# 1 General Introduction

## 1.1 BONE TISSUE

Bone is a multifunctional organ involved in structural and physiological processes. It provides support to the body and creates a protective barrier for the vital organs of the cranial and thoracic cavities. It is actively involved in body movements with muscles, being part of the musculoskeletal system. Bone tissue is composed of a peripheral cortical part, the compact bone or cortex, and an internal and spongy part, or trabecular bone, which harbors bone-marrow cavities that are essential for hematopoiesis. In addition, bone tissue is involved in metabolic processes, as it stores and mobilizes calcium and phosphate, regulating mineral homeostasis in the blood [1]. It also acts as reservoir of signaling molecules contributing to the endocrine regulation of energy metabolism [2].

The various different types of cells in the bone are essential for bone homeostasis and are surrounded by an extracellular matrix (ECM), which is hardened by mineral deposits and makes bone a very rigid and robust type of connective tissue (Figure 1). Bone is constantly rebuilt during remodeling and cells continuously receive inputs from the microenvironment in a complex interplay that makes bone tissue far from being inert [1].

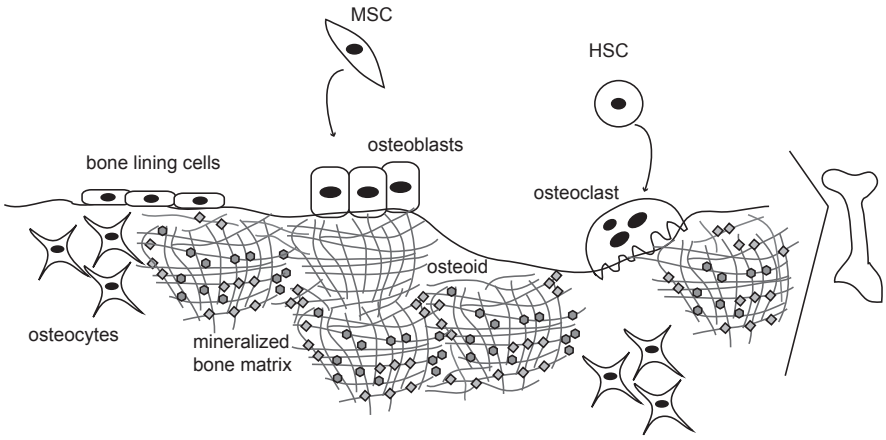


**Figure 1. Composition of bone tissue.** Bone tissue is composed of bone ECM and bone cells in a tight interplay. Osteoblasts originate from MSCs and secrete and mineralize bone ECM. They mature further towards osteocytes, entombed in the bone ECM and responsible for sensing mechanical strains, or the resting bone lining cells. Osteoclasts arise from HSCs and are responsible for bone resorption by degrading the ECM. MSC: mesenchymal stem/stromal cell; HSC: hematopoietic stem cell.

### 1.1.1 Bone extracellular matrix

Bone ECM is the key component of bone tissue and responsible for its strength, elasticity and plasticity. It creates a scaffold for bone cells and guides mineralization, promoting bone formation (Figure 2). Bone ECM is composed for almost 70% of an inorganic part of calcium hydroxyapatite (HA) crystals [$Ca_{10}(PO_4)_6(OH)_2$], whereas proteins, proteoglycans and water form the remaining organic part (Table 1) [3]. The inorganic part determines the strength of the bone, inducing low elasticity but robustness, and contributes to the metabolic role of bone in maintaining ion homeostasis. The organic part of bone ECM includes mainly collagen fibers and non-collagenous proteins (NCPs), such as proteoglycans and glycoproteins [4, 5]. Type-1 collagen represents about 90% of the organic bone ECM. Collagen fibers are essential for bone structure and flexibility, providing a structural support for osteoblast progenitors and promoting osteoblast differentiation. Collagen fibers are key mediators of matrix mineralization, creating a framework for matrix vesicles during mineral deposition [6, 7].

Non-collagenous proteins mainly consist of proteoglycans, glycoproteins, small integrin binding ligand N-linked glycoprotein (SIBLING) family members, Gla-containing proteins, but also matricellular proteins and matrix metalloproteinases (MMPs). They have regulatory roles, controlling the ECM organization but also modulating the mineralization process and cell-ECM interactions. Proteoglycans such as biglycan and decorin are very abundant in the bone ECM. They promote collagen fibrillogenesis and bind growth factors, for instance decreasing transforming growth factor β (TGF-β) availability [8]. Moreover, osteonectin (SPARC) is a glycoprotein that can bind calcium, collagen and growth factors, thus being essential for matrix mineralization and eventually regulating osteoblast attachment and differentiation

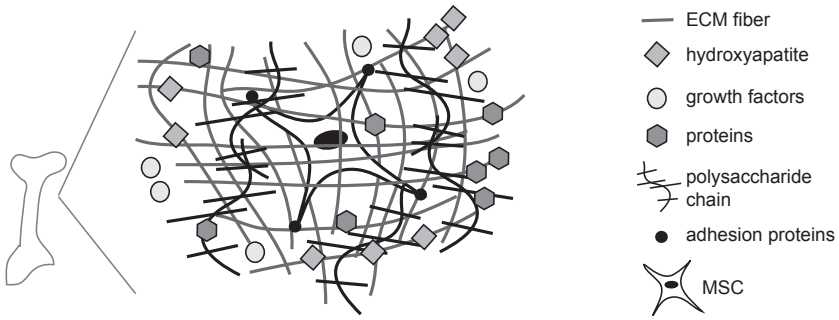


**Figure 2. Schematic representation of bone ECM.** Bone ECM has a rich composition of collagen fibers and many non-collagenous proteins, but also other molecules such as growth factors and polysaccharides and it is hardened by hydroxyapatite crystals. In bone tissue, bone ECM is essential to physically support bone cells but also actively influence their behavior. MSC: mesenchymal stem/stromal cell.

