nel (neural tissue protein containing Egf-like domains), Nel-like 1 and 2, Kielin/chordin-like protein and crossveinless-2 and are

responsible for the interaction with BMPs [16, 19]. In most cases, the CR domain-containing proteins act as BMP antagonist. However, in the case of kielin/chordin-like protein, and unlike previously described proteins containing CR domains, the CR domains are responsible for its potent effect as a BMP enhancer. Kielin/Chordin-like protein increases the affinity of the ligand to the receptor and/or enhances the stability of the ligand-receptor complex [20]. Additionally, crossveinless-2 can either potentiate or antagonize BMP signalling [21]. This indicates that not all proteins containing CR domains have an inhibitory effect on BMP signalling. Chordin-like 1 differs from chordin in the number of CR domains and in the spatial and temporal expression. Mouse chordin-like 1, sharing 93% homology with human chordin-like 1, is predominantly expressed in mesenchyme-derived cells and chondrocytes of the developing skeleton as well as in adult marrow stromal cells [18]. This expression pattern suggests that chordin-like 1 may play a role in skeletal development.

Recently, a novel member of the chordin family was identified and due to its homology to chordin-like 1, it was designated as chordin-like 2. The expression of chordin-like 2 is restricted to chondrocytes of various developing joint cartilage surfaces and connective tissues in reproductive organs [22, 23].

Chordin-like 1 sequesters the BMP in the extracellular space, thus inhibiting binding to its receptor [8, 15, 18, 24]. Moreover, chordin-like 1 contains potential BMP-1 target sites between CR2 and CR3. BMP-1 is a zinc metalloprotease which has been shown to cleave the chordin protein at defined residues, inactivating its biological activity with the concomitant release of free active BMP [25, 26].

Given the fact that BMPs are potent inducers of bone formation it is important to deliver them in the right place at the right time to avoid undesirable side effects. In tissue engineering, a lot of effort has been put on systems to deliver, in a controlled manner, BMPs [27-30]. As mentioned previously, BMP activity can be controlled by antagonists at different levels. Some proteins, such as noggin and chordin, bind directly to the molecule, thereby inhibiting binding to the receptor. Other molecules, such as heparin, sequester it so it will be available at a later time-point [31]. The reversible interaction antagonist-BMP and the subsequent release of active BMPs opens the possibility for tissue engineers to use the knowledge gathered from the studies involving BMP to engineer systems able to release not only the active BMP but also BMP antagonists that could fine-tune the signalling pathway. In this way, spatially complex patterns of BMP activity can be achieved to generate tissue engineered grafts.

In this manuscript, we analyzed the role of the BMP inhibitor chordin-like 1 on proliferation and osteogenic differentiation of MC3T3-E1 cells, a model cell line commonly used in bone tissue engineering, and hMSCs, multipotent cells used in tissue engineering applications and with potential clinical application [32].

Materials and methods

Cell culturing

Bone marrow aspirates (5-15 mL) were obtained from patients who had given written informed consent. hMSCs were isolated and proliferated as described previously [33]. hMSC proliferation medium was composed of minimal essential medium (α-MEM; Gibco), 10% foetal bovine serum (FBS;

Lonza), 0.2 mM ascorbic acid (Asap; Sigma), 2 mM L-glutamine (L-glut; Gibco), 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco) and 1 ng/mL basic fibroblast growth factor (bFGF; Instruchemie, Delfzijl, The Netherlands). Basic medium was composed of hMSC proliferation medium without bFGF and hMSC osteogenic medium was composed of hMSC basic medium supplemented with 10^{-8} M dexamethasone (dex; Sigma).

The MC3T3-E1 cell line was purchased from Riken Cell bank (RCB 1126) and cultured in MC3T3-E1 basic medium consisting of α-MEM (Biowhittaker), 10% foetal calf serum (FCS; Hyclone), 2 mM L-glutamine (Invitrogen), 100 U/mL penicillin (Life Technologies) and 100 µg/mL streptomycin (Life Technologies). MC3T3-E1 control medium was composed of basic medium supplemented with 50 µg/mL Asap (Sigma) and 10 mM BGP. MC3T3-E1 osteogenic medium was composed of control medium supplemented with 100 ng/mL rhBMP-2 (R&D systems).

Assessment of cell proliferation was performed using an Alamar blue assay according to the manufacturer's protocol. Briefly, culture medium was replaced with medium containing 10% (vol/vol) Alamar blue solution (Biosource, Camarillo, CA, USA) and cells were incubated at 37ºC for 4h. Fluorescence was measured at 590 nm on a Perkin Elmer LS50B plate reader.

Alkaline phosphatase flow cytometry and biochemical analysis

The effect of different concentrations of chordin-like 1 on alkaline phosphatase (ALP) expression of hMSCs was studied using a biochemical assay and flow cytometry. For the biochemical assay, hMSCs were seeded at 1000 cells/well in a 96-well plate for 5 days. After 5 days, cells were lysed in 0.2% Triton X-100 buffered with 0.1 M potassium phosphate at pH 7.8. Subsequently, 40 µL of CDP-star reagent (Roche) was added to a 10 µL aliquot of cell lysate and incubated for 30 minutes in the dark. Chemoluminescence was measured in a Victor plate reader (Perkin Elmer, Wellesley, MA, USA). Alternatively, samples were lysed with 0.2% Triton X-100 in PBS and ALP activity was determined in 100 µL aliquots of cell lysate using 100 mL of 20 mM pNPP (*p*-Nitrophenyl phosphate; Sigma) as a substrate. The resulting absorbance at 450 nm was recorded after 30 min incubation at 37ºC.

In the case of ALP measured by flow cytometry, hMSCs were seeded at 1000 cells/cm² and grown for 5 days in basic or osteogenic medium. Chordin-like 1 (100 nM; R&D systems) and BMP-2 (3.85 nM; R&D systems) were added to the medium alone or in combination. After 5 days, an Alamar blue assay was performed and ALP expression was analyzed using FACS as previously described [34].

For MC3T3-E1 cells, a biochemical assay was used to analyze the effect of different concentrations of chordin-like 1 on ALP expression following the instructions of the manufacturer of the CDP -star reagent (Roche Diagnostics, Almere, The Netherlands). Acidic phosphatase (ACP) activity was used to correct for the cell number. To this end, 5 µL of cell lysate was combined with 100 µL of buffered substrate solution (2.7 mM 4-nitrophenyl phosphate/0.1 M NaAc, pH 5.5). After 90 minutes, the reaction was terminated by addition of 10 µL of 1M NaOH and the absorbance at 405 nm was determined.

Transfection and reporter assay

C2C12 cells were seeded at 20,000 cells/cm² in a 6-well plate and transfected with the BRE-Luc and CMV-renilla reporters [35]. Briefly, 2 µg of BRE-Luc and 80 ng of CMV-Renilla were mixed with 4.16 µL of Fugene6 (Roche) in Optimem (Invitrogen) in a final volume of 100 µL. After 30 minutes, medium was discarded, and DNA-Fugene6 mix was added. After 4 hours, the medium was discarded and DMEM with 10% FBS was added. The next day, cells were trypsinized and seeded in a 96-well plate (10,000 cells per well). After 8h, chordin-like 1 and BMP-2 were added to the medium. Next day, a dual-luciferase assay was performed according to the manufacturer's protocol (Invitrogen).

Microarray analysis

To study gene expression as a function of osteogenic differentiation, MC3T3-E1 cells were cultured in osteogenic medium. Every three days, the medium was replaced with fresh osteogenic medium. For a second batch of cells, osteogenic medium was added for the first three days and substituted by control medium for the remaining culturing period. Cells were harvested at 0, 16, 32, 56, 72, 144, 176 and 200 hours and RNA was isolated using the Trizol reagent according to the protocol supplied by the manufacturer (Invitrogen, Breda, The Netherlands). cRNA labeling, hybridization of Affymetrix Mouse Genome 430A Genechips and data processing was performed as described previously [36].

Statistical analysis

The data was analyzed using Student's paired t-test ($P<0.05$).

Results

Chordin-like 1 is regulated during osteogenic differentiation of MC3T3-E1 cells and inhibits ALP expression

MC3T3-E1 cells were differentiated into the osteogenic lineage using BMP-2 and ascorbic acid and gene expression levels were monitored over time using microarrays. Temporal microarray analysis indicated that the expression of chordin-like 1 was up-regulated during early osteogenic differentiation of MC3T3-E1 cells (Figure 1A). Chordin-like 1 expression increased simultaneous with the markers of osteogenic differentiation such as alkaline phosphatase and osteocalcin during the first three days, after which its expression decreased. However, when BMP-2 was removed from the medium after three days, its expression continued to increase up to six days. The fact that chordin-like 1 expression was up-regulated early in osteogenic differentiation led us to analyze the role of human recombinant chordin-like 1 protein on proliferation and ALP expression of MC3T3-E1 cells. As shown in Figure 1B, human chordin-like 1 inhibited BMP-2 induced ALP expression of MC3T3-E1 cells, dose dependently (Figure 1B). Acidic phosphatase activities did not change with the chordin-like 1 treatment, indicating that cell numbers were not affected by the protein (Figure 1C). It should be noted that a more than 60-fold excess of chordin-like 1 to BMP-2 was necessary to detect an effect on ALP expression (Figure 1B).

Chordin-like 1 effect on ALP expression and proliferation of hMSCs

Our next goal was to analyze the effect of chordin-like 1 on osteogenic differentiation of hMSCs. We exposed hMSCs to increasing concentrations of chordin-like 1 in basic and osteogenic medium for 5 days and measured ALP expression using a biochemical assay. Addition of chordin-like 1, either to basic (data not shown) or to osteogenic medium, did not affect ALP expression (Figure 2A). Interestingly, when hMSCs were exposed to chordin-like 1 for 5 days, a dose-dependent increase in proliferation was observed compared to hMSCs exposed to dexamethasone alone (Figure 2B). This effect on proliferation was observed for 6 different donors (data not shown). For the concentration of chordin-like 1 that showed the maximum effect in proliferation (100 nM), one out of four donors showed an increase in ALP activity (Figure 2A and 2C). The unexpected increase in proliferation in the presence of chordin-like 1 led us to analyze the effect of BMP-2 on proliferation and differentiation of hMSCs.

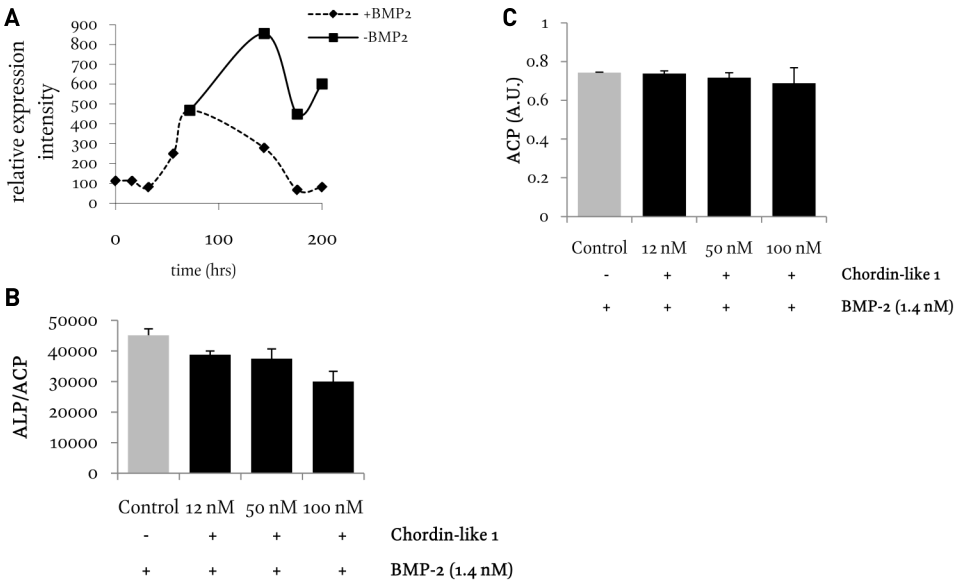

Figure 1 – Regulation of chordin-like 1 during BMP-induced osteogenesis of MC3T3-E1 cells and its effects on proliferation and ALP expression. A. Chordin-like 1 expression upon BMP induction of MC3T3-E1 cells. Note the differential expression of chordin-like 1 during early and later stages of osteogenic differentiation B. ALP expression by MC3T3-E1 cells cultured for three days in osteogenic medium in the presence of increasing concentrations of chordin-like 1. Note that ALP expression decreased, dose-dependently, in the presence of chordin-like 1 C. Proliferation of MC3T3-E1 cells cultured for three days in osteogenic medium in the presence of increasing concentrations of chordin-like 1. Note that there was no difference on proliferation compared with the control.

