UNIVERSITAT DE BARCELONA

# Cooperation of calcium, BMP and WNT signalling for optimal osteoblast differentiation. Application for bone tissue engineering

Rubén Francisco Aquino Martínez

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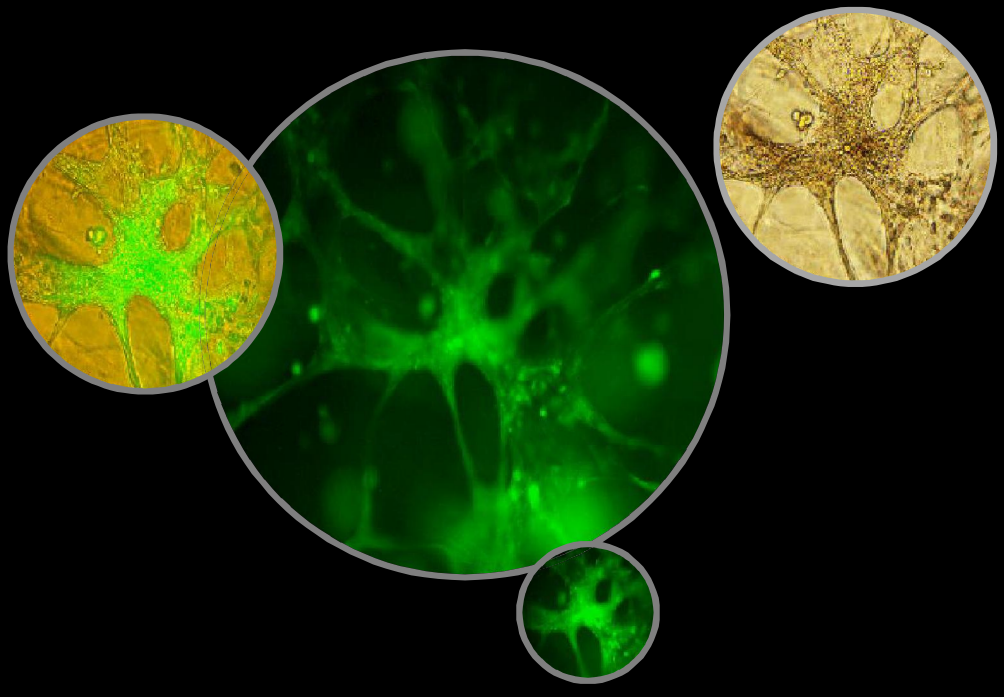

PhD Thesis

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## Application for bone tissue engineering.

PhD Thesis by
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TO

God source of provision, inspiration……

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## ABSTRACT

At least two specific features differentiate bone from other tissues, a blend of organic and mineral components, and a cellular organization with a hierarchical commitment degree. During bone remodeling osteoclasts dissolve the mineral and degrade the organic bone components, releasing the degradation products to the extracellular space.

Bone is the hardest connective tissue in the human body. However, skeletal tissue can be injured by trauma, atrophied by tooth loss, destroyed by pathological conditions or incompletely formed during congenital craniofacial bone defects, such as cleft palate. Autogenous bone is considered the "*gold standard"* to regenerate bone defects. However, wherever bone is harvested undesirable and detrimental effects are produced at the donor area. To avoid these drawbacks, bone tissue engineering has emerged to provide an alternative to autogenous bone harvesting drawbacks. It has been shown by several *"in vitro*" an *"in vivo"* studies that BMP-2 promotes osteogenic differentiation and bone formation. High doses are required (e.g. 1-45 μg/ml in animal models) to obtain acceptable outcomes. However, several side effects, such as inflammation and ectopic bone formation, have been reported after using elevated amounts of BMP-2.

In this work we employed a composite Gelatin/$CaSO_4$ scaffold that allows for an early expansion of seeded MSC´s, which is followed by an increased level of osteogenic differentiation after 10 days in culture. Furthermore, this seeded scaffold enhanced bone formation in a mouse model of critical-size calvarial defects. More importantly, *ex vivo* pretreatment of MSC´s with low amounts of BMP-2 (2nM) and Wnt3a (50 ng/ml) for 24 hours cooperatively increases the expression of osteogenic markers *in vitro* and bone regeneration in the critical-size calvarial defect in the mouse model.

Moreover, we determined the molecular mechanisms involved in cooperation between $Ca^{2+}$ and BMP-2 in MSC´s at early and late differentiating points. Early, at 24 hours, we observed an intracellular network activation which is antagonistic to BMP-2/Smad signalling. More importantly, a significant cooperative effect between $Ca^{2+}$ and BMP-2 is observed after 10 days. $Ca^{2+}$ promotes an autocrine/paracrine feed-forward loop that reinforces the BMP-2 osteogenic input. Of note, $Ca^{2+}$ alone induced similar osteogenic effects as BMP-2 alone in long-term cell culture.

In conclusion, cytokine signalling (such as BMP-2) and signalling from the mineral component (such as $Ca^{2+}$) signals interact during bone remodeling. Early on, $Ca^{2+}$ inhibits BMP-2 differentiation effect but later amplifies and reinforces the osteogenic BMP-2 effect.

## RESUMEN

Al menos dos características distinguen al hueso de otros tejidos, es un compuesto con elementos orgánicos y minerales y además tiene una organización celular con diferentes niveles de diferenciación. Durante el remodelado óseo los osteoclastos disuelven el componente mineral y degradan la fase orgánica liberando sus productos de degradación hacia el espacio extracelular.

El hueso es el tejido conectivo con mayor dureza en el cuerpo humano. Sin embargo, el tejido óseo puede ser dañado después de un trauma, puede sufrir atrofia por la pérdida de dientes, podría ser destruido por condiciones patológicas o incompletamente formado durante defectos óseos craniofaciales congénitos, tal como el paladar hendido. El hueso autólogo ha sido considerado la mejor alternativa para regenerar defectos óseos. Sin embargo, de cualquier sitio de donde se obtenga produce morbilidad en la zona donante. Para evitar esa desventaja, la ingeniería de tejido óseo ha surgido como una alternativa al hueso autólogo. Ha sido demostrado por estudios *in vitro* e *in vivo* que BMP-2 favorece la diferenciación de osteoblastos y la formación ósea. Altas dosis son necesarias (por ejemplo 1-45 μg/ml) para obtener resultados aceptables. Sin embargo, varios efectos adversos tal como inflamación y formación ectópica de hueso han sido publicados después de usar elevadas dosis de BMP-2.

En este trabajo hemos utilizado una combinación de Gelatina/$CaSO_4$ como andamiaje para cultivar células madre mesenquimales (MSC´s). Este andamiaje promueve inicialmente una amplificación de las células cultivadas, lo cual es seguido por una mayor diferenciación osteoblástica después de 10 días de cultivo. Además, este andamiaje cultivado con MSC´s incrementó la formación de hueso en un defecto óseo de tamaño crítico en cráneo de ratón. Lo más notable, pretratamiento de MSC´s "*ex vivo*" con dosis bajas de BMP-2

(2nM) y Wnt3a (50 ng/ml) durante 24 horas incrementó cooperativamente la expresión de marcadores osteogénicos *"in vitro*" y la regeneración ósea en los defectos de tamaño critico en cráneo de ratón.