**Table 1.** List of bone ECM components.

| Component | | | | Function |
|---|---|---|---|---|
| Inorganic (70-80% of wet weight of bone) | | | Hydroxyapatite crystals | Bone strength<br>Ion homeostasis |
| Organic (20-30% of wet weight of bone) [3, 6] | Collagenous proteins (90% of total ECM) | | Collagen type 1 | Primary ECM structural component<br>ECM mineralization<br>Osteoblast differentiation [7] |
| | Non-collagenous proteins (10% of total ECM) [4, 5] | Proteoglycans | Biglycan<br>Decorin | Collagen fibrillogenesis<br>Growth factor availability [8] |
| | | Matricellular proteins | Osteonectin | Collagen fibrillogenesis<br>ECM mineralization<br>Cell attachment and differentiation [9] |
| | | | Thrombospondin | ECM assembly<br>Growth factor availability [11] |
| | | | Fibronectin | ECM assembly<br>Cell adhesion [13] |
| | | | Periostin | ECM assembly<br>ECM mineralization [14] |
| | | SIBLING proteins | Bone sialoprotein | ECM mineralization<br>Cell adhesion [17] |
| | | | Osteopontin | ECM mineralization<br>Cell adhesion, proliferation and differentiation [18] |
| | | Gla-containing proteins | Osteocalcin | Osteoblast/osteoclast interaction<br>Glucose metabolism [20] |
| | | Growth factors | BMPs, TGF-β | Regulator of cell behavior and bone formation [22, 23] |
| | | Enzymes | Alkaline phosphatase | ECM mineralization [16] |
| | | | Matrix metalloproteinases | Regulator of bone formation and bone remodelling [96] |

[9, 10]. Similarly, thrombospondins (TSP) facilitate collagen cross-linking and fibrillogenesis and regulate TGF-β availability [11]. Fibronectin (FN) is expressed also in other connective tissues, it interacts with other ECM proteins, being crucial for collagen-matrix assembly. It can bind integrin proteins, modulating cell adhesion [12, 13]. Periostin (POSTN) is a matricellular protein secreted by osteoblasts that is essential for ECM organization, as it regulates collagen cross-linking and interacts with other ECM proteins. Its role in mineralization is still controversial [14]. Bone ECM also contains enzymes, such as the tissue-nonspecific isoform of alkaline phosphatase (TNAP or ALPL), which is expressed by differentiating osteoblast progenitors as membrane-bound form or released into the bone ECM. This enzyme is one of

the key regulators of mineralization as it increases the level of phosphate [15, 16]. Members of SIBLING proteins such as bone sialoprotein (BSP) and osteopontin (OPN) are detected in bone ECM. They contain Arg-Gly-Asp (RGD)-domains favoring integrin-mediated cell adhesion and controlling cell proliferation and differentiation. While BSP is osteoinductive and promotes matrix mineralization [5, 17], OPN regulates hydroxyapatite nucleation and crystal growth, controlling bone quality [18, 19]. Osteocalcin (BGLAP) belongs to the γ-carboxylated protein family or Gla-containing proteins. It is a very abundant ECM protein expressed during late stages of osteoblast differentiation, inhibiting matrix mineralization. Its function is still under investigation, but as it promotes osteoclast progenitor recruitment, it might be involved in osteoblast-osteoclast interaction. In its uncarboxylated form, it mediates bone metabolic function by controlling lipid and glucose metabolism [20, 21]. Bone ECM is also rich of growth factors such as bone morphogenic proteins (BMPs), TGF-β, insulin-like growth factor (IGF) and tumor necrosis factor alpha (TNF-α), which are crucial regulators of bone formation, but also cytokines and enzymes [3, 22, 23]. Most of these proteins were detected in human bone samples by mass spectrometry [24] and are listed in Table 1. Overall, bone ECM is a very dynamic structure that modulates bone formation and bone remodeling, directly by ECM-cell interactions but also by regulating the availability of signaling molecules.

### 1.1.2 Bone cells

Bone is actively maintained by different cell types: osteoblasts, osteocytes, bone lining cells and osteoclasts (Table 2). Osteoblasts are cuboidal cells that originate from mesenchymal stem cells (MSCs) and produce ECM, resulting in bone formation (Figure 1) [25, 26]. After bone formation phase, majority of osteoblasts undergo apoptosis, whereas some of them become inactive bone lining cells, which are elongated flat cells that cover the surface of the quiescent bone [25, 27, 28]. Moreover, some osteoblasts terminally differentiate toward osteocytes, which are the most abundant cells in bone [29]. Osteocytes are highly specialized cells em-

**Table 2.** List of bone cells and their functions.

| Cell type | Function |
|---|---|
| Osteoblasts [25, 26] | Anabolic cuboidal cells arising from MSCs<br>Bone forming cells by secreting and mineralizing bone ECM |
| Osteocytes [30] | Most abundant cells buried in the mineralized bone ECM, derived from osteoblasts<br>Mechanical sensors via cytoplasmatic processes to maintain bone structure<br>Key regulators of phosphate metabolism by secretion of FGF23 |
| Bone lining cells [29] | Inactive flat cells derived from osteoblasts, that delimit the bone surface |
| Osteoclasts [34] | Multinucleated giant cells arising from HSCs<br>Bone resorbing cells by degrading bone ECM |

bedded in lacunae in the mineralized ECM and are interconnected with each other and osteoblasts by cytoplasmatic extensions and paracrine signaling. They serve as mechanical sensors to maintain bone structure, translating mechanical forces into biochemical signaling [30]. They express specific markers such as dentin matrix proteins (DMP-1), phosphate-regulating gene with homologies to endopeptidases on the X-chromosome (PHEX), matrix extracellular phosphoglycoprotein (MEPE), sclerostin (SOST) and fibroblast growth factor 23 (FGF23) [30-32]. Osteocytes direct bone formation both by providing paracrine signaling toward osteoblasts but also by undergoing apoptosis to recruit osteoclasts, thus regulating bone turnover. They are key regulators of phosphate homeostasis by producing FGF23 [30].