Effects of chordin-like 1 on BMP signalling

We cultured hMSCs in basic or osteogenic medium for 5 days. BMP-2, in combination with dexamethasone, resulted in a small but significant reduction in proliferation of hMSCs grown in osteogenic medium (10% reduction). Moreover, chordin-like 1 again enhanced the proliferation of hMSCs independently of the presence of dex or BMP-2 (60% increase, Figure 3A). As shown in Figure 3B, we observed a significant reduction in ALP expression upon exposure of hMSCs to BMP -2. Although contra-intuitive for an osteogenic protein, we and others have observed this before

(data not shown and refs [37, 38]). Moreover, in this donor, chordin-like 1 did not have a significant effect on ALP expression, in either basic or osteogenic medium. Nevertheless, the addition of chordin-like 1 significantly rescued the negative effect of BMP-2 on ALP expression by hMSCs.

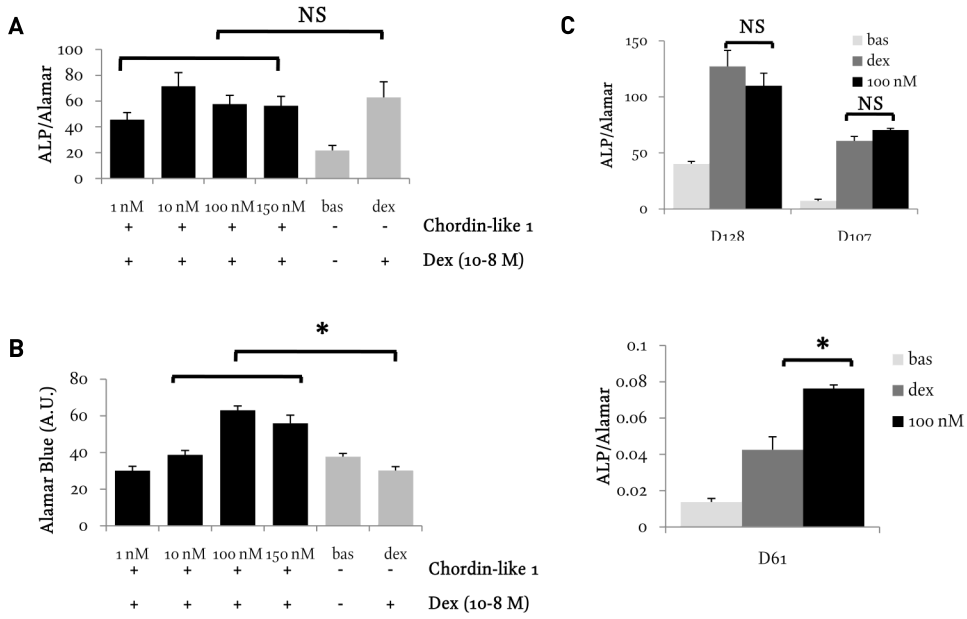

Figure 2 – Osteogenic potential and proliferation of hMSCs exposed to chordin-like 1. A. ALP expression by hMSCs exposed for 5 days to increasing concentrations of chordin-like 1 in osteogenic medium B. hMSCs were cultured in the presence of chordin-like 1 for 5 days upon which proliferation was analyzed. Note that there was a dose-dependent increase on proliferation of hMSCs exposed to increasing concentrations of chordin-like 1 C. ALP expression for three different donors upon exposure to 100 nM chordin-like 1. Note that only one of three donors showed a significant increase in ALP expression.

In order to analyze whether the effect of chordin-like 1 on proliferation of hMSCs and differentiation of MC3T3 cells is due to a potential BMP antagonizing effect, we analyzed the interaction of BMP-2 and chordin-like 1 using the BRE-luc reporter assay as a direct read-out of BMP signalling. The BRE-Luc reporter consists of five repeats of a BMP-responsive element from the ID1 gene, driving the expression of the luciferase gene (figure 4A). Upon exposure of C2C12 cells to 4 nM BMP-2, the BRE-Luc reporter showed a 5-fold increase in its activity. When we used increasing concentrations of chordin-like 1 in the presence of the same concentration of BMP-2 we did not observe a significant effect up to a concentration of 500 nM chordin-like 1 (stoichiometry 1:125). Importantly, at the concentration of chordin-like 1 where we observed an effect on proliferation of hMSCs (100 nM), there was no significant effect on the activity of the reporter. Only at 1000 nM we observed an inhibitory effect of chordin-like 1 on BMP-2 induced BRE-luc activity. This points towards a possible BMP-2 independent effect of chordin-like 1 on proliferation of hMSCs and osteogenesis of MC3T3 cells (Figure 4B).

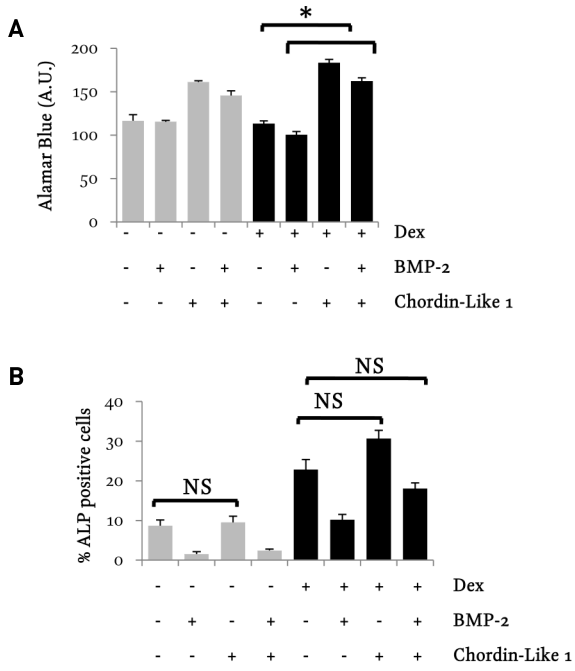

Figure 3 – Synergistic effect of BMP-2 and chordin-like 1 on proliferation and ALP expression of hMSCs. hMSCs were culture for 5 days in the presence of BMP-2, chordin-like 1 or the combination in the presence of basic and osteogenic medium. The effect of both proteins, either alone or combined, on the proliferation of hMSCs was analyzed (A). Note that independently of the medium chordin-like 1 enhanced the proliferation of hMSCs. The effect of chordin-like 1 was partially inhibited in the presence of BMP-2 B. hMSCs were cultured for 5 days in basic or osteogenic medium in the presence of BMP-2, chordin-like 1 or a combination of both. Note that exposure of hMSCs to BMP-2 resulted in a decrease on ALP expression in contrast with chordin-like 1 which did not affected ALP expression. The combination of chordin-like 1 and BMP-2 resulted in a decrease on ALP expression when compared to chordin-like 1 alone.

Discussion

The tight regulation of morphogens such as BMPs is of critical importance not only during embryonic development but also in the adult organism. Compounds like BMPs control several cellular functions in the adult organism but, as the name indicates, they are commonly known for their role in skeletal development. Since BMPs are able to induce bone ectopically, care should be taken when using them as therapeutic agents. One way the organism devised to control BMP activity is by producing BMP antagonists. For example, it has been shown that bone marrow-derived cells treated with dexamethasone or with BMP increase the expression of noggin most likely to control the activity of BMPs [38]. In our case we observed that upon exposure to BMP-2, MC3T3-E1 cells downregulate the expression of chordin-like 1, another BMP antagonist, even though the upregulation of BMP antagonists has been described as a mechanism to limit excessive exposure to BMP [39].

Although BMP signalling is conserved between species, different effects were observed between murine and human cells in response to BMP and to BMP antagonists. For example, it has been shown that noggin suppression enhances osteogenesis, both in vitro as well as in vivo, in the case of mouse cells whereas in the case of hMSCs, noggin enhanced in vitro osteogenesis pointing at a negative role for BMP signalling in hMSC in vitro osteogenesis [40, 41].

Besides studying this potential transcriptional mechanism regulating BMP signalling we proceeded to analyze the role of chordin-like 1 recombinant protein on BMP-induced differentiation of MC3T3-E1 cells. We observed that exposure of MC3T3-E1 cells to chordin-like 1 resulted in a dose-dependent decrease in ALP expression. These results are in line with previously published data showing that noggin is a negative regulator of osteogenesis and, upon suppression of noggin, there was an increase in markers of osteogenesis for MC3T3-E1 cells. These results implicate chordin-like 1 in osteogenic differentiation of MC3T3-E1 cells, potentially by controlling BMP availability. Nevertheless, we would like to stress that an excess of chordin-like 1 relative to BMP-2 is needed in

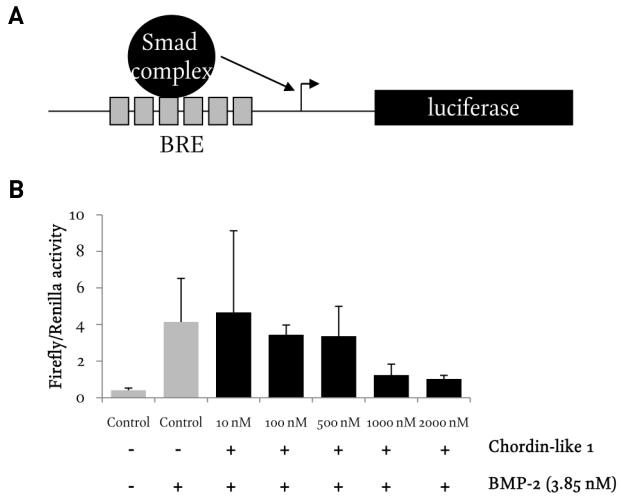

Figure 4 – Effect of chordin-like 1 on BMP signalling. A. BMP response element (BRE) – luc reporter system. The activity of the luciferase gene is under control of a promoter consisting of repetitive domains from the ID1 gene which are activated upon SMAD binding B. C2C12 cells were transfected with the BRE-Luc reporter and cultured for 24h with BMP-2 in the presence of increasing concentrations of chordin-like 1. Note that only at the highest concentrations of chordin-like 1 there was a marked decrease in the activity of the reporter.

order to observe a significant effect on ALP expression by MC3T3-E1 cells (stoichiometry BMP-2:chordin-like 1 1:72 to observe a nearly 50% reduction on ALP expression). It should be noted that ALP is not a direct BMP target gene. When we analysed the direct effect of chordin-like 1 on BRE activity, we did not observe an effect when BMP and chordin-like 1 were added at the 1:72 stoichiometry. This suggests that the negative effect of chordin-like 1 on BMP-induced ALP expression is a BMP-independent process, which warrants further investigation.

When we exposed hMSCs to increasing concentrations of chordin-like 1 there was no significant effect on dex-induced ALP expression. These results are in contrast with MC3T3-E1 cells highlighting the fact that the osteogenic program differs between the two cell types [37]. Indeed, we showed that exposure of hMSCs to BMP-2 inhibits ALP expression as previously shown by others [37,38]. In the case of MC3T3-E1 cells, BMP-2 was used to trigger the differentiation whereas in the case of hMSCs we used a well known corticosteroid (dexamethasone) to induce differentiation. Since ALP expression by hMSCs is not enhanced by BMPs it was not surprising that the exposure of hMSCs to a BMP antagonist did not affect ALP expression [37]. Nevertheless the addition of chordin-like 1 rescued the inhibition of ALP expression by BMP-2.