Además, determinamos el mecanismo molecular involucrado en la cooperación entre $Ca^{2+}$ y BMP-2 a corto y largo término durante la secuencia de diferenciación de las MSC´s en osteoblastos. Al inicio a las 24 horas, observamos la activación de una red de señalización intracelular la cual es antagónica a la vía BMP-2/Smad. A los 10 días, un efecto cooperativo entre $Ca^{2+}$ y BMP-2 es observado. $Ca^{2+}$ promueve la secreción endógena de BMP-2 lo cual produce un efecto autocrino y paracrino que refuerza la acción osteogénica inicial de BMP-2. Notablemente, un efecto similar en la diferenciación osteoblástica fue observado en MSC´s tratadas únicamente con $Ca^{2+}$ comparado con las tratadas solo con BMP-2.

En conclusión, señalización por citoquinas como BMP-2 y $Ca^{2+}$ (componente mineral) interactúan durante el remodelado óseo. $Ca^{2+}$ regula el estímulo osteogénico de BMP-2 mediante un mecanismo secuencial. Inicialmente, $Ca^{2+}$ inhibe la diferenciación celular producida por BMP-2 pero después de 10 días amplifica y refuerza el efecto osteogénico de BMP-2 favoreciendo la diferenciación de osteoblastos y la formación ósea.

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| | |
|---|---|
| *Alpl* | Alkaline Phosphatase |
| APC | Adenomatous polyposis coli |
| BLC | Bone lining cells |
| BMMSC | Bone marrow mesenchymal stem cells |
| BMP | Bone morphogenetic proteins |
| BRC | Bone remodeling compartment |
| BrdU | Bromodeoxyuridine |
| CaR /CaSR | Calcium sensing Receptor |
| $CaSO_4$ | Calcium Sulfate |
| DAG | Diacylglycerol |
| DKK1 | Dickkopf 1 |
| DOPC | Determined osteogenic precursor cells |
| FDA | Food and drug administration |
| FGF | Fibroblast growth factor |
| F-OST | Osteoblasts |
| F-RET | Subendosteal reticulocytes |
| GFP | Green fluorescent protein |
| GPCR | G protein coupled receptor |
| GSK3 | Glycogen synthase kinase 3 |
| IGF | Insulin growth factor |
| IOPC | Inducible osteogenic precursor cells |

| | |
|---|---|
| $IP_3$ | Inositol 3,4,5 trisphosphate |
| Ki-67 | marker for proliferation |
| MGP | Fetuin-Matrix Gla protein |
| MSCs | Mesenchymal stem cells |
| *Osx* | Osterix |
| PCP | Planar cell polarity |
| PDGF | Platelet-derived growth factor |
| PKC | Protein kinase C |
| SMA | Smooth muscle actin |
| SOST | Sclerostin |
| TGFβ | Transforming Growth Factor β |
| VEGF | Vascular endothelial growth factor |
| WNT | Acronym from wingless and int |
| μCT | Micro computed tomography |

PUBLICATIONS

- **Aquino-Martinez, R**., E. Rodriguez-Carballo, B. Gamez, N. Artigas, P. Carvalho-Lobato, M. C. Manzanares-Cespedes, J. L. Rosa and F. Ventura (2016). "Mesenchymal Stem Cells Within Gelatin/CaSO4 Scaffolds Treated Ex Vivo with Low Doses of BMP-2 and Wnt3a Increase Bone Regeneration." Tissue Eng Part A **22**(1-2): 41-52.

- **Aquino-Martínez, R**., Artigas,N. Gamez, B. Rosa, J.L. Ventura, F. "Extracellular calcium promotes bone formation from bone marrow mesenchymal stem cells by amplifying the effects of BMP-2 on SMAD and AKT signalling. 2016. Submitted

# 1. CHAPTER I: INTRODUCTION

## 1.1 BONE BIOCHEMICAL COMPOSITION AND CELLULAR ORGANIZATION

At least two specific features differentiate bone from other tissues, a particular arrangement of organic and mineral components and a cellular organization with a hierarchical commitment degree. This living biocomposite with a particular structure, composition and cellular disposition provide the skeleton with singular regenerative, mechanical and metabolic characteristics. In addition, the skeletal tissue allows a protective function of vital structures in thorax and in cranium.

### 1.1.1 Bone as biomaterial

Bone is a heterogeneous composite material consisting in decreasing order, of a mineral phase 65% (hydroxyapatite), an organic phase 25 % (collagen 90%, non- collagen proteins 5-10% and lipids 2%) and water 10% (Olszta, Cheng et al. 2007, Boskey 2013). Bone is commonly considered as a biphasic structure. A biocomposite with a mineral and an organic components. The bone mineral has been idealized as calcium hydroxyapatite, $Ca_{10}(PO4)_6(OH_2)_2$ (Kay, Young et al. 1964). However, biologic apatites contain minor and trace elements. The most important minor elements are carbonate ($CO_3$), magnesium (Mg) and sodium (Na) (LeGeros 2008). Indeed, approximately 99% of body calcium, 85% of the phosphorus, and between 40-60% of total body sodium and magnesium are associated with bone crystals (Buckwalter M. J. Glimcher et al 1995).The organic phase corresponds mostly to collagen Type I and to a lesser degree to non-collagenous proteins. This latter fraction is composed by osteocalcin, osteonectin, bone sialoprotein, osteopontin and growth factors such as TGFβ, IGF, FGF, BMP, VEGF and PDGF (Buckwalter M. J. Glimcher et al 1995, Linkhart, Mohan et al. 1996).Therefore, individually each bone component could act as relevant local biochemical signals for bone cells.

Bone can be depicted as a combination of a rigid inorganic component with a flexible collagen organic matrix. In this biocomposite the mineral provides

stiffness and strength to the bone tissue, whereas the collagen provides ductility or flexibility (Wilson, Awonusi et al. 2005). The small amounts of lipids, such as fatty acids, cholesterol, phospholipids, contained in mineralized bone tissue itself might play an important role in bone cell survival, critical signalling molecules and biomineralization (During, Penel et al. 2015). In addition, water may serve to couple the mineral to the organic matrix and may play a role in deformation (Wilson, Awonusi et al. 2005). With aging, there is a marked decline in the content of water (Triffitt, Terepka et al. 1968) and the mechanical behavior, morphology, bone cells, the matrix they produce and mineral deposited on this matrix are modified (Boskey and Coleman 2010). Therefore, the relative amounts and properties of the mineral and organic matrix in bone, as well as the organization at both the microscopic and macroscopic scales determine its mechanical strength (Boskey, Wright et al. 1999). A trabecular bone model as a nanocomposite material with hierarchical structure has been suggested. The model involves a bottom-up multi-scale approach, starting with nanoscale (mineralized collagen fibril) and moving up the scales to mesoscale (trabecular bone) levels (Hamed, Jasiuk et al. 2012).Figure 1.1.