Bone formation is contrasted by osteoclasts, which are large multinucleated bone-resorbing cells of hematopoietic origin (Figure 1). Osteoclasts strongly adhere to the fully mineralized bone matrix, seal a selected area and acidify it, eventually dissolving the inorganic part. Subsequently, proteases are secreted to degrade the organic part of the matrix, eventually leading to bone resorption. For the complete osteoclast activation, physical interplay with osteoblast is required via the receptor activator of nuclear factor kappa B (NF-kB) ligand (RANKL) cytokine [33-35]. Bone is renewed through life by a coordinated balance between bone resorption and bone deposition.

## 1.2 BONE FORMATION

During morphogenesis, bone formation can follow two processes. MSCs directly differentiate into osteoblasts, during intramembranous ossification such as in skull bones. Alternatively, in long and short bones, MSCs differentiate into chondrocytes that secrete an intermediate cartilaginous template. Chondrocytes become hypertrophic and are replaced by invading osteoblasts that proceed with ossification [36, 37]. Both processes require vascularization to recruit new cells and osteoblast differentiation to secrete and mineralize the osteoid or unmineralized bone.

### 1.2.1 Osteoblast differentiation and mineralization

MSCs in the bone marrow give rise to multiple cell lineages. Osteoblast differentiation is tightly regulated by specific transcription factors such as Runt-related transcription factor 2 (RUNX2) and osterix (OSX), that act in a controlled temporal sequence and promote skeletal development [38-40]. After osteogenic-lineage commitment of MSCs, osteoprogenitors start proliferating and then differentiate toward pre-osteoblasts, which express genes of bone matrix proteins, and further develop into mature osteoblasts, that mineralize the bone matrix (Figure 3).

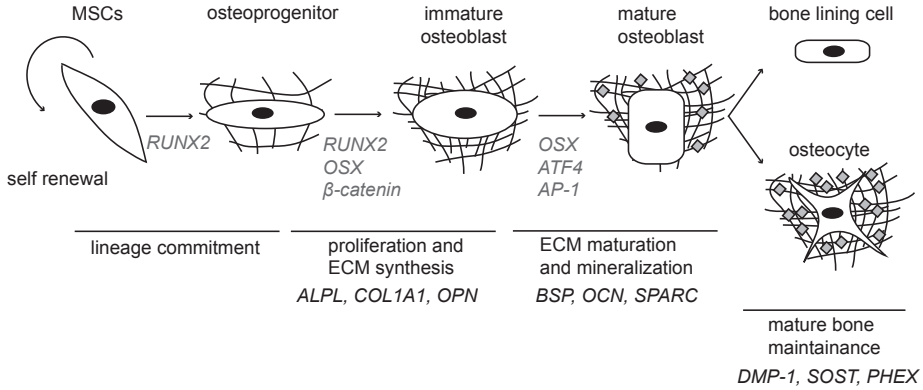


**Figure 3. Osteogenic differentiation of MSCs.** MSCs are self-renewal cells that can be differentiated toward osteoblasts. After lineage commitment, osteoprogenitor cells start to proliferate and differentiate becoming immature osteoblasts, which synthesize the immature ECM or osteoid. These cells further differentiate toward mature osteoblasts, that mineralize the bone ECM. Osteoblasts can undergo apoptosis, or become bone lining cells or further mature toward osteocytes. Signaling molecules activate several signaling pathways that results in a temporal sequence of transcription factors (the most important are indicated in grey), inducing the expression of cell-specific markers (indicated in black and resulting in an efficient osteogenic differentiation.

RUNX2 is the master regulator of skeletogenesis, essential for starting the osteogenic lineage commitment but also to keep a reservoir of immature osteoblasts [41, 42]. RUNX2 controls the expression of other osteoblast-specific genes, thus its expression is tightly regulated by cofactors such as distal-less homeobox-5 (DLX5) and msh-homeobox homologue-2 (MSX2), and inhibitors, such as twist homolog 1 (TWIST1). RUNX2 is upstream of OSX, which is the second important transcription factor that guides the transcriptional activity toward mature osteoblasts [43]. These transcription factors, together with others such as Activator protein 1 (AP-1), induce the expression of bone ECM proteins, e.g. collagen type 1 α1 (COL1A1), BSP and OPN [44]. Subsequently, osteoprogenitor cells continue the differentiation toward pre-osteoblasts and start synthesizing the osteoid. Activating TF4 (ATF4) has an important role in regulating mature osteoblast function, especially by regulating amino acid import into osteoblasts for ECM protein synthesis [45]. Eventually, they become mature osteoblasts that express high level of ALP and osteocalcin, and mineralize the matrix via mineral deposition. Although matrix mineralization is still not fully understood, it is driven by matrix vesicles and it proceeds in two phases [31, 46]. First, crystals of calcium and phosphate are formed inside matrix vesicles and secondly, they continue growing disrupting matrix vesicle membranes and creating a mineral nucleation site. Matrix mineralization is controlled by different proteins, such as NCPs, annexins and ALP that tightly regulate the balance between activators and inhibitors [4, 47, 48]. Mature osteoblasts undergo apoptosis or differentiate further toward os-

teocytes or bone lining cells. The transition toward osteocytes is still not fully known. However, osteoblasts need to reduce their volume and undergo cytoskeletal changes to develop dendritic morphology, together with upregulation of osteocyte-specific markers, prior of remaining embedded in the calcified matrix [29, 32].

### 1.2.2 Regulation of osteoblast differentiation and mineralization

Bone is continuously influenced by the surrounding microenvironment in an autocrine, paracrine and endocrine signaling. In more detail, cytokines and growth factors in the circulation, but also hormones and signaling molecules that are released by bone cells and by ECM-degradation, actively control bone formation. Osteogenesis is finely regulated by a crosstalk of signaling pathways that control the transcriptional regulators, leading to a tight regulation of osteoblast differentiation (Figure 4) [40].