Although chordin-like 1 did not affect ALP expression of hMSCs, we observed a clear effect on proliferation of these cells. It has been shown previously that inhibition of chordin results in a decrease in proliferation of hMSCs [42]. Here, we show that proliferation of hMSCs was enhanced, dose-dependently, in the presence of chordin-like 1. It is not likely that the positive effect of chordin-like 1 is mediated through inhibition of BMP signalling for several reasons. First, BMP-2 has a negative effect on dex-induced ALP expression whereas chordin-like 1 has no effect on ALP expression in 3 out of 4 donors (Figure 2). Second, we show here that chordin-like 1 can interfere with BMP-2 activity in osteogenic assays but the concentration of chordin-like 1 at which we observed an effect on hMSC proliferation was not sufficient to inhibit BMP signalling in C2C12 cells. Third, since exogenous BMP-2 did not affect proliferation, whereas it did affect osteogenesis of hMSCs, we can deduce that chordin-like 1 effect on proliferation is independent from BMP-2. The signalling pathway involved in this phenomenon is not known but it seems to be independent of BMP signalling.

In conclusion we showed that chordin-like 1 has different effects on MC3T3-E1 cells and hMSCs. Moreover, we showed that chordin-like 1 can interact with BMP-2 and by doing so affect osteogenic differentiation of both cell types. Interestingly, chordin-like 1 enhanced proliferation of hMSCs, dose-dependently, without affecting ALP expression. This effect can be further explored for tissue engineering applications where the number of cells is of paramount importance. Moreover, by controlling BMP signalling and by having the ability not only to bind BMP but also to be cleaved by a protease such as BMP-1, chordin-like 1 can be further explored as a molecule able to aid drug delivery systems for BMPs.

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Chapter 7

OSTEOINDUCTIVE CERAMICS AS A SYNTHETIC ALTERNATIVE TO AUTOLOGOUS BONE GRAFTING

"Some men see things as they are and say, 'why?' I dream of things that never were and say, 'why not?' "

George Bernard Shaw

Osteoinductive ceramics as a synthetic alternative to autologous bone grafting

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Abstract

There is increasing evidence that biomaterials can be endowed with biologically instructive properties by changing basic parameters such as elasticity and surface texture. However, translation of these so-called "smart" materials from *in vitro* proof of concept to clinical application is largely missing. Porous calcium phosphate ceramics are currently used to treat small bone defects but lack the critical ability to induce stem cell differentiation required for regenerating bone in large defects. In this study, we prepared a family of calcium phosphate ceramics with varying physico-chemical and structural characteristics. Micro-porosity correlated to their propensity to stimulate osteogenic differentiation of stem cells *in vitro* but also to the induction of bone formation in muscle tissue of sheep and dogs. Implantation of our materials in a large bone defect in sheep unequivocally demonstrated that osteoinductive ceramics are equally efficient in bone repair as autologous bone grafts. As such, osteoinductive ceramics provide pre-clinical proof of concept for the application of "smart" biomaterials.

Introduction

The role of biomaterials as medical devices is changing from a biologically passive, structural role to one in which the properties of the material will orchestrate the process of tissue regeneration. The change is fed by an increasing number of reports demonstrating that the behaviour of cells can be modulated by basic material properties such as surface texture, material elasticity and chemistry [1-5]. For instance, in the area of biomaterials for the restoration of bone defects it has been reported that surface topography influences osteogenesis and proliferation of bone marrow derived multipotent mesenchymal stromal cells (MSCs) *in vitro* [6,7]. Although tissue instructive materials hold great potential as off-the-shelf bio-active medical devices, so far the concept has not progressed much beyond the proof of concept phase in which *in vitro* assays demonstrate an effect on cellular differentiation or proliferation [8]. As a consequence, transplantation of autologous bone is still the golden standard in bone repair strategies. Autografts guide the in-growth of osteoblasts, the primary cell type responsible for bone matrix apposition, from the adjacent tissues into the defect, a process referred to as osteoconduction. In addition, autologous bone induces *de novo* bone formation by triggering the differentiation of undifferentiated progenitor cells into the osteogenic lineage, referred to as osteoinduction [9,10]. The latter phenomenon is essential for the repair of larger, so-called, critical size bone defects. Drawbacks of autografting are the limited availability of autologous bone and the negative side effects of bone harvesting, which make the search for bone graft substitutes an area of intense research [11].

The discovery that osteoinduction can also be accomplished by devitalised demineralised bone matrix (DBM) and the subsequent identification of bone morphogenetic proteins (BMPs) provided an alternative to bone autografts [9]. Both DBM and BMPs are broadly applied in the clinic, but their biological nature has implications for the production process leading to rather high batch variability and high production costs [12-15]. Moreover, the *in vivo* delivery of soluble molecules such as BMPs is inefficient. An alternative to the biological approach to bone regeneration would be the development of a synthetic material with intrinsic osteoinductive capacity. In line with this, Winter and Simpson were the firsts to report bone formation upon implantation of a polyhydroxyethylmethacrylate (poly-HEMA) sponge under the skin of pigs, an experiment that was performed in an attempt to explain incidences of hard tissue formation upon implantation of synthetic breast implants [16]. In the past thirty years, several porous calcium phosphate biomaterials as well as some metals were reported to possess osteoinductive capacity [17]. The underlying mechanism leading to bone induction by synthetic materials remains largely unknown. However, osteoinductive potential of biomaterials can be controlled by tailoring material characteristics such as chemical composition, surface topography and geometry. Resorption rate and cell-material interactions are likely to play a role.

The aim of this study was to correlate the osteogenic potential of a family of porous ceramic materials *in vitro* to ectopic bone formation *in vivo* and to demonstrate that synthetic materials present a *bona fide* alternative to autograft and BMP therapy with equal performance in the healing of a critical size bone defect.

Materials and methods

Synthesis and characterization of calcium phosphate ceramics

HA ceramics were prepared from HA powder (Merck, Germany) using the dual-phase mixing method and sintered at 1250°C for 8 hours according to a previously described method [25]. BCP ceramics were fabricated using the H_2O_2 method using in-house made calcium-deficient apatite powder and sintered at 1150°C (BCP1150) and 1300°C (BCP1300), respectively [26]. The method used to synthesize the BCP ceramics was also used in the case of TCP. TCP ceramics were prepared from TCP powder (Plasma Biotal, UK). Ceramic particles (1-2 or 2-3mm) or ceramic discs (Ø 17 X 8 mm) were prepared, cleaned ultrasonically with acetone, 70% ethanol and demineralised water, dried at 80ºC and sterilized by gamma irradiation prior to use.

The macro and micro structure of the different ceramics was evaluated using a scanning electron microscope (SEM; XL30, ESEM-FEG, Philips, The Netherlands). Composition of the ceramics was determined by X-Ray Diffraction (XRD; Miniflex, Rigaku, Japan). Specific surface area of the different ceramics was analyzed with mercury intrusion (Micromeritics Intrument Incorporation, USA).

The calcium release profile of the ceramics was determined by immersing 0.5 ml of 1-2 mm ceramic particles in 100 mL of simulated physiological saline (0.8% NaCl, 50 mM HEPES, 37°C, pH 7.3) and monitoring the calcium concentration using a calcium electrode for 200 minutes.

To calculate the concentration of protein adsorbed, 1 cm^3 of ceramics was incubated for 3 days in 1% foetal bovine serum in PBS at 37°C. Protein adsorption was measured using a protein assay kit (micro BCA™, Perbio) according to the manufacture's protocol.

RNA isolation and quantitative PCR

To analyze the effect of the different ceramics in the gene expression profile of hMSCs, $2x10^5$ cells were seeded per three particles and culture for 7 days in osteogenic medium. As a control we seeded 5000 cells/cm^2 on tissue culture flasks either in basic or osteogenic medium for 7 days. Total RNA was isolated using Trizol and the Nucleospin RNA isolation kit (Macherey-Nagel) according to the manufacturer's protocol. The quality and quantity of RNA was analyzed by gel electrophoresis and spectrophotometry. Six hundred and fifty ng of RNA was used for cDNA synthesis using iScript cDNA synthesis kit (BioRad) according to the manufacturer's protocol. 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. Fold induction relative to cells grown on tissue culture plastic in osteogenic medium was calculated using the ΔCT method after normalization with 18S as a housekeeping gene.

Ectopic bone formation by hMSCs

hMSCs were isolated from adult bone marrow and cultured as previously described [27]. To evaluate the effect of different calcium phosphate ceramics on ectopic bone formation by hMSCs, we seeded $2x10^5$ cells per three particles of approximately 2-3 mm. Cells were cultured *in vitro* for 7 days in the presence of osteogenic medium. Prior to implantation, the tissue-engineered constructs

were washed with PBS. Six immuno-deficient mice (HsdCpb :NRI-nu, Harlan, Horst, The Netherlands) were anesthetized using isoflurane, surgical sites were cleaned with ethanol and subcutaneous pockets created. Three particles of each of the calcium phosphate ceramics were implanted on these pockets for 6 weeks.

Posterolateral spinal fusion model in dog

The surgical operation was performed under general anesthesia (30 mg pentobartital sodium per kg body weight) and sterile conditions. Firstly, the spinous processes of L3 an L4 were found, and small incisions were made on both sides. Secondly, the spinous processes and the vertebral body between L3 and L4 were exposed in both sides by blunt separation. After injuring the exposed bone using a scraper, materials (HA or TCP, 1-2 mm, 5 mL) were placed in both sides. Finally, the muscles from both sides were tightly closed with sutures. After surgery, penicillin was intramuscularly injected for 3 consecutive days to prevent infection. Twelve weeks after surgery, the animals were sacrificed and samples were harvested with surrounding tissues. The samples were then fixed, dehydrated, embedded in MMA and un-decalcified sections were made parallel to the posterolateral processes and stained with methylene blue and basic fuchsin for histological and histomorphometrical analysis. Sections obtained 5 mm away from the end of spinous processes were used for histomorphometry and the area between the two processes and 5 mm from the middle was selected as region of interest. The area percentage of bone in the region of interest and in available space, and area percentage of materials in region of interest were calculated and data obtained from 8 animals was pooled for quantitative analyses.

Sheep model (intramuscular implantation and iliac implantation)

All the experiments were performed following approval of the University Animal Care and Ethics Committee from the University of New South Wales.

In the case of intramuscular implantation (ectopic implantation) ten adult female sheep (3 years old) were used. Different calcium phosphate ceramics (BCP1300, BCP1150 and TCP) particles with a size of 1-2 mm were implanted intramuscularly for 12 weeks. For the iliac implantation (orthotopic implantation), twenty two adult female sheep (3 years old) were sedated using an intramuscular injection of Zoletil and anesthetized using a mixture of O_2 (4 L/min) and isoflurane (1.5% to 2.5%). Pain relief was administered prior to commencement of the surgical procedure. An incision was made over the exposed length of the iliac crest, through the periosteum. Upon exposure of the Os ilium a 17 mm defect was created and the implanted materials were placed in the defects. The periosteum, muscles, fat tissue and skin were closed over the iliac crest by suturing layers using resorbable sutures (3-0 Dexon, Davis & Geck, North Ryde, Australia). In the case of autograft the bone was harvested at the same time as the creation of the defect and reduced to 1-2 mm particles using a rongeur. Recombinant human BMP-2 (Medtronic, Memphis, TN) at a concentration of 0.4 mg/mL (0.72 mg/defect) was soaked on 17 mm diameter Helistat absorbable collagen hemostatic sponges (Integra LifeSciences Corporation, Plainsboro, NJ) for 15 minutes prior to stacking in the defect.

During the implantation period animals were fed with a standard diet and had continuous access to water.

Histology and histomorphometry

Implants were retrieved and fixed in 0.14 M cacodylic acid buffer pH 7.3 containing 1.5% glutaraldehyde. Fixed samples were dehydrated in ethanol series and embedded in MMA. Sections were processed on a histological diamond saw (Leica SP1600, Wetzlar, Germany) and stained with 1% methylene blue (Sigma) and 0.3% basic fuchsin solution (Sigma).

Sections (one section per sample across the middle, or otherwise specified) were scanned using a digital scanner (Dimage Scan Elite 5400II, Konica Minolta Photo imaging, INC) in order to have an overview and representative images were used for histomorphometrical analysis using Photoshop software (Adobe). Either the area percentage of bone in the samples or the area percentages of bone in the available space were obtained for quantitative analyses.

Statistical analysis

Statistical analysis was performed using a One-way ANOVA followed by a Tukey's multiple comparison test ($P<0.05$).