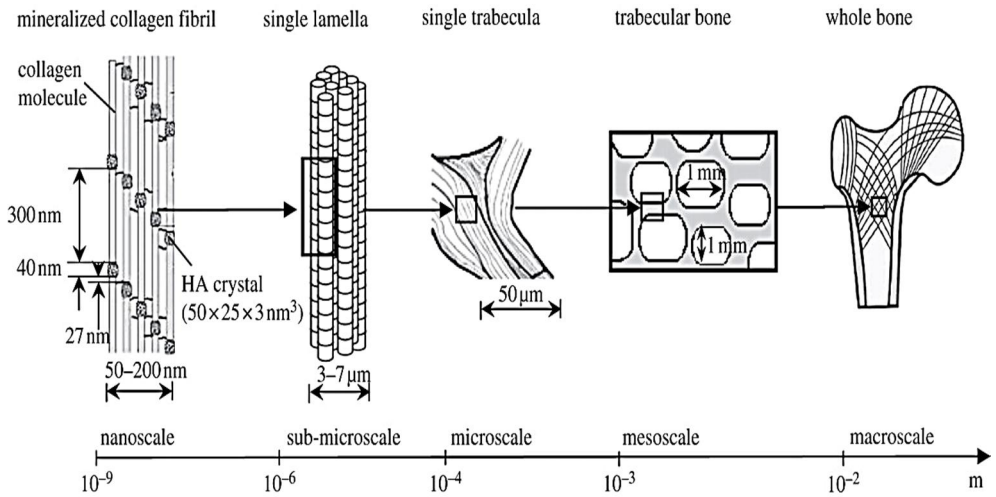



Figure 1.1.Simplified bone composition and hierarchical structure of trabecular bone. Taken from (Hamed, Jasiuk et al. 2012)

### 1.1.2 Anatomical cellular relationship between bone and bone marrow.

Bone marrow and trabecular bone are closely related. Wherever true bone is formed, irrespective of the initiating mechanism, it generally leads to new hematopoietic marrow (Triffitt 1987). They are adjacent tissues changing gradually without a clear separation as a whole anatomical structure, but each one with different and specific functions. The bone marrow can be seen as two mayor compartments, the stroma and the hematopoietic. Figure 1.2.

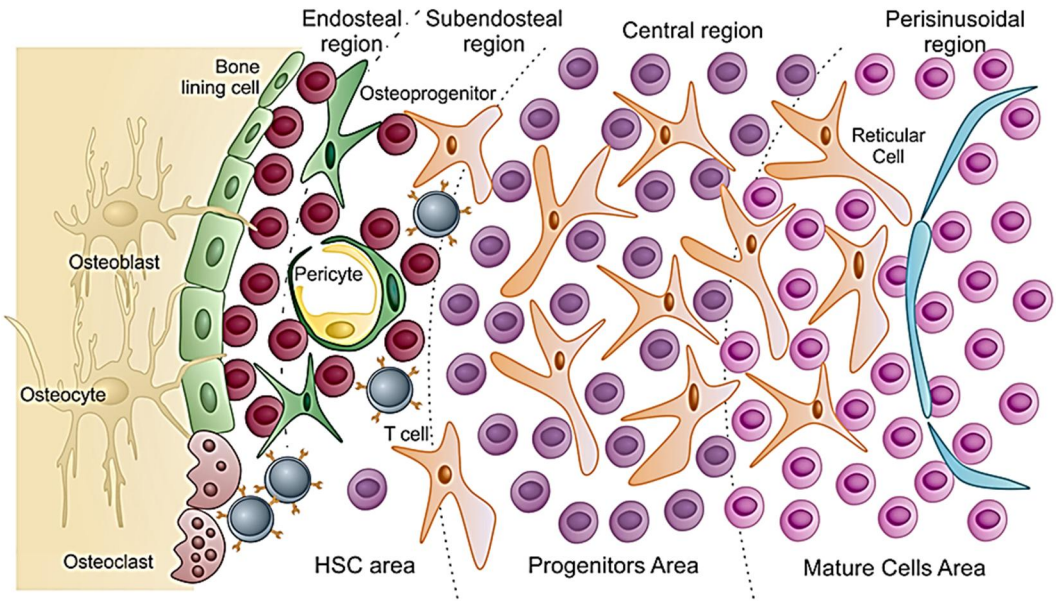



Figure 1.2. Bone and bone marrow cells relationship. Taken from (Bonomo, Monteiro et al. 2016)

Anatomically, bone marrow stroma includes all non-hematopoietic reticular cells, adipocytes, endothelial cells, macrophages and osteoblasts (Weiss 1976, Balduino, Hurtado et al. 2005, Krause, Scadden et al. 2013). The stroma of marrow consists mainly of a network of reticular cells and fibers together with the endothelial cells lining the walls of the sinusoidal vessels (Owen 1980). The parenchyme is composed of all the different immature and

mature blood cell precursors (Fliedner, Graessle et al. 2002). Stromal derived mesenchymal stem cells can, under suitable conditions, differentiate into osteoblasts, whereas the bone resorbing osteoclasts arise from the hematopoietic fraction.

By morphological/histochemical criteria and proliferative ability five maturational stages in osteoblast lineage could been identified: skeletal progenitors, preosteoblasts, osteoblasts, osteocytes and bone lining cells (BLC) (Aubin 1998, Bianco 2011). Osteoblasts in trabecular bone are derived from mesenchymal stem cells present in bone marrow stroma. These cells with bone forming potential localized in marrow can be divided in two groups. First, those which are capable of spontaneous differentiation and have been called determined osteogenic precursor cells (DOPC) (Owen 1978). Second, undifferentiated mesenchymal cells which will form bone in the presence of an inducing agent, they are called inducible osteogenic precursor cells (IOPC) (Owen 1978). Inductive agents such as decalcified bone matrix, transitional epithelium of the bladder and bone morphogenetic proteins (BMP´s) have been reported (Friedenstein 1968, Huggins 1968, Urist and Strates 1971). DOPC have been shown to be present only in the stromal tissue of marrow and bone surfaces, whereas IOPC are found widespread throughout many connective tissues and in marrow (Owen 1980).

Associated with this, it has been reported that the endosteal/ subendosteal environment comprise two distinct stroma-cell populations, subendosteal reticulocytes (F-RET) and osteoblast (F-OST). Balduino and Hurtado et al. (2005) found that F-OST cells cultured in standard culture medium spontaneously formed extensive mineralized nodules, whereas F-RET did not. The authors suggest that F-RET cells are mostly osteoprogenitor cells and F-OST represent a population of osteoblasts.

The preosteoblast is considered the immediate precursor of the osteoblast and is identified by its localization in the adjacent one or two cell layer from the osteoblasts lining bone formation surfaces (Aubin 1998). The

osteoblast is a specialized cell that produce bone by secreting an organic extracellular matrix which becomes a mineralized tissue. The percentage of trabecular bone surface covered by osteoblasts comprise around a 5%, but a gradual reduction is observed with age in samples of human cancellous bone from the iliac crest (Merz and Schenk 1970). Expressing Osteocalcin, Osterix and Runx, mature osteoblasts line the bone surface meanwhile an undifferentiated stromal subpopulation preferentially reside close to and around the sinusoidal wall in the subendosteal region (Nakamura, Arai et al. 2010, Cordeiro-Spinetti, Taichman et al. 2015). Figure 1.3.