The most important regulators of osteoblast differentiation are the members of the TGF-β superfamily, namely BMPs, TGF-β itself and activins (Figure 4 and 5) [49,

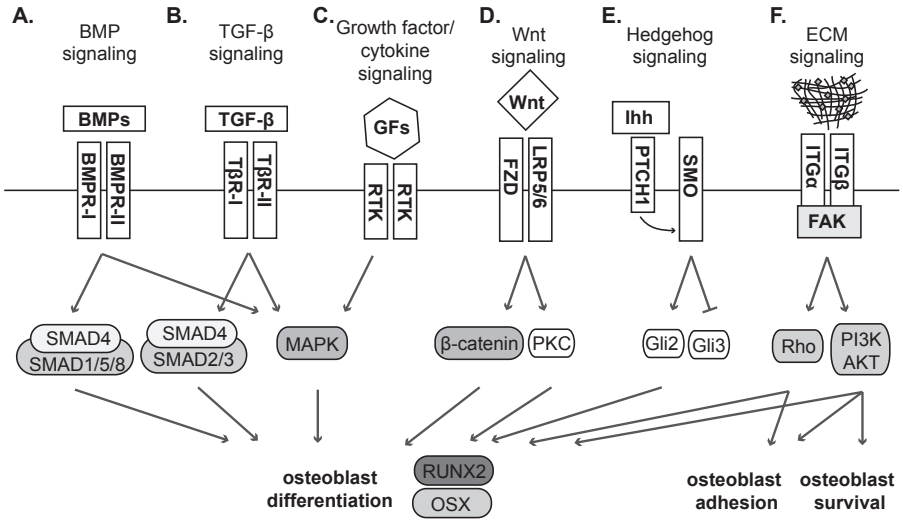


**Figure 4. Signaling pathways regulating osteoblast differentiation, adhesion and survival.** A) BMP signaling induces osteogenic differentiation via canonical (SMAD) and non-canonical (MAPK) signaling pathway. B) TGF-β increases the pool of osteoprogenitors and promotes early osteogenic differentiation via canonical and non-canonical pathway. C) Growth factors and cytokines signal through RTKs to phosphorylate MAPK signaling activating osteogenic transcription factors such as RUNX2 and OSX. D) Osteogenic differentiation is promoted by canonical (β-catenin) and non-canonical (PKC) Wnt signaling. E) Ihh signals through PTCH1 and SMO to activate the transcription of RUNX2 and OSX. F) ECM mechanotransduction via integrin-mediated signaling, which activates Rho family GTPase that mediates cytoskeletal rearrangements, MAPK and β-catenin signaling pathways promoting osteogenic differentiation, and PI3K/AKT signaling pathway that promotes osteoblast survival, adhesion and differentiation. Simplistic view, as pathways are interconnected in a complex network and inhibitors are not shown. Arrowheads indicate stimulation, lines with bars represent inhibition.

50]. BMPs bind to the heterodimeric receptors BMPR-I and BMPR-II, inducing the phosphorylation of SMAD1/5/8 and recruitment of SMAD4, that eventually activate the transcription of osteogenic transcription factors (Figure 4A). BMPs, especially BMP2 and BMP4, promote osteogenic differentiation and maintain the function of mature osteoblasts, thus being essential for *in vivo* bone formation. Signaling molecules such as gremlin, noggin, chordin, but also SMAD6 act as regulators by inhibiting BMP signaling. BMPs exert their osteoinductive role also irrespectively of SMAD proteins, in a non-canonical signaling pathway through p38 and JNK MAPK (Figure 4A) [22, 50].

Moreover, BMP signaling is also modulated by Hedgehog signaling that modulates SMAD proteins. Hedgehog binds to membrane receptors Patched (Ptc) and Smoothened (Smo), inducing Smo release and regulation of transcription factor Gli2 and Gli3, that modulate hedgehog target-gene expression (Figure 4E). Indian hedgehog (Ihh) is important to promote the early stages of osteoblastogenesis in endochondral ossification, by activating RUNX2 and OSX [40, 51].

At the early stages of osteoblast differentiation, BMP action is strengthened by TGF-β, which facilitates the proliferation, chemotaxis and differentiation of osteoprogenitors. However, TGF-β inhibits the late stages of differentiation toward osteocyte and matrix mineralization [52-55]. TGF-β binds to the tetrameric receptor complex of TβR-I and TβR-II, which phosphorylates SMAD2/3, that translocates into the nucleus with SMAD4 and regulate the transcription of target genes (Figure 4B). SMAD2/3 also recruits HDAC that inhibit RUNX2. As BMPs, TGF-β also signals through a SMAD-independent pathway, that involves p38 and ERK-MAPK signaling, that activate RUNX2 [54, 55]. Bone formation is also regulated by activins, as further elucidated in section 1.2.3.

Wnt signaling is also crucial for osteogenic-lineage commitment of MSCs and for bone metabolism. Wnt signals through 2 types of pathways. Wnt canonical pathway involves β-catenin, which is stabilized by the binding of Wnt to its Frizzled receptor (FZD) and co-receptors Lipoprotein receptor-related protein 5 and 6 (LRP5/6), and can induce the transcription of target genes such as RUNX2 after having bound T cell factor/Lymphoid enhancer binding factor (TCF/LEF) (Figure 4D). When Wnt is not present, β-catenin is degraded by Glycogen synthase kinase 3β (GSK-3β)/Axin/Adenomatous polyposis coli (APC) complex. Wnt signaling can also act through a non-canonical β-catenin independent pathway, which involves PKC and intracellular calcium release. Wnt signaling is inhibited by molecules such as secreted frizzled-related proteins, members of SOST family sclerostin and Dickkopf-1 and Wnt-inhibitory factor 1 (DKK1). Despite canonical pathway has been the major focus, non-canonical pathway is also important in bone cells. Wnt signaling stimulates the replication of osteoprogenitors, inducing osteoblastogenesis, but also inhibits

osteoblast apoptosis, positively correlating with bone mass [39, 56, 57]. However, Wnt signaling pathway also showed a negative regulation of osteogenic differentiation in human MSCs, illustrating that further studies are needed to investigate Wnt crosstalk with other pathways in MSC differentiation [58, 59]. At gene transcription level, these signaling pathways are interconnected between each other in an intricate crosslink that tightly control osteoblast differentiation and bone homeostasis.