Results

Synthesis and characterization of calcium phosphate ceramics

In order to produce porous calcium phosphate ceramics with varying biological activities, we either used calcium phosphate powder with different chemical compositions (hydroxyapatite [HA], tricalcium phosphate [TCP], or a mixture thereof [biphasic calcium phosphate, BCP]) or we exposed the ceramics to different post-synthesis sintering temperatures (BCP1150 and BCP1300). Using XRD analysis, we observed the presence of β-TCP in BCP and a trace of HA (<10%) in TCP, whereas HA was phase-pure (Figure 1A). High magnification scanning electron microscopy imaging showed no differences in macrostructure among different ceramics (data not shown), though their microstructure (pores and grains <50 μm) varied. The grain size of BCP1150 and TCP was smaller and the number of micropores larger as compared to HA and BCP1300 (Figure 1B). As a consequence, the specific surface area of the four ceramics varied from 0.1 m^2/g for HA to 1.2 m^2/g for TCP, resulting in differences in adsorption of serum protein, with TCP adsorbing more proteins per volume of material than BCP1150 and BCP1300 (Figure 1C). In contrast, when adsorption of protein was expressed per surface area, BCP1150 and TCP bound less proteins than BCP1300 and HA. We assessed the rate of calcium release from the four ceramics and found that it was significantly faster for TCP compared to the other three ceramics (Figure 1D). An overview of the materials characteristics is given in Table 1.

Material properties control stem cell differentiation

The effect of material properties on tissue development is mediated via cell-material interaction and we therefore decided to analyze the effect of the four different materials on osteogenic differentiation of human multipotent marrow stromal cells (hMSCs) *in vitro* and *in vivo* [18,19]. First, we

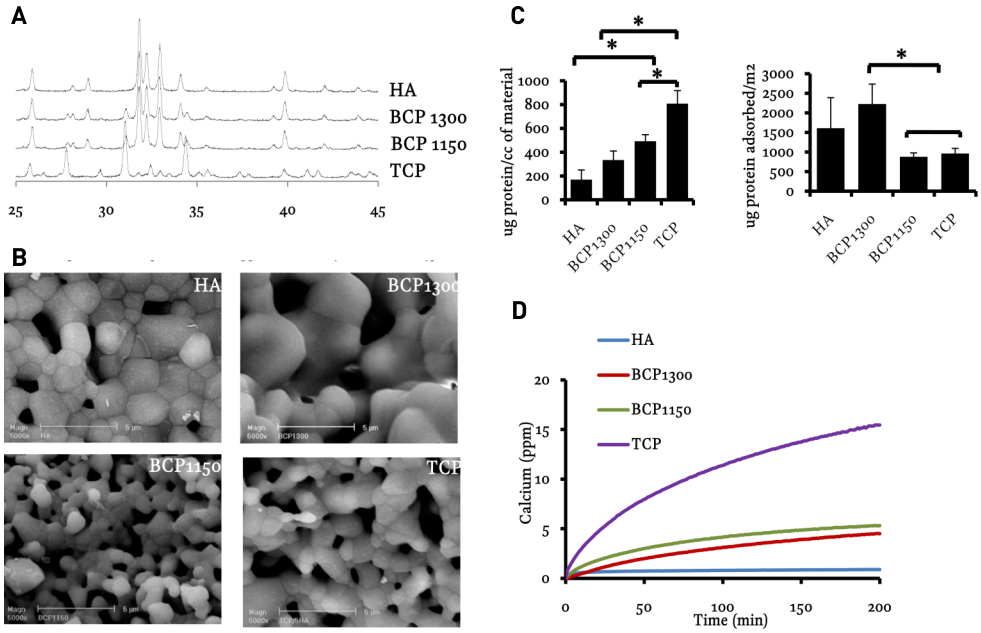


Figure 1 – Characterization of calcium phosphate ceramics. A. XRD analysis showing the composition of the four different ceramics with their characteristic peaks indicated. B. ESEM photographs depicting their microstructure. C,D. Protein adsorption and calcium release profile of the different ceramics, respectively. The error bars represent standard deviations. An asterisk (*) denotes statistical difference (One-way Anova and Tukey's test, $P<0.05$).

seeded hMSCs on the four different ceramics and cultured them for 7 days in osteogenic differentiation medium after which gene expression was analyzed using quantitative PCR on a panel of genes indicative for osteogenic differentiation. As a control, we grew hMSCs on culture plates in control and osteogenic medium and observed the well documented increase in alkaline phosphatase (ALP) expression in osteogenic medium (Figure S1) [20]. With the exception of ALP and collagen type I, gene expression of all genes was higher on the four ceramics than on culture plastic, suggesting that

	HA	TCP	BCP1150	BCP1300
Chemistry	HA	5HA+95TCP	20TCP/80HA	20TCP/80HA
Particle size (mm)	1-2	1-2	1-2	1-2
Specific surface (m^2/g)	0.1	1.2	1	0.2
Percentage of materials	46.4±2.4	49.9±1.8	45.6±2.2	N/A
Cumulative Ca release (ppm)	0.9±0.1	15.3±0.2	5.4±0.1	4.2±0.4

Table 1 – Overview of the characteristics of the calcium phosphate ceramics used in the study

the ceramics favor osteogenic differentiation (Figure 2A and Figure S1). Furthermore, marked differences in expression levels of genes encoding osteocalcin, bone sialoprotein and osteopontin were found in hMSCs cultured on the different ceramics. A similar, but less profound effect for S100A4 and Runx2 was also observed. Interestingly, hMSCs on TCP consistently displayed the most osteogenic profile, and on HA the least (Figure S1).

Next, we assessed bone apposition by hMSCs on the different ceramics. Porous calcium phosphate ceramics are frequently used in bone tissue engineered constructs, in which culture expanded MSCs are seeded onto the ceramic *in vitro* and then implanted. Upon implantation, MSCs will differentiate into osteoblasts and deposit bone tissue onto the ceramic surface [21]. We cultured hMSCs *in vitro* for 7 days on the four calcium phosphate ceramics (HA, BCP1300, BCP1150 and TCP) in osteogenic medium. The constructs were implanted subcutaneously into immuno-deficient mice for 6 weeks after which formation of new bone tissue was assayed using histomorphometry. No bone formation was on HA scaffolds seeded with hMSCs (Figure 2B and 2C). In contrast, we did observe apposition of bone tissue on grafts of BCP1300, BCP1150 and TCP ceramics seeded with hMSCs. On TCP, we observed a five times higher amount of bone than on BCP1300 and BCP1150 (2.7 ± 1.6% in TCP, 0.7 ± 1.3% in BCP1300 and 0.6 ± 0.9% in BCP1150) demonstrating that ceramics stimulated osteogenic differentiation *in vitro* and bone formation *in vivo* depending on their physico-chemical and structural characteristics.

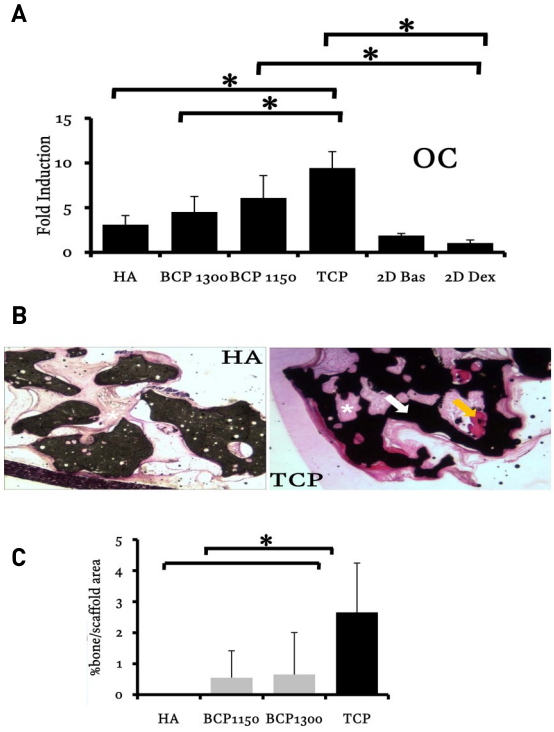
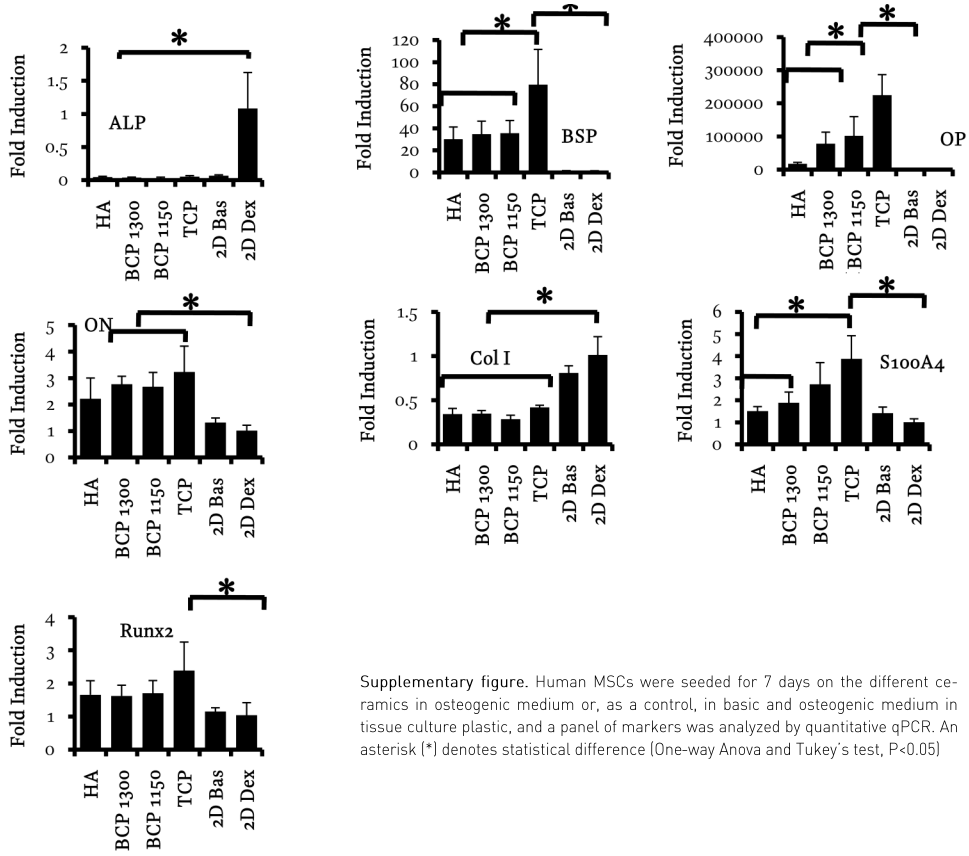


Figure 2 – Osteogenic differentiation of hMSCs on ceramics of different composition. A. Expression of the bone related protein osteocalcin by hMSCs seeded in the different ceramics. Expression levels were normalized with 18S. Fold induction was calculated using the ΔCT method relative to dex-treated hMSCs in tissue culture plates. The error bars represent standard deviations. B,C. Bone forming potential of hMSCs seeded in different ceramics. Histological sections (B) and quantification of bone area per scaffold area (C) are shown. Basic fuchsin stains bone red (orange arrow), methylene blue stains fibrous tissue blue (asterisk) and the ceramic is shown in black (white arrow). The error bars represent standard deviations. An asterisk (*) denotes statistical difference (One-way Anova and Tukey's test, P<0.05).

In vivo osteoinduction by different calcium phosphate ceramics

As mentioned before, osteoinduction is a critical parameter of any bone graft in large bone defects. To analyze the osteoinductive potential of our ceramics, we used both ectopic implantation in muscle tissue of dogs and the clinically relevant posterolateral spinal fusion model in which two materials can be compared in a paired manner. For this purpose, we selected the two extremes from

the previous experiments, HA and TCP. Histomorphometric analysis after 12 weeks of implantation showed that the area percentage of bone in available pore space was 5 times larger in TCP as compared to HA, both in muscle and in the spine, demonstrating that calcium phosphate ceramics with different chemical composition have different osteoinductive potential, which was in accordance with *in vitro* results (Figure 3A and Figure 3B). We observed a resorption of 77% of the implanted TCP after 12 weeks of implantation, whereas no detectable resorption of HA was observed (Figure 3C).