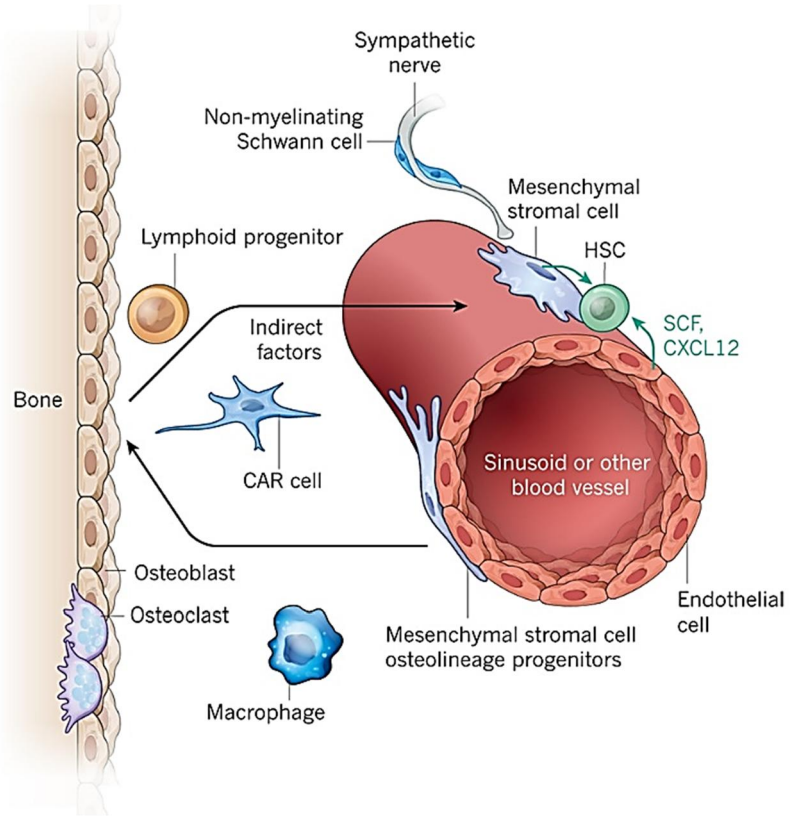



Figure 1.3.Mature osteoblastic cells line the bone surface, whereas mesenchymal osteoprogenitor cells reside around bone marrow microvessels. Taken from (Morrison and Scadden 2014)

Osteoblasts trapped by the secreted extracellular matrix become osteocytes. This transformation involves a range of morphological changes such as decrease in cell body size, increase of cell processes and change in intracellular organelles (Franz-Odendaal, Hall et al. 2006). Osteocytes form an intricate network of cytoplasmic prolongations interacting with one another and with those cells covering the bone surface. Osteocytes compose 90 to 95% of all bone cells in adult bone (Bonewald 2011) and they are considered the most differentiated cells in the osteoblast lineage.

About 75% of the surface of cancellous bone is quiescent (Parfitt 2003) and is covered by bone lining cells (BLC). These cells in the outer surface undergo a morphological change from a cuboidal to an “inactive” flattened shape. BLC separate the bone surface from the bone marrow. However, the mineralized bone surface is not in contact with the bone lining cells directly. They are separated from each other by the Lamina Limitans, a layer always present in “inactive” bone surfaces. The BLC layer forms an essential ionic partition between a bone fluid compartment, which includes the lacunae and their canaliculi, and an extracellular fluid compartment which includes the marrow and vascular space (Menton, Simmons et al. 1984).

### 1.1.3 Endosteum a frontier between bone and bone marrow

Although osteoblast, preosteoblast and postosteoblast stages can be identified, together they constitute an anatomically and physiologically linked system. The organization of bone from Lamina Limitans, endosteal bone lining cells to periendothelial mesenchymal stem cells in bone marrow maintain a functional connection. At the peritrabecular region the endosteum is placed as a border between the bone and bone marrow (Nakamura, Arai et al. 2010). Endosteal region is an enriched region in microvessels. Endosteum is the site in which a rich anastomotic network connects the bone and marrow circulation, which may contribute to defining a specific ionic environment at this site (Bianco

2011). A characteristic of the endosteum where active bone remodeling takes place is an increased extracellular calcium ion concentration (Adams, Chabner et al. 2006).

Four zones relative to endosteal surfaces and the wall or the central venous sinus have been suggested: endosteal, subendosteal, central and intermediate zones (Lambertsen and Weiss 1984). Figure 1.4. The endosteal and subendosteal zones compose the endosteal niche, where at least three stromal cell types can be identified: osteoblast, non-perivascular reticular cells (probably most pre osteoblasts) and perivascular cells, probably mesenchymal stem cells (Cordeiro-Spinetti, Taichman et al. 2015).

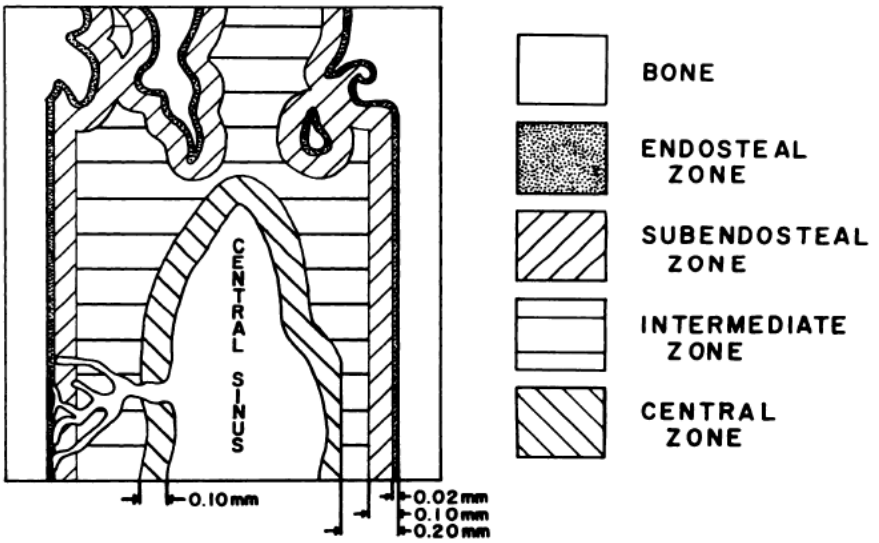



Figure 1.4. Model of bone and bone marrow zones. Taken from (Lambertsen and Weiss 1984)

### 1.1.4 Bone remodeling

Bone remodeling is a temporal and localized renewal of damaged or aging bone. Figure 1.5. This bone replacement mechanism is made by coordinated and sequential actions of bone resorting osteoclasts and bone

forming osteoblasts. Although both osteoclast and osteoblast activity could be considered as isolated or disconnected events, they are interdependent actions. Both represent events within the same process carried out at the same location, the eroded bone surface.

Under physiological conditions, bone remodeling is a tightly regulated sequence of cellular events in response to physical and biochemical signals. Osteocytes and their canalicular network provide a mecanosensory detection system by which the bone remodeling sequence is not an aleatory process. Consequently, bone remodeling takes place only where it is needed in response to damage, to change in loading or to remove old bone (Sims and Martin 2014). The normal trabecular bone remodeling sequence can be represented by successive stages: quiescence, activation, resorption, reversal, formation and back to quiescence or termination (Parfitt 1984, Parfitt 1994, Rucci 2008, Raggatt and Partridge 2010).

A site where the bone surface is quiescent with respect to remodeling, meaning a resting region where neither resorption nor formation is currently in progress, is selected for initiating the process (Parfitt 1994). Osteocyte death by microdamage has been suggested to be the major event leading in the initiation of osteoclastic bone resorption (Eriksen 2010). Before osteoclast activation, the cement line or Lamina Limitants is degradated by osteoblastic lining cells in response to osteocyte signalling. This event produces a detachment between bone lining cells and the bone surface. This separation forms a specialized micro anatomical structure called Bone Remodeling Compartment (BRC) (Hauge, Qvesel et al. 2001).

#### 1.1.4.1 Osteoclasts degrade the mineral and organic bone components

Osteoclasts are multinucleated cells derived from circulating monocyte progenitors. They are specialized cells that remove damaged or aged bone. Quiescent or resting bone surfaces are covered by the Lamina Limitans which BLC must digest before osteoclasts degrade bone.