Other signaling molecules such as insulin-like growth factor 1 (IGF1), vitamin D3, parathyroid hormone (PTH), steroids, glucocorticoids, leptin and calcitonin regulate mineral homeostasis and modulate bone formation, generally exerting a stimulatory action, but their effect is highly dependent on experimental factors (Figure 4C) [60].

### 1.2.3 Activin signaling

Bone cells are surrounded not only by the ECM but also by many signaling molecules such as activins, which play an active role in modulating bone formation. Activins are dimers of 2 inhibin β subunits and belong to the TGF-β superfamily. Activin-A for instance is formed by the dimerization of 2 inhibin βA subunits [61, 62]. The binding of Activin-A to transmembrane receptor type 2 (ACVR2A and ACVR2B) activates receptor type 1B (ACTVR1B=ALK4), eventually leading to the transcription of target genes via cytoplasmatic SMAD2/3 signal transduction (Figure 5) [63, 64]. Activin-A signaling regulates several cell functions such as cell proliferation and differentiation [65]. Activin-A signaling is modulated by several regulatory molecules and it is interconnected with TGF-β- and BMP-signaling, creating a complex cross-talk between these pathways [66, 67].

Activins target many cell types and different tissues, including bone. Activin-A is detected in bone matrix, secreted by osteoblasts and osteoclasts [68-70]. It showed a pro-osteoclastogenic effect *in vitro* [71-73]. Despite this, its role in bone metabolism and especially on osteoblast behavior is still not fully clear [74]. In some studies, Activin-A was shown to promote osteogenic differentiation *in vitro* and bone formation *in vivo* [68, 71, 75]. However, we and others reported Activin-A to inhibit matrix mineralization *in vitro*, by altering ECM composition and matrix vesicle production [69, 76-78]. Moreover Activin-A inhibition induced an increase in bone mass [79, 80]. These controversial findings are most likely due to differences in cell culture systems or in the different species that were employed [69, 74, 77]. Further studies are thus needed to understand the molecular mechanism of action of Activin-A during osteoblast differentiation, in order to use Activin-A for clinical treatment of bone-related diseases.

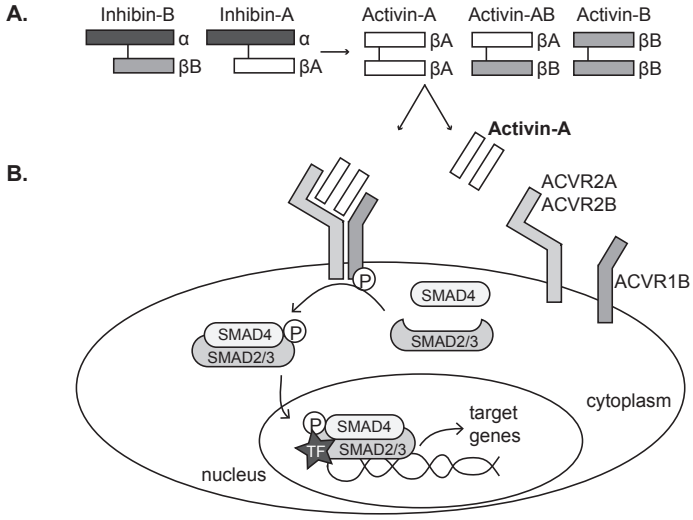


**Figure 5. Activin signaling.** A) Dimerization of 2 inhibin subunits forms Activin A, AB, B. B) Activin-A binds to receptors type 1 and 2, inducing the phosphorylation of SMAD2/3. After binding of SMAD4, the phosphorylated complex migrates into the nucleus, it recruits transcription factors and promotes the transcription of target genes.

### 1.2.4 How ECM regulates osteoblast differentiation

Bone ECM proteins have physical cues that actively influence cell behavior via cell-matrix interactions. As mentioned in section 1.1.1, bone ECM proteins such as collagen, fibronectin and thrombospondins express RGD motifs that recruit osteoblasts via adhesion molecules. Integrin-mediated adhesion activates intracellular signaling pathways eventually altering gene expression [81-83]. The affected genes are involved in cell adhesion, proliferation and differentiation and therefore regulate osteoblast and osteoclast behavior [84-87]. In detail, upon osteoblast adhesion, integrins transduce mechanical forces into biochemical signals, inducing the activation of focal adhesion kinase (FAK) and Src family kinases, that phosphorylate and activate an intricate network of signaling pathways, such as PI3K/AKT, ERK1/2 MAPK, PKC and Rho family, eventually modulating cell behavior (Figure 4F) [88]. FAK-mediated signaling activates PI3K/AKT that promotes osteoblast survival and osteogenic differentiation, but also Rho family GTPase that modulate cytoskeletal rearrangements for cell adhesion and migration. Moreover, RhoA/ROCK activate MAPKs, which are central transducers of integrin signal to the nucleus, controlling osteogenic transcription factors such as RUNX2 and ATF4, eventually promoting osteogenic differentiation [83, 89, 90]. Wnt/β-catenin signaling is also activated by FAK upon integrin-activation induced by ECM-mechanical strain [91, 92]. In addition, bone ECM tightly controls growth factor availability, both by sequestering growth

factors or protecting them from degradation. For instance, TGF-β is stored as latent protein in the ECM. It is non-covalently bound to latency associated peptide (LAP) and bound to the ECM via latent TGF-β binding proteins (LTPBs) [23]. Its availability is controlled by thrombospondins, small leucine rich proteoglycans and proteases [93], and its active form is released by osteoclast-mediated bone resorption [23, 55, 93, 94]. Bone ECM degradation by proteases such as Membrane type 1 matrix metalloproteinase (MT1-MMP) is also a key modulator of osteoblast to osteocyte differentiation, but also of bone remodeling, modulating osteoblast/osteoclast interactions and osteoclast-mediated resorption [95-97].