Supplementary figure. Human MSCs were seeded for 7 days on the different ceramics in osteogenic medium or, as a control, in basic and osteogenic medium in tissue culture plastic, and a panel of markers was analyzed by quantitative qPCR. An asterisk (*) denotes statistical difference (One-way Anova and Tukey's test, P<0.05)

To demonstrate that not only chemistry but also structural characteristics can influence the osteoinductive potency of ceramics, we implanted BCP1150, BCP1300 and TCP in muscle of sheep. Unfortunately, HA was omitted from this study due to technical difficulties. Twelve weeks after implantation in paraspinal muscles, we observed that bone induction had occurred in all three calcium phosphate ceramics. However, the amount of bone formed varied among the different ceramics (Figure 4A). Bone apposition was again highest in TCP (28.7 ± 4.8% of available pore space) followed by BCP1150 (17.7 ± 5%). Significantly less bone was observed in BCP 1300 (11 ± 7.5%) indicating that both chemistry and structural properties can influence the *in vivo* osteoinductive potential of the ceramics (Figure 4B).

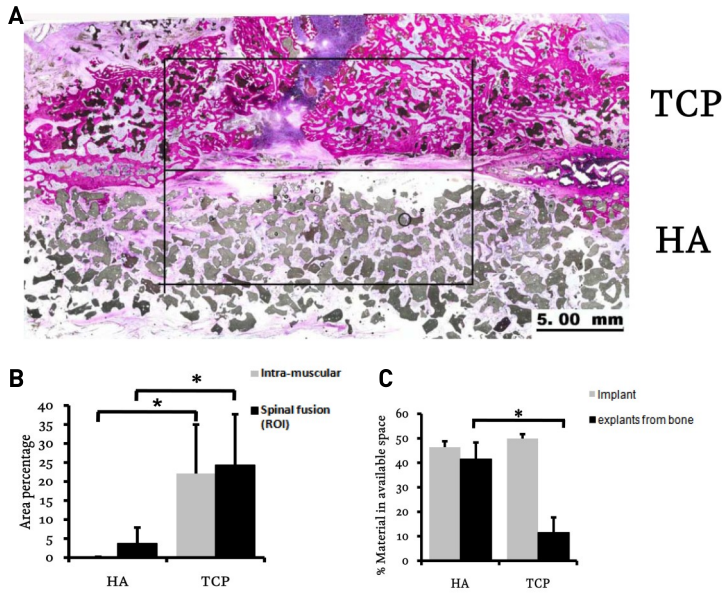


Figure 3 – Posterolateral spinal fusion in dogs. A. Histological overviews showing newly formed bone in TCP and HA implants. B. The area percentage of bone for HA and TCP ceramics in the case of intramuscular implantation (grey bars) and in the spinal fusion (black bars). C. The percentage of material available before implantation (grey bars) and upon explantation (black bars). Note that HA ceramic was not resorbed during the 12 weeks implantation in contrast with TCP. The error bars represent standard deviations. An asterisk (*) denotes statistical difference (Student paired t-test, P<0.05).

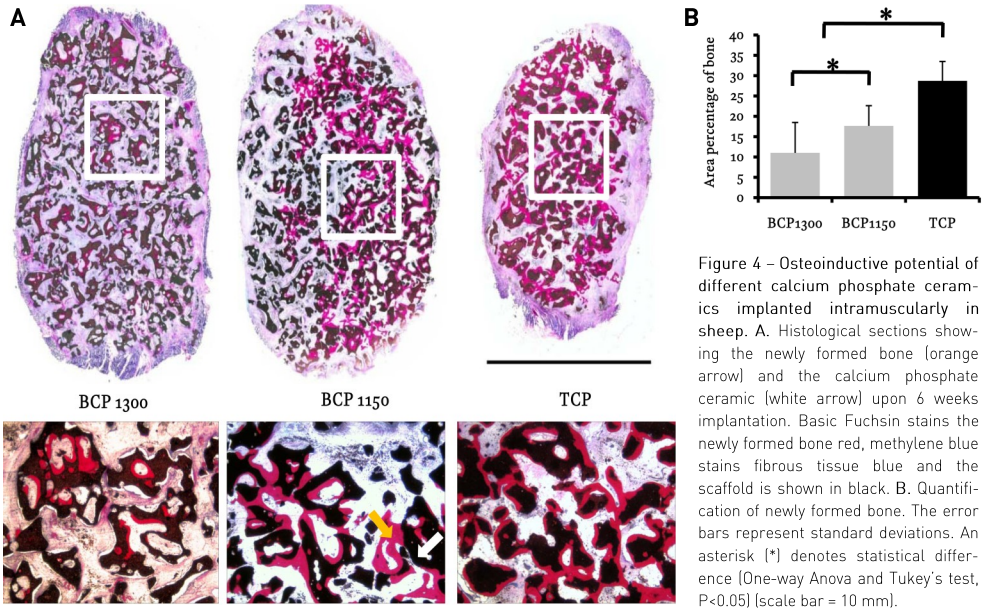


Figure 4 – Osteoinductive potential of different calcium phosphate ceramics implanted intramuscularly in sheep. A. Histological sections showing the newly formed bone (orange arrow) and the calcium phosphate ceramic (white arrow) upon 6 weeks implantation. Basic Fuchsin stains the newly formed bone red, methylene blue stains fibrous tissue blue and the scaffold is shown in black. B. Quantification of newly formed bone. The error bars represent standard deviations. An asterisk (*) denotes statistical difference (One-way Anova and Tukey's test, P<0.05) (scale bar = 10 mm).

Calcium phosphate ceramics as a bone graft substitute in a critical-size defect

The studies above indicate that TCP is a superior ceramic with respect to stem cell differentiation and osteoinduction *in vivo*. We next tested the ability of TCP to heal a critical-sized orthotopic defect in comparison with standard treatments. To this end, we implanted TCP in a bilateral iliac wing defect in sheep with a critical size diameter of 17 mm. As a negative control, we included a

group in which the defect was left empty and as a positive control we used two groups of sheep in which the defect was either treated with autologous bone or with a preparation of recombinant human BMP-2 (rhBMP-2) delivered in a collagen sponge. The latter treatment is a commercially available product used for spinal fusion surgery [22, 23].

Twelve weeks after implantation, we observed that the iliac wing defect was not able to heal spontaneously, although some newly formed bone could be seen along the host bone bed (3.1±1.8% of the defect was covered by new bone, Figure 5 and Figure6), confirming that the defect was critically-sized. In contrast, when autologous bone was used as an implant, newly formed bone was found throughout the defect (32.2±6.5% new bone). As it is difficult to distinguish between implanted bone autograft and newly formed bone, the percentage of bone measured represents both residual bone autograft and newly formed bone. Histological analysis revealed tight bonding between new bone and the host bone bed, without interspersed fibrous tissue layer (Figure 6). However, in the centre of the defect, less bone was observed than in the defect periphery, and large areas were filled with fibrous tissue in all animals tested. Results obtained with implantation of rhBMP-2 were similar to those of autologous bone. In the rhBMP-2 treated defects, 22.8±10.1% of the defect was filled with new bone. Fibrous tissue was observed in the centre of the defect of all animals tested. In addition, ectopic bone formation outside the defect area was found in 8 out of 10 animals, probably due to diffusion of rhBMP-2 from the graft into the adjacent soft tissue (Figure 5).

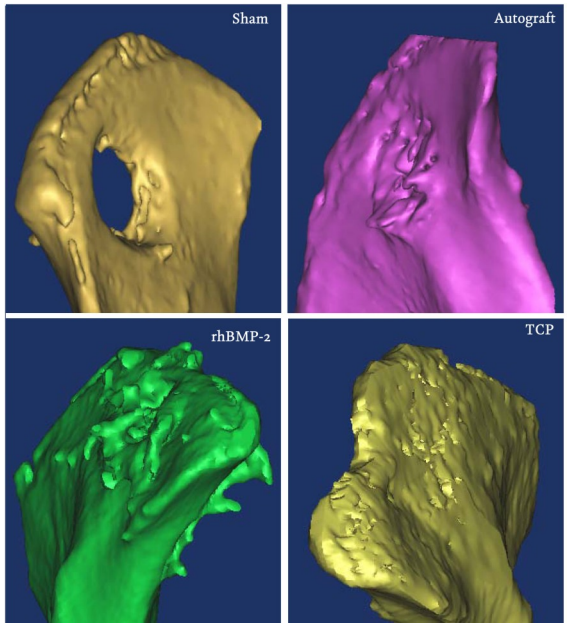


Figure 5 – Illium defect. Figure presents three dimensional models of the Os Ilium after 12 weeks implantation are shown. Bone formation outside the margins of the defect was found in the rhBMP-2 group while this was not a feature with the TCP group where the material remained within the defect with new bone formation and implant resorption observed at 12 weeks.

Interestingly, implants made of TCP ceramic showed similar performance to autologous bone regarding bone formation. With an area of new bone covering 33.9±6.8 % of the defects, they outperformed the rhBMP-2 group (an overview of the histology and quantification of bone formation can be seen in Figure 6A and 6B, respectively). Significant resorption of the ceramic material could be observed: the percentage of ceramic area in the defect area decreased from 56% to 21% after 12 weeks of implantation (Figure 6C). Similar to autograft, new bone formed in TCP treated defect formed a tight bond with the host bone, and no fibrous tissue was observed in the periphery of the defect. In the central area of the defect, only two out of 10 animals showed the presence of fibrous connective tissue.

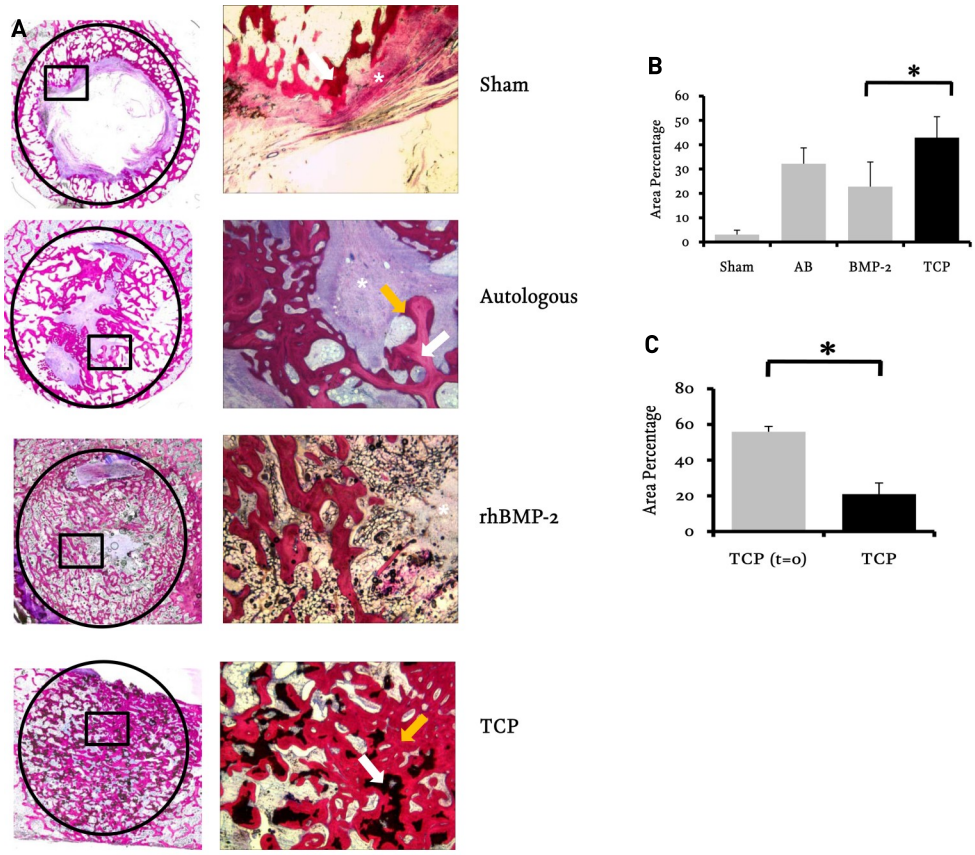


Figure 6 – Performance of calcium phosphate ceramics, autologous bone and rhBMP-2 in a critical-size defect in the illium opf sheep. A. Histological sections depicting the newly formed bone within the defect created in the illium of sheep. The defect margins are indicated by the black circle on the left panel and on the right panel (2x magnification) the demarked region can be seen in detail. Basic fuchsin stains bone red, methylene blue stains fibrous tissue blue and the scaffold is shown in black. Newly formed bone is indicated by an orange arrow, autologous bone or the ceramic material are indicated by a white arrow and fibrous tissue is indicated by an asterisk (*). B,C. Area percentage of bone per available area between the different conditions (B) and the resorption of the ceramic after 12 weeks implantation (C). The error bars represent standard deviations. An asterisk (*) denotes statistical difference (One-way Anova and Tukey's test, $P<0.05$ (B) and Student paired t-test, $p<0.05$ (C))

Discussion

In this manuscript, we demonstrate that a fully synthetic implant based on calcium phosphate ceramic is at least equally successful to autograft and rhBMP-2 in the treatment of a critical-sized bone defect. Unlike many other synthetic bone graft substitutes, which are considered solely osteoconductive, the ceramic presented here possesses intrinsic osteoinductivity, comparable to autograft, DBM and BMPs, which could explain its performance orthotopically and represent a paradigm shift in the treatment of bone defects.