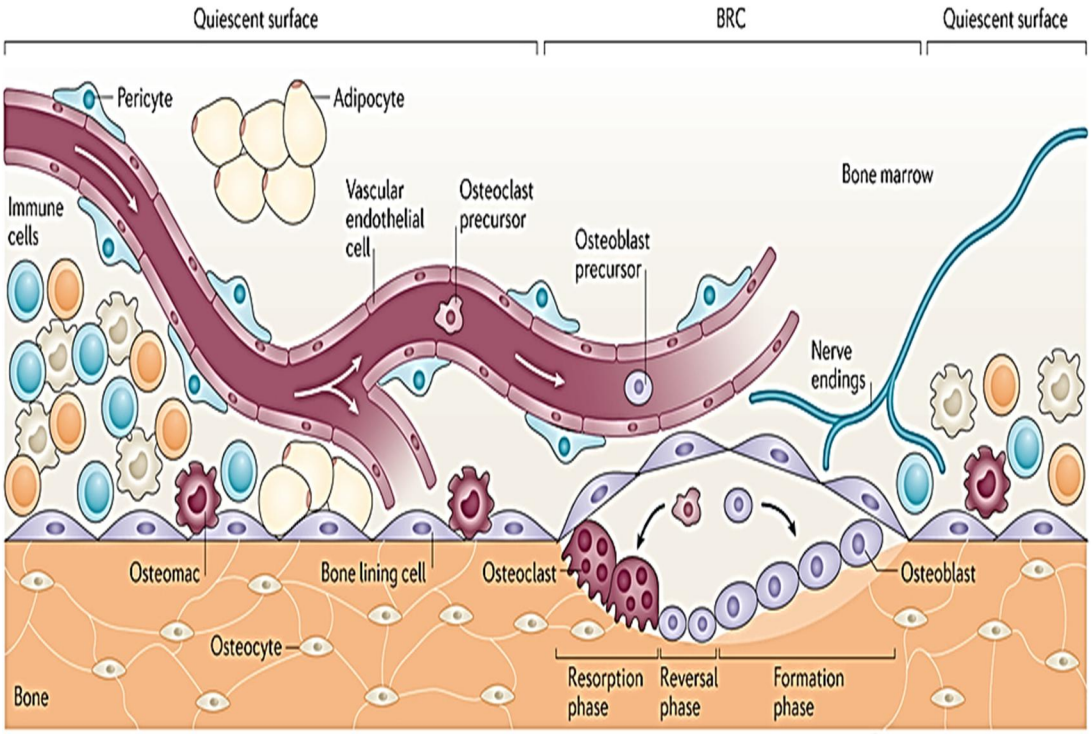



Figure 1.5. Bone remodeling is a temporal and localized cellular event. Taken from (Croucher, McDonald et al. 2016)

BLC predispose the bone surface to osteoclast resorption by exposing the mineral component of bone (Chambers and Fuller 1985). This mineral exposure promotes osteoclasts' attachment to the denuded bone surface by forming a sealed zone. Osteoclasts dissolve the mineral portion of bone by acid production while the organic component is degradated partially by proteolytic activity. Acidic conditions are created by hydrochloric acid (HCl) secretion by osteoclasts releasing calcium, phosphate and water from the basic hydroxyapatite that comprises bone mineral (Blair 1998). Figure 1.6.

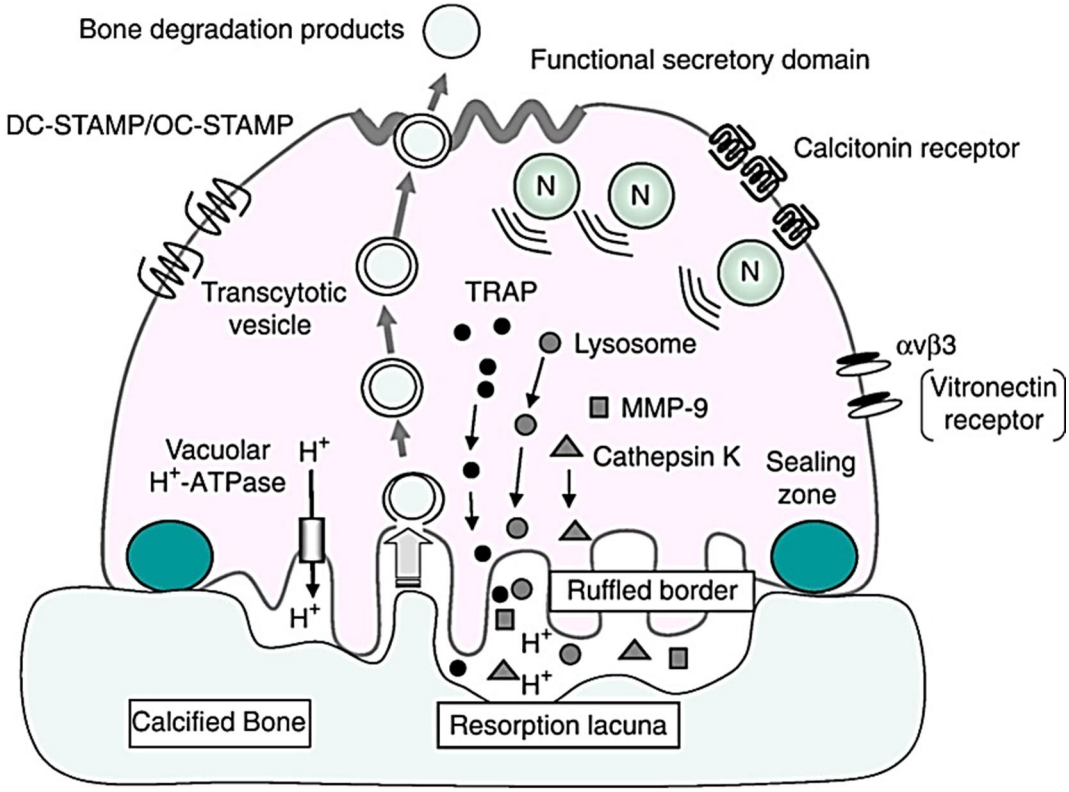



Figure 1.6. Osteoclast release bone degradation products by transcytosis toward the extracellular space. Taken from (Takahashi, Udagawa et al. 2014)

The mineral phase is solubilized before collagen degradation (Bonucci 1974, Blair, Kahn et al. 1986). Collagen fragments are produced and released by a partial degradation of the organic component. In addition, this incomplete breakdown leaves behind demineralized collagen bundles covering the resorption site walls after osteoclasts leave. Both, mineral (calcium ions) and organic degradation products are released toward the extracellular space by transcytosis (Salo, Lehenkari et al. 1997, Yamaki, Nakamura et al. 2005).

#### 1.1.4.2 Bone remodeling compartment (BRC)

As mentioned above, endosteal lining cells serve as a border between bone surface and the marrow space. This lining cell barrier persists during bone remodeling but is released from the cancellous bone surface by a disruption of the junctions between lining cells and embedded osteocytes (Hauge, Qvesel et al. 2001). BRC provides a ready access for regulatory factors produced outside bone diffusing through the canopy layer from the bloodstream to the interior of BRC (Eriksen, Eghbali-Fatourechi et al. 2007). In addition, this particular microenvironment facilitates an adequate concentration of a myriad of bone degradation products that promote bone formation after resorption. Therefore, bone lining cells would function as a selective membrane that controls the traffic of different substances inside BRC creating a restricted and proper milieu for the recruitment of osteogenic precursors.

The relevance of the cellular and molecular events occurring during bone remodeling is that under normal conditions osteoclast resorption is always followed by bone formation. In fact, previous remodeling and resorption is a common mechanism that takes place before the formation of mature bone during bone healing. There are at least three prerequisites that must be accomplished before bone formation is initiated during trabecular bone remodeling: Bone Remodeling Compartment formation, physical and biochemical signals and finally osteoprogenitor cell recruitment.