### 1.2.5 Bone remodeling and bone repair

Bone is constantly renewed through lifetime to maintain bone stability and quality, in a process called bone remodeling. It arises from a coordinated balance between bone resorption and bone deposition and is controlled by biochemical and biophysical stimuli [60, 98, 99]. Firstly, osteoclasts degrade bone matrix and secondly osteoblasts secrete and mineralize new ECM, leading to newly formed bone. The processes of bone formation and resorption are tightly coupled and regulated by paracrine and endocrine signaling [100]. Locally, bone remodeling is directly controlled via secreted molecules. For instance, osteoblasts and osteocytes secrete RANKL and macrophage colony-stimulating factor (M-CSF) to initiate the osteoclast-mediated resorption, and they shut off the signal by producing osteoprotegerin (OPG), a RANKL decoy receptor [34]. Bone remodeling is also indirectly controlled via osteoblast stimulating growth factors entrapped in the matrix, that are released after osteoclast-mediated ECM degradation [60, 101]. Osteocytes also participate in this balance by sensing mechanical strains and releasing SOST.

Bone has also the ability to self-repair after injuries. In these cases, the vasculature surrounding the injured site forms a hematoma that induces the release of Vascular endothelial growth factor (VEGF), BMP, FGF and platelet-derived growth factor (PDGF) that promote bone regeneration. Moreover, osteocytes undergoing apoptosis recruit macrophages that remove tissue debris, osteoclasts resorb the damaged bone and eventually fibroblasts secrete a temporary collagen scaffold to hold the fractured bone extremities (callus formation). This creates a structural support for progenitors, that migrate and differentiate toward osteoblasts and chondrocytes. These cells deposit new immature ECM which will be then ossified and remodeled in a correct structure, eventually leading to newly formed bone [102, 103].

The optimal balance between bone formation and resorption is essential for bone quality, stability and physiological tissue renewal. An imbalance in the mineral density and in collagen fiber orientation will decrease bone-quality and mechanical properties, leading to a tissue that is not functional. If the delicate balance between bone formation

and bone resorption is lost, this results in pathological conditions such as osteoporosis or osteopetrosis [60, 104]. Moreover, ectopic calcification of vascular walls represents a major issue of unwanted mineralization. Vascular smooth muscle cells undergo an osteoblast-like differentiation, inducing matrix mineralization of vessel walls, leading to this pathological condition [105, 106]. Therefore, a better understanding on matrix biology and how the microenvironment influences cell behavior would be useful to control bone quality to improve health and quality of life of patients.

## 1.3 CURRENT THERAPIES FOR SKELETAL DEFECTS AND BONE TISSUE ENGINEERING

Bone has the capacity to self-repair, as it is highly vascularized and constantly remodelled [100, 102]. However, bone healing capacity is not sufficient in case of large skeletal defects such as with large fractures or in primary tumours and bone-metastasis. Moreover, self-healing capacity of bone decreases with age, as in osteoporosis [107]. To date, the gold standard approach is treating critical-sized defects with autologous bone transplantation, as it is osteoconductive and immunocompatible. However, major drawbacks are the limited amount of bone that can be safely transplanted, second surgery-related risks and costs [108]. Allografts are also considered as alternatives, despite the potential immunogenicity and the risk of bacterial and viral transmission [109, 110].

However, effective strategies are needed for large bone defects, as current treatments are ineffective and limited. Bone tissue engineering has been proposed as valid alternative to mimic the structure of native bone matrix and to enhance the osteoinductivity of bone grafts [111-113]. Bone tissue engineering combines autologous bone-forming cells, osteogenic growth factors and scaffolds to recruit and stimulate the osteogenic differentiation of local progenitors. These cells should deposit new bone matrix while scaffolds are being resorbed, eventually driving new bone formation at the injured site [111, 114].

### 1.3.1 Mesenchymal stromal cells for bone tissue engineering

Bone-marrow derived MSCs, also defined as skeletal stem cells (SSCs) can differentiate toward different cell types such as osteoblasts, adipocytes, chondrocytes and myoblasts (Figure 6) [115-118]. MSCs can be isolated from bone marrow and other tissues based on their ability to adhere to plastic culture dish and on the expression of markers such as CD73, CD90, CD105, and the absence of hematopoietic markers (CD34, CD45, CD14/11b, CD79a/CD19 and HLA-DR), though a definitive set of markers that identifies the mesenchymal stem cell is still lacking [119, 120].

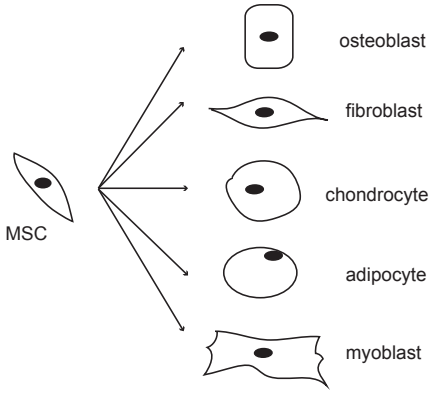


**Figure 6. Multilineage differentiation of mesenchymal stem cells.** MSCs are multipotent stem cells that can give raise to different cell lineages, such as osteoblasts, fibroblasts, chondrocytes, adipocytes and myoblasts. MSC: mesenchymal stem/stromal cell.

MSCs are promising candidates for bone tissue engineering due to their intrinsic ability to differentiate toward osteoblast progenitors and to support bone turnover and hematopoiesis [121, 122]. Moreover, they extensively proliferate *in vitro*, secrete trophic factors and exhibit immunomodulatory properties [116, 123]. MSCs have been successfully infused in patients with degenerative bone conditions such as osteogenesis imperfecta [124, 125]. They represent ideal candidates to be used for cell-based therapies to treat osteoporosis, following the promising results in murine models of osteoporosis (reviewed in [126]). Moreover, *ex-vivo* expanded MSCs in combinations with scaffolds have been extensively investigated in preclinical studies and have been successfully used in bone defects for bone tissue engineering applications [112, 127, 128]. However, low frequency in the bone marrow, heterogeneity of the population and donor-to-donor variation are major limitations. Furthermore, MSCs spontaneously lose their proliferative capacity and multi-differentiation potential during the *ex-vivo* expansion that is required to reach a proper yield for clinical applications [129-132]. In addition, the frequency and the osteogenic potential of stromal cells decrease with aging, representing a major issue in elderly patients [133, 134]. Alternative substrates that maintain differentiation capacity of the MSC population are thus needed.