So far, porous ceramics are used as bone fillers for small bone defects, where osteoconduction is sufficient. Autografting and BMPs are still the prime choice in treatment of complex and large bone defects, primarily because they are osteoinductive. Annually, around 300,000 patients undergo

spinal fusion procedures in US alone with estimated costs of $11.25 billion involving mainly autologous bone grafting and BMPs. In our study, TCP proved equally efficient as autologous bone in inducing bone apposition in the pre-clinical iliac wing defect model in sheep, with even less fibrous tissue in the central regions of the implant than autologous bone. Fibrous tissue formation is one of the main reasons for unsuccessful healing of large bone defects and non-unions as well as for implant failure. Bone formation induced by TCP remained within regions of the defect, whereas we observed BMP-induced bone formation in soft tissue surrounding the defect as well. Based on the findings reported in this manuscript, we have embarked on clinical trials to evaluate the possibility to use TCP as a true bone graft in large bone defects.

Our data show that the ability of ceramics to instruct cell and tissue development can be controlled merely by changing either the chemical composition or structural properties. The high osteoinductive potential of TCP begs questions about the cellular and molecular mechanism behind it. Although we have not pinpointed a particular signal transduction pathway yet, the fact that TCP shows higher expression of osteogenic markers by hMSCs *in vitro* and more *de novo* bone formation *in vivo* as compared to other investigated ceramics suggests a mechanism in which TCP triggers osteogenic differentiation. Our working hypothesis is that pericytes, the smooth muscle cells aligning the invading capillary blood vessels, encounter a milieu in which the cells differentiate into osteoblasts [24]. We are testing the hypothesis by focusing on the interaction between TCP and hMSCs *in vitro* and in the ectopic bone formation model using micro-array analysis and genetic interference studies. Another remaining question concerns the other side of the molecular interface: which biomaterial properties play a role in osteoinductivity and how do they influence the osteogenic process? Differences in dissolution behavior of the ceramic, which can be obtained either by changes in chemical composition (calcium phosphate phase) or by changes is structural properties (crystallinity, grain size, porosity, specific surface area) seem to be associated with osteoinductive potential *in vivo* [17]. We observe that the ceramic with the strongest osteoinductive potential displays the most pronounced dissolution *in vitro* and *in vivo.* Similarly, we observe differences in protein adsorption with TCP as the ceramic with the highest protein adsorption per volume of all ceramics. However, neither of these observations necessarily has a causal relationship with osteoinductive potential *in vitro* and *in vivo.*

Resolving the molecular mechanism of osteoinduction will offer tools to develop new osteoinductive materials, e.g. based on polymeric materials, in order to meet other requirements for successful bone repair, such as mechanical and handling properties. Furthermore, by understanding biological processes involved in osteoinduction by biomaterials, we will obtain more fundamental insight into biomaterial-tissue interactions.

Data presented in this manuscript on ceramic biomaterials in the area of bone graft substitution provide pre-clinical proof of concept for a new generation of "smart" materials, displaying superior biological performance through modulation of cell behavior. Bio-instructive scaffolds can find their way in a plethora of applications, from biodegradable sutures to contact lenses and stents and in all instances, the challenge lies in finding the optimal parameters for the particular biomedical application. For this, *in vitro* bio-assays that predict the performance of a material for intended application in the human body are essential. In this manuscript, the prospective knowledge of the osteoinduc-

tive capacity of different ceramics helped us in identifying a suitable *in vitro* cell system. For other biomaterials and other clinical applications, cell biologists and material scientist need to team up to streamline the process of identifying tissue instructive biomaterial properties. With the ever increasing power to produce libraries of biomaterials using combinatorial chemistry, nanotechnology and rapid prototyping technology, biomaterials are expected to gain full access in the clinic as bioactive devices.

Acknowledgments

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Chapter 8

CONCLUSION

"Happiness lies in the joy of achievement and the thrill of creative effort"

Franklin D. Roosevelt

General discussion and conclusion

Bone is a complex and dynamic tissue composed of different cell types contributing to the maintenance of homeostasis throughout life. Diseases affecting the musculoskeletal system are common in our society and are a major cause of disability in developing countries. Fractures are one of the most common occurrences associated with the musculoskeletal system. They can result from trauma or they can be a side effect of a bone-related disease such as osteoporosis or bone cancer, which weakens the bone and compromises its integrity. When a fracture occurs there is loss of skeletal integrity which has to be treated as soon as possible. The process of fracture healing involves a coordinated response of distinct cell types. Initially, cells from the immune system are recruited followed by a cascade of signals that will lead to blood vessel infiltration and concomitant migration of mesenchymal stem cells (MSCs) to the site of injury [1]. Fracture healing can occur directly or indirectly. In the first case, after internal fixation, discrete remodelling units attempt to reestablish new Haversian systems, without the formation of a callus, to restore mechanical continuity. In the second case, new bone formation occurs via endochondral ossification with callus formation prior to ossification. This process is generally enhanced by motion and inhibited by fixation [2]. In some cases, the process of fracture healing is impaired and there is the need to artificially restore mechanical continuity with the help of prostheses. Nevertheless, due to poor integration or even rejection by the body, this option is far from optimal.

In an attempt to improve tissue integration, some prostheses are treated with biomimetic coatings to guide and support bone regeneration. The prosthesis and the biomimetic coating can be considered as components of the tissue engineering approach described earlier in this thesis. In this case, the prosthesis can be considered the scaffold and the biomimetic coating as a signal to enhance tissue integration. The addition of cells into the equation is an attempt to further enhance integration and bone formation. The use of pre-differentiated cells and scaffolds aims at restoring as early as possible the skeletal continuity. Desirably, the implanted scaffold should be replaced over time by native tissue without compromising the mechanical integrity. In the case of bone, this implies the production of a mature collagen type I matrix and subsequential mineralization.

It is believed that the use of collagen type I might shorten the process of bone regeneration by providing one of the main components of bone. Nevertheless, as a natural-derived polymer, the quality of collagen as a scaffold is batch dependent and, due to poor mechanical and degradation properties, post-processing methods are needed [3].

In chapter 2, I analyzed in detail the lessons learnt from nature and how to improve scaffold design inspired by extracellular matrix (ECM). ECM plays a key role in several biological processes such as cell adhesion, proliferation and differentiation [4,5]. In contrast to soluble factors which are commonly used to differentiate cells in vitro but which are difficult to release in vivo, the use of ECM in vivo is less complicated. This makes collagen type I a topic of intensive research in osteogenic differentiation. Until recently, individual components of ECM were used in tissue engineering applications. Considering the intricate composition of ECM, this is a very reductionist approach to mimic natural ECM. More recently, research is focused on the integration of different components of the matrix. For example, incorporation of RGD peptides or other collagen motifs can enhance adhesion of cells to synthetic ECM. Furthermore, incorporation of cleavage sites for matrix

degrading enzymes allows control over degradation of synthetic ECM [4,6-9]. To identify which components of the matrix can decisively influence differentiation, preliminary research to identify them is needed. In chapter 3, I analyzed the role of the main component of bone ECM (collagen) on the differentiation of MSCs. The rational behind it is that during fracture healing the presence of MSCs in the defect plays a key role in bone formation [10]. A crosstalk between osteoblasts derived from MSCs and newly deposited collagen matrix has to occur for mineralization to proceed. I show that interfering in collagen production by depleting ascorbic acid (Asap) from the medium (a cofactor for an enzyme involved in collagen synthesis) affects both the proliferation as well as the differentiation of MSCs into the osteogenic lineage, in vitro as well as in vivo bone formation. Gene expression profiling revealed that in the absence of Asap there was a downregulation of pro-mitogenic genes and an upregulation of genes involved in adipogenic and chondrogenic differentiation. Interestingly, in the presence of Asap there was an increase in the expression of genes involved in matrix remodelling indicating that matrix production and remodelling are prone to regulatory mechanisms that contribute to tissue homeostasis. In addition to the role in proliferation and osteogenic differentiation, I analysed the role of collagen in the maintenance of lineage commitment. Asap is mainly known as a pro-osteogenic factor [11,12]. Nevertheless, since it has a pro-mitogenic effect and since the number of cells is of key importance for tissue engineering applications, I studied if it would be possible to add Asap starting from the isolation of MSCs without compromising their multilineage potential. Indeed, I showed that the presence of Asap not only has a positive effect on proliferation without interfering in the differentiation of the cells towards the chondrogenic or adipogenic lineage.

Next, in chapter 4, and based on the results from the previous chapter indicating that the presence of collagen has a positive effect on the number of uncommitted cells, I analyzed if we could interfere with post-translational modifications of collagen in an attempt to enhance the differentiation of MSCs into the osteogenic lineage. Collagen type I is present in several tissues of the body but only in bone we find mineralized collagen. This is puzzling since collagen biosynthesis is common to all the other tissues in the body. As such, we analyzed if structural changes in collagen might be related to mineralization. I evaluated the role of collagen crosslinking, an extracellular post-translational modification of the collagen molecules, in osteogenic differentiation. Using an irreversible inhibitor of the enzyme responsible for the crosslinking – lysyl oxidase – I analyzed how its absence affects differentiation. A cell line commonly used in tissue engineering (MC3T3-E1 cells) and a clinical relevant cell type (hMSCs) were used to test the response to uncrosslinked collagen. In the case of MC3T3-E1 cells I showed that inhibition of crosslinking inhibits osteogenic differentiation both by reducing the ALP activity and by inhibiting calcium accumulation, an early and late marker of osteogenic differentiation, respectively. In contrast, inhibition of collagen crosslinking did not affect ALP activity in hMSCs. Remarkably, it enhanced calcium accumulation despite the fact that no effects on gene expression or in integrin signalling could be observed when crosslinking was inhibited compared to the control. It is my hypothesis that this increase in calcium accumulation is due to an increase in the available space for crystal growth. Mineralization of collagen molecules occurs in the gap junctions between tightly packed collagen molecules [13]. Inhibiting collagen crosslinking might loosen these compact molecules, thus providing more space for crystal growth.

The possibility to manipulate the level of crosslinking in order to increase the pre-mineralization of tissue engineered constructs seeded with hMSCs opens a new possibility for treatment of bone defects. So far, cell-based tissue engineering results in insufficient amounts of bone deposition in vivo [14, 15]. Moreover, current therapies are mostly focused on manipulation of hMSCs using soluble factors which mostly limits their application to the in vitro expansion phase. By manipulating in vitro the collagen structure and subsequent implantation of the cell-based bone graft, we can interfere with a physical signal. Based on our in vitro data, this might enhance bone formation in vivo. Interestingly, distinct results were observed in both cell types in response to inhibition of crosslinking. This indicates that the extrapolation of knowledge gathered on one cell type cannot always be extrapolated to another cell type as the same signal can have two disparate biological outcomes.