BRC formation coincides with the initiation of bone resorption (Hauge, Qvesel et al. 2001).Figure 1.7. As we mention above, BRC is a space interposed between the bone surface undergoing remodeling and the bone marrow (Andersen, Sondergaard et al. 2009). Osteoid degradation and bone lining cell detachment from the bone surface allow the creation of a distinguished three dimensional space. Under the canopy formed by the detached lining cells, an adequate gradient concentration of soluble signals and other degradation products are confined.

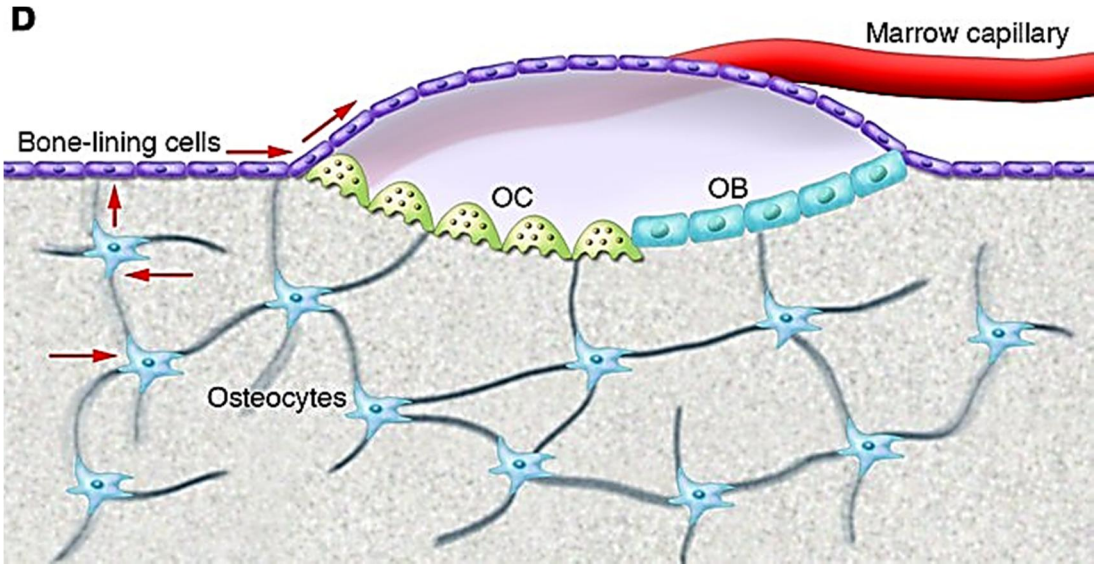



Figure 1.7. Bone remodeling compartment (BRC) formation coincides with the initiation of bone resorption. This particular microenvironment facilitates an adequate concentration of a myriad of bone degradation products. Taken from (Khosla, Westendorf et al. 2008)

Mature osteoblasts produce an organic matrix mostly of type I collagen. These collagen fibrils, together with mucopolysaccharides, proteoglycans and other locally or distally produced molecules, form a substrate on which inorganic salts are deposited (Centrella, McCarthy et al. 1988). This organic and inorganic matrix ossifies and becomes subject to the catabolic or resorptive efforts of the osteoclasts (Centrella, McCarthy et al. 1988). During osteoclast resorption the inorganic fraction is gradually dissolved and the demineralized collagen matrix is not completely degraded. These organic and mineral debris are co-released toward the extracellular space acting as signalling molecules.

Polypeptide growth factors are molecules that are specialized for intercellular communication (Taipale and Keski-Oja 1997). These soluble signalling molecules (growth factors and cytokines) are produced and stored in the bone matrix by mature osteoblasts and released during resorption. Of note, these intercellular signalling proteins act in picomolar to nanomolar

concentrations to regulate host cell function and their physiological role is to coordinate the modeling and remodeling of tissues (Nathan and Sporn 1991). Among the main growth factor families stored in bone matrix and having significant effects on bone remodeling are Insulin Growth Factor (IGF), Transforming Growth Factor β (TGFβ), Platelet Derived Growth Factor (PDGF), Bone Morphogenetic Proteins (BMP´s), Fibroblast Growth Factor (FGF) (Hauschka, Mavrakos et al. 1986, Canalis, McCarthy et al. 1988). Because a myriad of cytokines and growth factors are stored in the bone matrix it follows that multiple signalling biochemical molecules are simultaneously present within the bone remodeling compartment.

Osteoclast activity causes a rise in the concentrations of calcium and phosphate in the aqueous solution of the BRC (Price, Caputo et al. 2002). In addition, this cellular activity could promote the liberation of carbonate, magnesium and sodium ions present in the inorganic bone component. However, it has been reported that a Fetuin-Matrix Gla Protein (MGP) prevents the growth, aggregation and precipitation of the mineral component. That is, MGP prevents the formation of the supersaturated concentrations of the released ions ($Ca^{2+}$, $PO_4^{3-}$, $Mg^{2+}$,$CO_3^{2-}$) within the BRC that lead to formation of crystal nuclei (Price, Caputo et al. 2002).

Calcium is the major component of the mineralized bone matrix which is, in its ionic form, released within the remodeling microenvironment (Dvorak and Riccardi 2004). Calcium levels are significantly different in the hemivacuole, where previous studies have reported values between 8-40mM and over the nonresorbing surface of the osteoclast, where direct measurements indicate values <2mM during resorption (Berger, Rathod et al. 2001). Osteoclasts respond to elevated calcium concentrations inside the resorbing compartment with morphological and functional changes triggering a dramatic cell retraction followed by a profound inhibition of bone resorption (Zaidi, Shankar et al. 1995). In contrast, exposure of MC3T3-E1 cells (which exhibit properties of osteoprogenitor cells and preosteoblasts) to high amounts of calcium (up to

4.8mM) stimulated both chemotaxis and proliferation (Yamaguchi, Chattopadhyay et al. 1998). However, both events have also been reported with 10 mM. (González-Vázquez, Planell et al 2014)

Osteoprogenitor recruitment is the result of a highly organized combination of cell activities including migration, proliferation, differentiation and apoptosis (Jensen, Andersen et al. 2015). These cellular events overlap in time and are located spatially within the BRC and nearby anatomical structures. The release and solubilization of a myriad of organic and mineral degradation debris and their diffusion through the canopy influence the adjacent tissues. This diffusion induces changes on the rich capillary subendosteal region and the lifted canopy cells. By these mechanisms, the BRC provides guidance to these progenitor cells by building up a high concentration of chemoattractans and through site specific anchorage (Andersen, Sondergaard et al. 2009).

The reversal phase couples bone resorption and bone formation by generating an osteogenic environment at remodeling sites (Delaisse 2014). Reversal cells colonize resorbed bone surfaces immediately after osteoclasts leave and prepare the eroded surfaces for bone formation, removing resorption debris left behind and depositing a cement line (Abdelgawad, Delaisse et al. 2016). Under these conditions, the covered and particularly prepared reversal surfaces becomes an osteoinductive cellular coating promoting the migration of preosteoblasts preceding the deposition of new bone (Yamaguchi, Chattopadhyay et al. 1998, Delaisse 2014).