### 1.3.2 Scaffolds and biomaterials for bone tissue engineering

Scaffolds for bone tissue engineering aim to reproduce the mechanical properties of native bone, by providing a structural template for osteoprogenitors and influencing their behavior to promote the reconstruction of their natural environment. Therefore, scaffolds should be biocompatible, osteoconductive and favor integration and vascularization of the graft [135]. Moreover, they should be biodegradable to be

replaced by the newly formed bone, and porous to allow the diffusion of nutrients for cell survival.

Metals, such as titanium, have good mechanical properties similar to native bone and can be tailored in porosity to allow cell attachment and proliferation. However, they poorly integrate with the surrounding bone tissue and are not biodegradable [136]. Ceramics, such as hydroxyapatite or beta-tricalcium phosphate (β-TCP), have a lower mechanical strength than metal scaffolds, they are mechanically brittle, but they are as osteoconductive as bone. They are used for dental and orthopedic surgery [137-139]. Polymers can be natural such as collagen, fibrin or alginate-based, or synthetic, such as poly-lactic acid (PLA), and can be easily tailored for their mechanical properties to improve their potential applications. Different types of scaffolds are also combined to create composite materials to improve their beneficial properties. For instance, metals are functionalized with calcium-phosphate to enhance osseo-integration and more recently with protein coatings or growth factors and cytokines to increase the osteoinductivity [140, 141].

Scaffolds have been successfully combined with MSCs for bone tissue engineering in several preclinical models. MSCs have been implanted with HA-based scaffolds in large bone defects in different animal models, favouring osseointegration and accelerating defect repair, eventually promoting bone formation [142-145]. Moreover, MSCs have been successfully applied in clinical applications, after *ex-vivo* expansion and implantation on HA and on titanium [112, 127, 146, 147]. Despite these few clinical trials that have shown the efficacy of MSCs implanted with scaffolds in bone defects and the safety of using MSCs, translation to the routine clinical application is still far. Studies are still needed to improve the amount and quality of bone formation and also to avoid the risk of ectopic bone growth [111, 128].

Scaffolds are important for mechanical support but cannot reproduce the complex architecture of native tissue ECM. Thus, native ECM-based scaffolds and biomaterials are ideal candidates for bone tissue engineering, as they aim to mimic the physiological functions of bone matrix *in vivo* and represent off-the-shelf and immune-compatible alternatives to living grafts. In this context, the use of native tissue/organ-derived ECM has been proposed as natural biomaterial, together with *in vitro* cell-secreted ECMs, aiming to maintain the osteogenic potential of MSCs that is lost during *ex-vivo* expansion [148-151].

### 1.3.3 ECMs for bone tissue engineering

ECMs are complex 3D frameworks of proteins, lipids and proteoglycans that are present in every organ, important for the structural properties of the tissues [152]. The ECMs have physical cues to actively influence cell adhesion, migration, proliferation and differentiation [153-155]. The ECM influences cell behavior via

its biomechanical properties such as stiffness, which was shown to influence MSC differentiation. Moreover, ECM not only provides mechanical support to cells but it is also a reservoir of signaling molecules [156-158]. In bone ECM for instance, presentation of osteoinductive signals embedded in the ECM regulates bone formation, angiogenesis and cell recruitment, as described in Section 1.2.4. Overall, the ECM in native tissues actively modulates cell behavior directly by activating signaling pathways, or indirectly by modulating cytokine bioactivity and transducing mechanical signals [157, 159, 160].

As the complex structure of physiologic ECM is difficult to reproduce but critical for tissue engineering, native tissue-derived ECMs and *in vitro* cell-secreted ECMs have been proposed. Native ECMs have been successfully obtained by decellularization of whole organs, to preserve tissue architecture/3D ultrastructure, composition and biomechanical properties of native organs. In the context of tissue engineering, several organs, such as rat heart, liver and lung, have been decellularized and reseeded with cells, that successfully acquired ECM-induced organ-specific properties [161-163]. *In vitro* cell-secreted ECMs are produced when cells are cultured, as they deposit a matrix underneath that can be used as new substrate, after cell removal by decellularization or devitalization treatments [164, 165]. These treatments aim to remove cellular components to minimize immune response, but leaving the bioactive ECM-cues functional and intact [166-169]. Physical treatments such as freeze/thaw cycles or sonication, aim to disrupt cellular components. Chemical agents such as acids, detergents, or enzymes such as proteases and nucleases, are also used for this purpose. In some cases, $NH_4OH$ followed by Triton-X and Phosphate-buffered saline (PBS) washings have been used [170], whereas in others deoxycholate [171], freeze-thaw cycles [172] have been efficiently employed. In most cases a combination of different decellularization treatments is used, as in the preparation of the devitalized ECMs presented in this thesis.

Since 1970s, many *in vitro* derived ECMs have been produced from different cell types, such as fibroblasts, hepatocytes and stem cells, and under different culture conditions, such as monolayer, aggregates or on scaffolds [165, 173-176].

The protein composition of cell-derived ECMs is still under investigation, as it is also depending on cell type and culture conditions [171, 177-179]. Despite this, cell secreted ECMs reproduce the complex composition of native ECM, better than single peptides or proteins coatings [180]. In the case of bone tissue engineering, cell-secreted ECMs have been studied to regulate stem cell functions as alternative substrate to improve cell culture conditions. Cell-derived ECMs produced with different devitalization treatments enhanced the osteogenic potential of seeded MSCs *in vitro* [172, 177, 181-185]. Moreover, MSC multilineage potential was maintained when MSCs were expanded on ECMs rather than on tissue culture plates, making

cell-derived ECMs suitable tools to support cell proliferation for *ex-vivo* expansion [170, 177, 186, 187]. ECM derived from early passage-MSCs was able to rescue the osteogenic potential of aged cells [187-189]. In addition, MSCs cultured on ECM retained better *in-vivo* bone-formation capacity than those expanded on standard culture plates [170, 186]. However, the *in vitro* properties have not always been reproduced *in vivo*. Despite some positive results showing that cell-secreted ECMs increased the osteoinductive properties of HA in critical-sized calvarial defects in rats [183, 190], maintaining MSCs at the injured site [191], other studies did not report an increased bone formation, though vascularization was improved [192-194].