Based on the results of chapter 4 we next tried to use the in vitro knowledge to develop a highly macroporous scaffold to incorporate crosslinked or uncrosslinked collagen and analyze its potential to form bone in vivo when seeded with rat or hMSCs. Chapter 5 describes the production of hydroxyapatite scaffolds coated with crosslinked or uncrosslinked collagen. We determined whether the presence of collagen has a positive effect on osteogenic differentiation and if crosslinking would further affect differentiation. The incorporation of crosslinked collagen affected the proliferation of both rat and hMSCs. This could be explained by the denser fibre meshes found in the sample when collagen was crosslinked which would impede the migration of cells into the inner parts of the scaffolds. One interesting finding in this study was the big difference in bone deposition between rat and hMSCs, which underlines the difference in bone forming potential of MSCs derived from man compared to that of MSCs derived from most of our experimental animal models. Proliferating cells are necessary to achieve ectopic in vivo bone formation and the differences might reflect the difference in the proliferative potential upon implantation [16]. We could not find a positive effect for collagen on the in vivo bone formation by rat MSCs. Surprisingly, the incorporation of collagen resulted in a decrease in in vivo bone formation by hMSCs, a result which was more pronounced in the case of crosslinked collagen. These results indicate that, in the case of rat MSCs, other factors rather than collagen contribute to the in vivo bone formation whereas in the case of hMSCs the presence of collagen impairs the in vivo bone formation. The reason for this could be the stabilization of the scaffold due to ECM production by the cells. Although I did not quantify it, I observed that the amount of ECM produced by rat MSCs was higher than by hMSCs, which may lead to a faster degradation of the fibers upon implantation in the case of hMSCs with concomitant decrease in the number of cells attach to the scaffold, which in turn lead to a decrease in bone formation. From chapter 4 and chapter 5 we can conclude that the presence of uncrosslinked collagen does not affect proliferation of hMSCs. In line with the results from chapter 4 we observed that hMSCs seeded on HA scaffolds with uncrosslinked collagen deposited significantly more bone than hMSCs on HA scaffolds with crosslinked collagen.

As a multidisciplinary field, tissue engineers use a combination of scaffolds, cells and signals to restore damage or loss of organ function. As such, besides the focus on controlling cell differentiation both in vivo and in vitro, I also studied the effects of the two other players in the tissue engineering cycle: soluble signals and scaffolds. In chapter 6, I focused on the modulation of BMP signalling in an attempt to control differentiation into the osteogenic lineage. In chapter 7, I described

the development of a new synthetic material able to induce the formation of such a complex tissue as bone. In chapter 6, the role of the BMP antagonist chordin-like 1 on proliferation and differentiation of both MC3T3-E1 cells as well as hMSCs was analyzed. As in the case of chapter 4 for the inhibition of collagen crosslinking, I also found differences between the two cells types in response to chordin-like 1. In MC3T3-E1 cells, chordin-like 1 inhibits BMP-induced ALP activity without affecting proliferation whereas in the case of hMSCs, chordin-like 1 consistently enhanced proliferation and did not affect differentiation. The mechanism by which chordin-like 1 enhances proliferation is not known but is most likely independent of BMP signalling. Because the number of cells is a key factor in any tissue engineering application the discovery of a protein that has such a pronounced effect on proliferation without compromising differentiation opens new possibilities. Furthermore, chordin-like 1 contains potential target sites for a protease which will inactivate it, releasing any free active BMP bound to it [17, 18]. This reversible binding between chordin-like 1 and BMPs can be a useful tool for tissue engineering application giving us the possibility to use it as a BMP drug delivery system.

In the last chapter I described a synthetic material able to restore bone continuity in a critical size bone defect with the same efficacy as the golden standards in the clinic. Currently, when bone is lost or damaged, the first option to replace it is autologous transplantation of bone from another site. Nevertheless, with the discovery of osteoinduction by BMPs a new alternative is available to treat bone defects [19-23]. Although currently used in patients to induce bone formation, BMPs are difficult to deliver in vivo and very costly because supra-physiological dosages are required to achieve the desired effect. As such, the work reported in this chapter presents a new, efficient and cost effective alternative to the previous methods. Based on previous work showing differences in osteoinductive capacity between distinct calcium phosphate ceramics, a range of materials was produced with distinct micro and macro structure, resorption rate, protein binding ability and calcium release [24, 25]. These materials showed osteoinductive potential when implanted ectopically in the muscle of sheep. This osteoinductive capacity is a good indication of the potential of the material to perform well in a bony defect [26]. Therefore, the performance of the best osteoinductive material produced was compared to the golden standard, autologous bone, as well as with BMP-2, in the treatment of a critical-size defect in the ilium of sheep. The result showed that the performance of the synthetic material was comparable to autologous bone and BMP-2. The mechanism of action of this synthetic material remains to be unravelled, requiring the creation of simplified models to overcome the need to use large animals. Two possible models were presented in this chapter which can be used in the future to study the mechanism of action of synthetic materials.

In conclusion, this thesis shows that a successful tissue engineering approach for a complex tissue such as bone should comprise knowledge obtained from research in the three main players of the tissue engineering cycle: cells, signals and scaffolds. As shown throughout this thesis, lessons obtained from cell biology can be used to develop new scaffolds incorporating new signalling cues. The search for suitable models that will allow us to move from the bench to the clinic still remains a challenge since some results gathered in one model do not hold in another.

Future perspectives

As mentioned earlier in this thesis, the combination of cells, signals and scaffolds in tissue engineering applications can bring additional benefits when compared to the separate implementation of these individual parts. The first step towards the rational combination relies on the analysis of the biological system in question. Before ethical issues are solved and the differentiation into the desired lineage is fully controlled in the case of embryonic stem cells, adult stem cells will be the model cell type for tissue engineering applications. As such, efforts should be made to standardize their culture conditions so results can be compared between different groups. Moreover, donor variation is a major concern in this model. Although other cell lines can be helpful to tackle some biological questions, efforts should be mainly concentrated on adult stem cells as this will be, most likely, the cell type to be used in future clinical applications. Genomic and proteomic databases of adult stem cells should be created, again with emphasis on using standardize protocols to overcome the issue of donor variation.

The next step would be to interfere with the differentiation of adult stem cells by modulating different pathways known to be involved in osteogenic differentiation. The use of genetically modified cells is far from being a reality in a clinical scenario due to associated risks for the recipient. Therefore, efforts should be directed to find molecules able to interfere with the molecular mechanism driving differentiation during the in vitro expansion phase. The use of high throughput screening technologies and libraries of compounds should be implemented and used routinely. We should carefully and critically analyze the results from these assays and the readout should be broadened. This means that the functional assays currently in use should be revised and maybe other readouts should be established. As collagen is a key component of osteogenic differentiation, maybe compounds with the potential to interfere with its production or structure should be further analyzed. The information gathered from these studies should allow us to improve scaffold design either by mimicking the structure of ECM or by biofunctionalizing the scaffolds with molecules or small peptides that can guide differentiation. Additionally, we should make efforts to spatially control the delivery of biological agents as well as topographical features within a scaffold. Moreover, the mechanical properties of scaffolds can be tailored to mimic the tissue to be replaced and by doing so controlling the differentiation of adult stem cells, as previously shown [27, 28]. It would be an alternative to the use of soluble factors and provides the potential to control cell differentiation by controlling cell shape [29-31].

Since the differentiation of adult stem cells into the desired lineage is far from trivial, we should put our efforts in the design of scaffolds that, upon implantation, can recruit the cell types necessary to restore the damaged tissue. This can be achieve by incorporating biomolecules or small peptides able to bind certain cell types, by controlling the degradation rate and by doing so control the release of any biological agent present in the scaffold or even by using molecular imprinting techniques that can recruit molecules of interest to the damaged area [32].

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Summary

The increase in life expectancy has led to a concomitant increase in the incidence of lost or damaged tissues associated with ageing or other malignancies, subsequently providing a boost to the field of tissue engineering. Tissue engineering aims to be an alternative when the intrinsic regenerative capacity of the body fails, either due to the extent of the damage or due to ageing. From the early 90's to the present, the field has been growing and incorporating expertise from more and more disciplines.

In this thesis I focused on the regeneration of bone tissue. Currently, when bone is damaged or lost and the natural regenerative process is impaired or absent, few alternatives are available to restore bone continuity. These alternatives rely on the use of autologous bone, bone morphogenetic proteins (BMPs), synthetic materials or a combination. However, these alternatives are far from ideal and as such the search for new alternatives has intensified in recent years.

Throughout this thesis I tried to analyze different alternatives that could eventually lead to new therapies. Extracellular matrix (ECM) holds an enormous potential not only as a physical support capable to restore mechanical integrity but also as a biological entity providing molecular cues to drive the differentiation of uncommitted cells into the desired lineage.

Hence, chapter 1 gives an overview of the field and the currently available tools with special emphasis on the cell types and differentiation pathways potentially involved in the process of bone formation. Chapter 2 discusses the role of ECM and its potential application in the field of tissue engineering. The main components of ECM are introduced with a special emphasis for the most abundant molecule in ECM – collagen. The latest developments regarding the technological advances in the use of ECM for tissue engineering applications are also included. Chapters 3 and 4 analyze how collagen affects the differentiation of human mesenchymal stem cells (hMSCs) towards the osteogenic lineage. Particular emphasis was devoted to the presence or absence of collagen during differentiation (chapter 3) and to the structure of collagen (chapter 4). A key role for collagen in proliferation of hMSCs and a new mechanism by which collagen crosslinking interferes with the in vitro differentiation of hMSCs into the osteogenic lineage was unraveled. Moreover, we drew attention to the response of different cell types to collagen structure. This raises some concerns regarding the extrapolation of results obtained with one cell type to another. In this case, opposing effects between MC3T3-E1 cells, a mouse calvarial cell line commonly used in bone tissue engineering, and hMSCs, a more clinical relevant cell type, are disclosed with respect to collagen crosslinking and osteogenic differentiation.

Chapter 5 results from what I learned from the previous chapters. A new family of macroporous ceramics incorporating crosslinked or uncrosslinked collagen was developed and their performance analyzed both in vitro as well as in vivo. Again, results showed differences between species with respect to collagen structure and in vivo bone forming potential of rat and human mesenchymal stem cells (MSCs) when seeded in the different ceramics. Chapter 6 analyzed the role of a component of the BMP signaling pathway (chordin-like 1, a BMP inhibitor) in osteogenic differentiation of mouse and hMSCs. A new role of chordin-like 1 in proliferation of hMSCs was discovered. This effect is observed solely in the case of hMSCs as mouse MSCs and MC3T3-E1 cells show a different response to chordin-like 1. Finally, chapter 7 introduces a new family of calcium phosphate ceramics

with osteoinductive potential. The performance of these new materials was characterized in several in vitro and in vivo models and a pre-clinical study was performed and unequivocally showed the potential of these new class of materials to substitute the current therapies. We showed that, compared to BMP-2 treatment or autologous bone transplantation, a fully synthetic material was capable of healing a critical-size bone defect in sheep. Although the mechanism by which synthetic materials are capable of inducing bone formation ectopically has not been unraveled yet, we showed that nevertheless they could be a valid alternative in the clinic.

In conclusion, I described new roles of ECM in osteogenic differentiation and disclosed a new class of ceramics with favorable characteristics to be a valid alternative to current bone therapies.

Samenvatting

Als gevolg van de toename in levensverwachting krijgen we steeds meer te maken met uitgevallen of beschadigde weefsels, die vaak in verband staan met veroudering of andere ziekten. Dit heeft geleid tot een sterke toename van de weefsel regeneratie. Het doel van weefsel regeneratie is een alternatief te bieden wanneer het lichaam zelf niet voldoende intrinsieke regeneratieve capaciteit bezit om de schade te herstellen omdat deze óf te groot is óf omdat het lichaam verouderd is. Al sinds de vroege jaren '90 tot heden is het veld groeiende en worden expertises van verscheidene disciplines hierin gecombineerd en benut.

In dit proefschrift heb ik me gericht op de regeneratie van botweefsel. Momenteel zijn er weinig alternatieven om het bot te herstellen wanneer het beschadigd of zelfs verdwenen is, en het natuurlijke regeneratie proces aangetast is. De huidige alternatieven zijn gebaseerd op het gebruik van autoloog bot, *bone-morphogenetic-proteins* (BMPs), synthetische materialen of een combinatie hiervan. Deze alternatieven zijn echter verre van ideaal en de zoektocht naar nieuwe alternatieven is dan ook sterk toegenomen gedurende de afgelopen jaren.