Kristensen *etal.* suggest a model in which osteoprogenitor recruitment may proceed along three concurrent routes: route 1 originates from BLCs and proceeds along the bone surface; route 2 originates from bone marrow envelope cells, which develop into the canopies covering the bone remodeling site and supply osteoblast progenitors to the reversal surfaces; route 3 probably originates from perivascular cells, reaching canopies along capillaries (Kristensen, Andersen et al. 2014). Figure 1.8. In addition, since bone remodeling occurs in a highly vascularized region, an alternative source has

been suggested. Eghbali-Fatourechi *et al* (2007) reported that cells destined to become osteoblasts on bone surfaces likely enter the BRC not directly from the bone marrow, but rather via the capillaries that penetrate the BRCs. These circulating osteoblastic cells contribute to the pool of osteoblastic cells entering the BRC (Eghbali-Fatourechi, Modder et al. 2007).Figure 1.9.

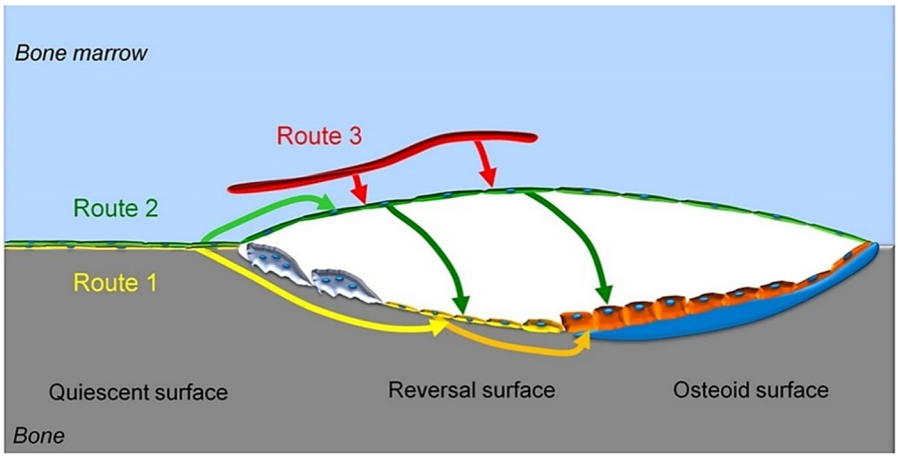



Figure 1.8. Model proposing three distinct routes for recruitment of osteoprogenitor cells during cancellous bone remodeling. (Kristensen, Andersen et al. 2014)

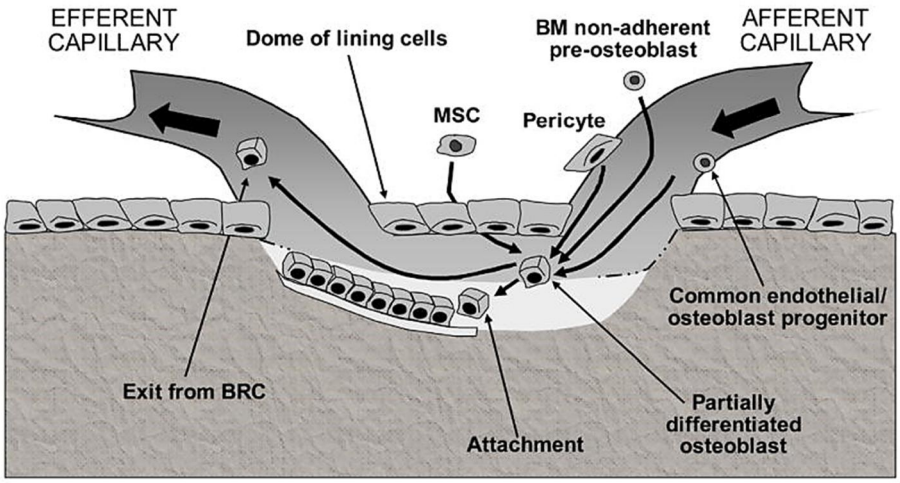



Figure 1.9. Because BRC is highly vascularized, circulating cells would be an additional source of osteoprogenitor cells. (Eghbali-Fatourechi, Modder et al. 2007)

Considering these recruitment routes of osteoprogenitors two separate but complementary episodes of cellular migration could occur within BRC. First, reversal cells follow the released signalling molecules and attach demineralized collagen fibrils left by osteoclasts. Second, osteoblast precursors are attracted chemotactically from the canopy and perivascular areas of the capillaries toward the reversal surface. Consistently, expression of Smooth Muscle Actin (SMA), a motility marker, has been detected in pericytes around the capillaries, the BRC canopy and reversal cells on eroded surfaces but not in mature osteoblasts (Kristensen, Andersen et al. 2013, Delaisse 2014). SMA may support the migratory function of osteoblast progenitors because it is a contractile protein involved in cell motility (Kristensen, Andersen et al. 2013). In addition, the motility of progenitor cells gradually decreases during differentiation. At a later stage, the differentiated cells have low motility and high adhesion ability (Ichida, Yui et al. 2011).

Several reports have demonstrated that degradation debris such as collagen fragments, relatively low doses of growth factors and extracellular calcium ions produce a common chemotactic response on mesenchymal stem cells (Godwin and Soltoff 1997, Dirckx, Van Hul et al. 2013, Krause, Scadden et al. 2013, Abdelgawad, Soe et al. 2014). Consequently, osteoprogenitor cells at an early differentiation stage could be targets for chemoattractive signalling molecules liberated from bone matrix and such cells could gradually differentiate into mature bone forming osteoblasts.

Given that bone formation occurs only above a given cell density (Kristensen, Andersen et al. 2014), attraction of osteoblast progenitors toward the reversal surface is required. A possible mechanism for providing sufficient bone forming osteoblasts is cell proliferation and regions of convergence between capillaries and canopies coincide with a higher prevalence of Ki-67 positive proliferative cells (Kristensen, Andersen et al. 2013). In addition, it has been proven that canopy cells are less differentiated than those of the reversal surface, as shown by the inverse levels of ki-67 versus osterix expression

(Kristensen, Andersen et al. 2014). Disruption or loss of canopies are associated with insufficient osteoblast progenitor recruitment on the reversal surface and absence of progression of the remodeling cycle to bone formation (Jensen, Andersen et al. 2015).

The need for a minimal cell number before cells can differentiate is also observed in skeletogenesis (Hall and Miyake 1992, Hall and Miyake 1995). Condensations represent a critical stage in skeletogenesis such that osteoblasts will only differentiate in high cell densities associated with condensations. Previously dispersed populations of mesenchymal cells gather together to differentiate. They form an aggregation or condensation, which is the earliest sign of the initiation of a skeletal element (Hall and Miyake 1992, Hall and Miyake 2000). Therefore, bone formation after resorption requires a cellular threshold which is reached by the attraction of cells from different sources in response to chemotactic signals toward the eroded bone surface.

## 1.2 BONE REGENERATION AND BONE GRAFTS

Bone is the hardest connective tissue in the human body. However, the skeletal tissue can be injured by trauma, atrophied by tooth loss, incompletely formed during congenital craniofacial bone defects or destroyed by pathological conditions. Understanding the basic principles of the cellular and molecular events regulating osteoblast differentiation is essential for the development of effective approaches to regenerate bone.

### 1.2.1 Autogenous grafts

Autologous bone grafts, that is bone taken from another anatomical area of the same patient, has been considered the "*gold standard*" for treating osseous defects. Autogenous bone are obtained with certain costs to the patient including additional surgical incisions; increased postoperative morbidity;

weakened donor bone sites; and potentially serious complications from any of the previous conditions (Burchardt 1983).