Cell-secreted ECMs have been proposed as coating for scaffolds, to ameliorate the osteoinductive properties of biomaterials. In addition to the supportive role of the scaffold itself, as the ECM is cell-instructive, it should guide resident cells toward endogenous tissue repair [183, 193, 195]. For instance, titanium surfaces have been modified by osteoblast-secreted ECM [196] and cell-secreted ECM have been already used to coat 3D scaffolds, maintaining their osteoinductive properties [182, 193].

In summary, despite cell-secreted ECMs are mechanically poorer than native decellularized ECM, they represent a valid alternative for bone tissue engineering, as they are readily available and more customizable, though studies aiming to im-

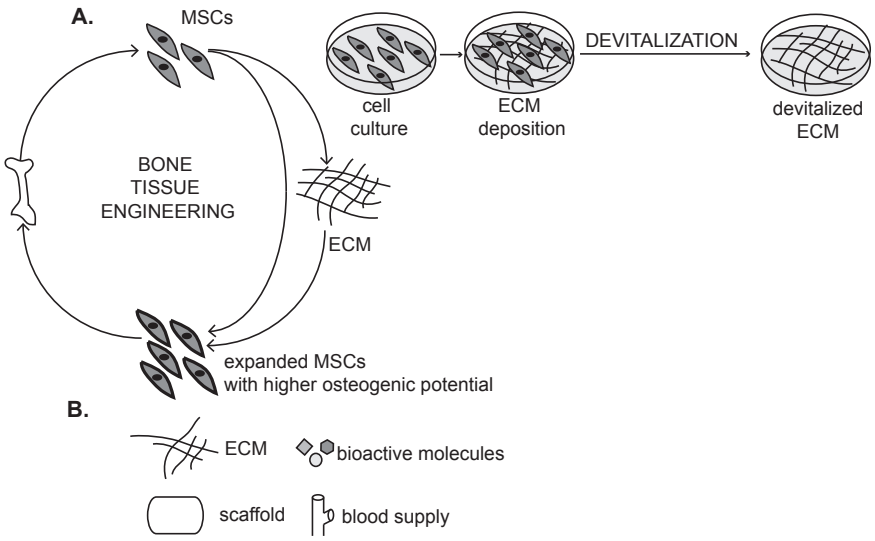


**Figure 7. Schematic use of cell-derived ECM for bone tissue engineering applications.** A) Bone marrow-derived MSCs are cultured to allow the deposition of the ECM and devitalized to obtain the cell-derived ECM. B) Cell-derived ECM can be used with MSCs to enhance their proliferative capacity and their osteogenic potential, together with scaffolds to provide mechanical cues, osteoinductive molecules and blood supply, to ameliorate bone tissue engineering applications.

prove the osteoinductivity are needed. Despite the successful results of bone tissue engineering in preclinical studies, further investigations are required to successfully introduce it in clinical practice (Figure 7). To this aim, cell-derived ECMs could help improving biomaterials as well as maintaining the stemness of MSCs during the *ex-vivo* expansion, eventually leading to successful application of bone tissue engineering in clinical practice.

## 1.4 AIM OF THIS THESIS

Cells are continuously influenced by tissue-microenvironment, receiving structural support from the surrounding extracellular matrix and inputs from signaling molecules. The ECM is a crucial structural component of tissue architecture, but beyond this, it acts as reservoir of signaling molecules and provides physiological cues that actively influence cell behavior. In the context of bone, MSC osteogenic differentiation is modulated not only by signaling molecules, but also by the extracellular matrix. The composition of the ECM is a key modulator of cell behavior, but studies that unravel ECM components to identify regulatory molecules are still lacking.

In the last years, in the context of bone tissue regeneration, the interest grew upon cell-secreted ECMs as alternative to whole tissue-derived ECMs to better understand the physiology of tissue microenvironment and how it influences cell behavior, aiming to ameliorate stem cell properties for tissue repair.

In this thesis, we aim to elucidate how the extracellular microenvironment influences osteoblast behavior. This to better understand matrix biology in bone-physiological context, but also to identify regulatory candidates (proteins or soluble factors) that could be possibly used to ameliorate stem cell properties for bone regeneration.

In **Chapter 2**, we present a model of devitalized osteoblast-derived ECM to study the functional effect on adhesion, proliferation, osteogenic differentiation and mineralization of MSCs *in vitro* and bone formation *in vivo*. We extensively characterize the proteomic composition of the devitalized ECM by mass spectrometry and compare it to the human bone proteome.

In **Chapter 3**, we modified the composition of the devitalized ECM presented in Chapter 2 to create 2 additional models of devitalized ECMs, produced by undifferentiated MSCs and osteoblast-derived MSCs with Activin-A, that result in extremely different MSC differentiation and mineralization phenotypes. We analyze the protein composition of each ECMs by mass spectrometry in a comparative proteome profiling, to investigate regulatory candidates involved in the osteopromotive action of the ECM.

In **Chapter 4**, we study the effect of a 2-days-Activin-A treatment on osteoblast behavior. As the impact of a signaling might vary in relation to a specific time period during differentiation, we investigate how Activin-A at early stages of osteoblast differentiation influences ECM mineralization by gene expression and biochemical analyses, but also how it modulates the expression pattern of miRNAs in osteoblasts.

In **Chapter 5**, we compare the cell secreted devitalized ECM presented in Chapter 2 to titanium and investigate how these different surfaces influence the kinome profile of MSCs by using PamChip Array technology and how this regulates cell adhesion.

Finally, the conclusions of the work are presented in **Chapter 6**, giving further insights into understanding how osteogenic differentiation and mineralization are influenced by the microenvironment. Limitations and pitfalls are also discussed, together with future perspective for clinical applications.

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