Gedurende dit proefschrift heb ik geprobeerd verschillende alternatieven te analyseren die uiteindelijk zouden kunnen leiden tot nieuwe therapieën. De extracellulaire matrix (ECM) heeft enorme potentie, niet alleen als fysieke ondersteuning die in staat is om de mechanische integriteit te herstellen, maar ook als biologische entiteit, waarbij de ECM signalen kan afgeven om cellen aan te sturen en te differentiëren naar het gewenste celtype.

In hoofdstuk 1 wordt een overzicht gegeven van het huidige veld en de beschikbare methoden waarbij speciale nadruk wordt gelegd op de verschillende celtypen en differentiatie mechanismes die mogelijk betrokken zijn gedurende de botformatie. Hoofdstuk 2 bespreekt de rol van de ECM en mogelijke toepassingen hiervan in de weefsel regeneratie. De componenten van de ECM worden geïntroduceerd met speciale aandacht voor de hoofdcomponent – collageen. Tevens worden hier de laatste ontwikkelingen wat betreft de technische vooruitgang in het gebruik van de ECM in weefsel regeneratie besproken. In hoofdstuk 3 en 4 wordt geanalyseerd hoe collageen de differentiatie van humane mesenchymale stamcellen (hMSCs) naar de osteogene lineage beïnvloedt. Nadruk ligt vooral op de aanwezigheid of afwezigheid van collageen tijdens de differentiatie (hoofdstuk 3) en de structuur van collageen (hoofdstuk 4). Hierbij is een sleutelrol gevonden voor collageen in de proliferatie van hMSCs en een nieuw mechanisme ontrafeld waarbij de crosslinking van collageen interfereert met de in vitro differentiatie van hMSCs naar de osteogene lineage. Daarnaast hebben we gekeken naar de responsie van verschillende celtypes op de structuur van collageen. Hierbij is het wel de vraag in hoeverre de resultaten met het ene celtype te extrapoleren zijn naar het andere celtype. Op het gebied van collageen crosslinking en osteogene differentiatie zijn in dit geval tegengestelde effecten gevonden tussen MC3T3-E1 cellen, een veel gebruikte cellijn in botweefsel regeneratie die is afgeleid van cellen uit het calvarium van muizen, en hMSCs, een klinisch meer relevant celtype.

Hoofdstuk 5 resulteert van wat ik heb geleerd uit de vorige hoofdstukken. Er is een nieuwe groep macroporeuze keramieken ontwikkelt die gecrosslinkt of ongecrosslinkt collageen bevatten en deze materialen zijn zowel in vitro als in vivo geanalyseerd. De resultaten laten wederom zien dat cellen van verschillende origine, bijvoorbeeld ratten- en humane MSCs anders reageren op de

structuur van collageen evenals het potentieel om bot te vormen, wanneer deze cellen gezaaid worden op de verschillende keramieken. Hoofdstuk 6 bespreekt de rol van een component van de BMP signaal transductie route (chordin-like 1, een BMP remmer) in de osteogene differentiatie van muis en humane MSCs. Hierbij is ontdekt dat chordin-like 1 een rol speelt in de proliferatie van humane MSCs. Dit effect is alleen waargenomen in het geval van humane MSCs, aangezien MSCs van muizen en MC3T3-E1 cellen anders reageren op chordin-like 1. Tot slot wordt er in hoofdstuk 7 een nieuwe groep calcium fosfaat keramieken met osteoinductieve eigenschappen geïntroduceerd. De eigenschappen van deze nieuwe materialen zijn gekarakteriseerd in een aantal in vitro en in vivo modellen en er is een pre-klinische studie uitgevoerd waarin onmiskenbaar het potentieel om de huidige therapieën te vervangen is aangetoond. We hebben laten zien dat, in vergelijking met behandeling met BMP-2 of transplantatie van autoloog bot, deze volledig synthetische materialen in staat zijn om een defect met bot te overbruggen dat te groot is om vanzelf te herstellen. Hoewel het mechanisme waardoor synthetische materialen bot vormen op een ectopische plaats niet ontrafeld is, hebben we desondanks laten zien dat deze materialen een goed alternatief kunnen zijn in de kliniek.

Ter conclusie; in dit proefschrift heb ik een nieuwe rol voor de ECM in osteogene differentiatie beschreven evenals een nieuwe groep keramieken met dusdanige eigenschappen dat ze een zeer goed alternatief vormen voor de huidige botweefsel regeneratie therapieën.

Sumário

O aumento da esperança média de vida resultou no concomitante aumento de lesões e danos irreparáveis em diversos órgãos, um fenómeno associado não só ao envelhecimento mas também a doenças características da terceira idade. Este quadro clínico-social instigou o aparecimento de uma nova disciplina: a engenharia de tecidos. A engenharia de tecidos evoluiu como uma possível alternativa para os casos em que a capacidade regenerativa do organismo não é suficiente, quer devido à extensão da lesão quer ao envelhecimento do mesmo. Desde o início da década de 90 que esta nova disciplina tem vindo a progredir, incorporando o conhecimento de diversas áreas científicas.

A presente tese incide na área da regeneração óssea. Actualmente, quando o tecido ósseo é danificado e a capacidade regenerativa deste tecido é limitada, poucas são as terapias disponíveis para restaurar a continuidade óssea. Entre elas encontra-se o uso de osso do próprio paciente, uma família de proteínas designada como proteínas morfogenéticas ósseas, materiais sintéticos ou uma combinação das opções anteriores. No entanto, estas alternativas estão longe de ser a opção ideal e, como tal, a busca de novas soluções tem vindo a intensificar-se nos últimos anos.

Ao longo desta tese foram estudadas alternativas que eventualmente poderão resultar na implementação de novas terapias clínicas. A matriz extracelular é uma entidade com enorme potencial não só capaz de agir como um suporte físico com capacidade de restaurar a integridade mecânica no caso de lesões ósseas mas também como uma entidade biológica apta para estimular a diferenciação de células estaminais mesenquimais em células da linhagem pretendida.

Assim sendo, no capítulo 1 é apresentada uma panorâmica geral desta nova disciplina e são abordadas as novas ferramentas disponíveis, com especial ênfase nos tipos de células e nas vias de sinalização potencialmente envolvidas no processo de formação de osso.

No capítulo 2 é discutido o papel da matriz extracelular e o seu potencial na área da engenharia de tecidos. Os principais componentes da matriz extracelular são aqui descritos e especial atenção é prestada à molécula mais abundante da matriz – colagénio. Os últimos desenvolvimentos no que respeita a avanços tecnológicos e ao uso de componentes da matriz extracelular em engenharia de tecidos estão aqui incluídos.

Os capítulos 3 e 4 analisam a forma como o colagénio influencia a diferenciação de células estaminais mesenquimais humanas na linhagem osteogénica. Especial atenção é prestada à presença ou ausência desta molécula durante a diferenciação (capítulo 3) e à estrutura da molécula (capítulo 4). Um papel essencial do colagénio na proliferação de células estaminais mesenquimais humanas e um novo mecanismo através do qual o arranjo destas moléculas poderá interferir com a diferenciação *in vitro* na linhagem osteogénica é aqui apresentado. Além disso, é também deixado um alerta para a resposta de diferentes tipos de células relativamente à estrutura do colagénio. Isto levanta algumas reservas no que diz respeito à extrapolação de resultados obtidos com um tipo de células para outro. Neste caso, efeitos opostos entre células MC3T3-E1, uma linha celular derivada do osso calvário de murganho e que é frequentemente utilizada em engenharia de tecidos, e células mesenquimais estaminais humanas, um tipo de células mais relevante do ponto de vista clínico, são descritos no que concerne à estrutura do colagénio e à diferenciação na linhagem osteogénica.

O capítulo 5 é o resultado do conhecimento adquirido nos capítulos anteriores. Uma nova família de materiais cerâmicos macroporosos incorporando colagénio com diferentes estruturas foi desenvolvido e a sua performance analisada *in vitro* e *in vivo*. Uma vez mais os resultados mostram diferenças entre espécies no que diz respeito à estrutura do colagénio e ao potencial de formar osso *in vivo* entre células estaminais mesenquimais de rato e humanas.

O capítulo 6 analisa o papel de um dos componentes da via sinalização das proteínas morfogenéticas ósseas (chordin-like 1, um inibidor da respectiva via) na diferenciação osteogénica de células de murganho e humanas. Um novo papel para esta proteína na proliferação de células estaminais mesenquimais humanas foi descoberto. Este efeito é observado somente no caso das células humanas uma vez que as células estaminais mesenquimais de murganho e células MC3T3-E1 revelaram um comportamento oposto na presença desta proteína.

Finalmente, o capítulo 7 revela uma nova família de materiais cerâmicos compostos por cálcio e fosfato com potencial osteo-inductivo. Estes novos materiais foram caracterizados em diversos modelos *in vitro* e *in vivo* que inequivocamente demonstraram a capacidade desta nova classe de materiais sintéticos para substituir as terapias actuais. Neste estudo foi demonstrado que comparativamente com o tratamento com a proteína morfogenética óssea-2 ou o transplante de osso do próprio, um material completamente sintético é capaz de curar um defeito ósseo crítico num modelo pré-clínico em ovelhas. Apesar do mecanismo através do qual o material sintético é capaz de induzir a formação de osso não seja totalmente revelado, é demonstrado neste estudo que esta nova família de materiais poderão constituir uma alternativa válida num cenário clínico.

Em conclusão, esta tese descreve novas funções da matriz extracelular na diferenciação osteogénica e é aqui apresentada uma nova classe de cerâmicos com características favoráveis constituindo uma alternativa válida às terapias actuais.

Acknowledgments

And finally here I am for the trickiest part of this thesis. Whereas it is easy to write about things you see, do or plan to do, it is not so easy to write about things you do not see, do not know but you do feel. So it is for the acknowledgments of a thesis. This thesis would not be without the help, support and incentives of several people that in one moment or the other succeed in helping me reaching the finishing line…I am afraid this part of the thesis will not make to much sense but I will try my best to organize my feelings….

When I decided to change from computer science to Applied Biology I was far from imagine where that would take me. From my first experience in a laboratory in Madrid, where I had the pleasure of finding people capable of guiding me in the lab as well as in an amazing city (thank you Chema and the others in the lab) to here, I have found many inspiring people. A special word to my immunology professor Jorge Pedrosa. You got me into this…Without you I would probably be away from labs.

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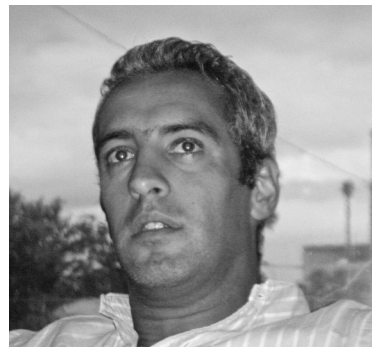
E claro que isto não é um esquecimento: poderias ter estado na parte dos portugueses, na parte dos colegas de doutoramento, na parte dos amigos de viagens, de festas e de desportos mas a verdade é que estás não só nesses todos como em muito mais. Ana, eu sei que esta tese foi quase tão difícil para ti como para mim, mas está feita....Estas são as ultimas linhas que escrevo e são só para ti! Daqui para a frente temos outros desafios, viagens e cumplicidades pela frente! Adoro-te!

Curriculum Vitae



Hugo Fernandes was born on October 16th, 1977 in Joane, Portugal. After spending the first years of his life in Joane he moved to Braga where he finished high school in Colégio Dom Diogo de Sousa in 1995. From 1995 to 1998 he was enrolled in the graduation of Systems Engineering and Informatics at the University of Minho, Braga. In 1998 he re-enrolled in Applied Biology and graduated in 2003 at the same University. The final graduation thesis was performed at the University Complutense, Madrid, Spain in 2002 within the Socrates/ Erasmus Program. There he worked in Immunopathologies associated with T lymphocytes under the supervision of Dr. José Ramon Regueiro.

In 2004 he accepted a PhD position at the University of Twente, Enschede, The Netherlands. The research was performed under the supervision of Dr. Jan de Boer and Prof. Clemens van Blitterswijk, and the results are presented in this thesis entitled "Instructive Elements for Bone Tissue Engineering" .

In 2009 he started a post-doc in the same group working in endothelial differentiation of mesenchymal stem cells.