Although this approach is associated with these drawbacks, this type of graft merges the required features to promote bone healing and regeneration. Autografts agglutinate a three-dimensional bone framework, cellular secreted and bone matrix derived signalling molecules and osteoprogenitor-osteocyte lineage cells. Indeed, autologous grafts promote bone defect regeneration as the result of the integration of three interdependent mechanisms. Namely, osteogenesis, osteoconduction and osteoinduction.

### 1.2.2 Osteogenesis, osteoconduction and osteoinduction

Osteogenesis occurs when viable osteoblasts and/or osteoblast precursors are transplanted with the bone graft. Since few mature osteoblasts survive transplantation the stem cells are responsible for a significant portion of new bone formation (Cypher and Grossman 1996). Osteoconduction is the mechanism through which trabecular autografts provide a three dimensional framework giving mechanical support and allowing the invasion of vascular and stromal tissue into the graft. This term means that bone growth on a surface or down into pores, channels or pipes (Albrektsson and Johansson 2001).

In this process, a bone graft serves as a scaffold or lattice, facilitating migration of host cells for osteogenesis and eventually leading to partial resorption of the graft. This process is known as creeping substitution (Lind and Bunger 2001). Several biomaterials have been manufactured reproducing specific attributes that promote bone formation. These graft substitutes are formed from a variety of materials that are designed to mimic the three dimensional characteristics of autograft (Matassi, Nistri et al. 2011). Osteoconduction depends to a fairly large extent on previous osteoinduction (Albrektsson and Johansson 2001).

Osteoinduction is the guided attraction of osteoprogenitor cells from the host bed responding to chemoattractant signals released from the implanted graft or biomaterial. In addition, osteoinduction could be defined as the process by which osteogenesis is induced and implies the recruitment of immature cells and the stimulation of these cells to develop into preosteoblasts (Albrektsson and Johansson 2001). The mechanism of osteoinduction occurs naturally to varying degrees during fracture healing and after the implantation of an autologous bone graft and human acid demineralized bone matrix (Ferretti, Ripamonti et al. 2010). This process is mediated by a cascade of signals and the activations of several extra and intracellular receptors (Giannoudis, Dinopoulos et al. 2005). Among these signals regulating the osteoinductive process are BMPs and other growth factors such as TGFβ, PDGF, IGF and FGF (Cypher and Grossman 1996, Lind and Bunger 2001).

In addition, it has been shown that osteoinduction by diverse calcium phosphate biomaterials, such as synthetic hydroxyapatite ceramic, coral derived hydroxyapatite ceramic, α and β tricalcium phosphate, biphasic calcium phosphate in various animal models (Habibovic and de Groot 2007, Barradas, Yuan et al. 2011). In the present day, the exact mechanism of osteoinduction by biomaterials is still not completely understood (Barradas, Yuan et al. 2011). The osteoinductive property of calcium based biomaterials induces osteoblast differentiation and bone formation without the addition of recombinant growth factors. Furthermore, it is questionable whether the mechanism of osteoinduction by BMPs and that of inorganic biomaterials are related and, if so, to what extent (Barradas, Yuan et al. 2011). At least one relevant difference in osteoinduction by BMPs and biomaterials is that bone formation induced by biomaterials starts directly as bone (intramembranous) (Yuan, Van Den Doel et al. 2002), while recombinant BMP induces endochondral bone *in vivo* (Reddi and Cunningham 1993, Wozney and Rosen 1998).

## 1.3 BONE TISSUE ENGINEERING

The term “tissue engineering” was officially coined at a National Science Foundation workshop in 1988 to mean “the application of principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function” (O'Brien 2011). This definition has evolved and several key elements have been included. The fundamental concept behind tissue engineering is to utilize the body´s natural biological response to tissue damage in conjunction with engineering principles (Porter, Ruckh et al. 2009).

The following definitions have been suggested by several authors:

*Tissue engineering is an interdisciplinary field that implies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain or improve tissue function. (Langer and Vacanti 1993).*

*Tissue engineering, one of the major components of regenerative medicine, follows the principles of cell transplantation, materials, science and engineering towards the development of biological substitutes that can restore and maintain normal function* (Atala 2004).

*Tissue engineering is the science of design and manufacture of new tissues for the functional restoration of the impaired organs and replacement of lost parts due to disease, trauma or tumors. Tissue engineering is based on principles of cellular and molecular developmental biology and morphogenesis guided by bioengineering and biomechanics (Reddi 2000)*.

Currently distinct anatomical zones are used to obtain autogenous bone grafts to reconstruct craniofacial bone defects, such as cleft palate, pathological conditions or after trauma. Between these sources are iliac crest, cranial bone, mandibular symphysis, rib and tibia (Rawashdeh and Telfah 2008). However, wherever bone is harvested undesirable and detrimental effects are produced at the donor area.

To avoid these drawbacks, bone tissue engineering has emerged to provide an alternative to autogenous bone harvesting. The three key ingredients for tissue engineering are inductive signals, responding cells and biomimetic biomaterials based on extracellular matrix (scaffolding) (Reddi 2000). Current efforts are aimed at developing smart three-dimensional scaffolds similar to the internal architecture of bone that are inherently osteoinductive by mimicking the geometry and biochemistry of the extracellular matrix of the tissue (Heliotis, Ripamonti et al. 2009).Bone tissue engineering strategies to regenerate bone generally fall into two categories: scaffolds seeded with cells and cell-free devices, which depend on the body´s natural ability to regenerate (Atala 2004, Bueno and Glowacki 2009).

The cell-based approaches require the isolation, culture and expansion of mesenchymal stem cells. These osteogenic progenitor cells are seeded into the scaffold and induced to differentiate into bone forming osteoblasts. This differentiation is promoted through an osteogenic media or a single recombinant growth factor or in combination. Finally, seeded scaffolds are implanted in the injured zone to regenerate the bone tissue.

In Contrast, cell-free or acellular approaches do not depend on previously isolated mesenchymal stem cells. Essentially, it is dependent on the body´s natural ability to regenerate and on the presence of the host´s osteoprogenitor cells (Atala 2004, Bueno and Glowacki 2009). The scaffolds gradually degrade upon implantation and are replaced and remodeled by new bone synthesized and secreted by ingrowing cells (Bueno and Glowacki 2009).

Since acellular scaffolds provide a three dimensional framework, they are frequently combined with soluble growth factors to promote osteogenic cells and vessel ingrowth. By combining osteoconductive and osteoinductive materials as composites, the aim is to achieve better material properties (Schieker, Seitz et al. 2006).

Therefore, tissue engineering constructs promote bone regeneration by the combination of a three dimensional provisional scaffold with osteoinductive signals, with or without cells. This construct allows mesenchymal stem cells to migrate from the host recipient site and attach to its surface, proliferate and differentiate into bone matrix secreting osteoblasts.

### 1.3.1 Mesenchymal Stem Cells (MSCs)

The concept of stem cells originated at the end of the19th century as a theoretical postulate to account for the ability of certain tissues to self-renew for the lifetime of an organism (Bianco, Robey et al. 2008). Stem cells are cells with the capacity for unlimited or prolonged self-renewal. Usually, between the stem cells and their terminally differentiated progeny, there is an intermediate population of committed progenitors with limited proliferative capacity and restricted differentiation potential (Watt and Hogan 2000). Figure 1.10. MSCs are pluripotent cells present in many adult mesenchymal tissues, such as synovium, muscle, adipose tissue and bone marrow (Jorgensen, Gordeladze et al. 2004). However, it has been reported that MSCs could reside in virtually all post-natal organs and tissues related to their existence in a perivascular niche or blood vessel walls (Caplan 2008, Crisan, Yap et al. 2008).

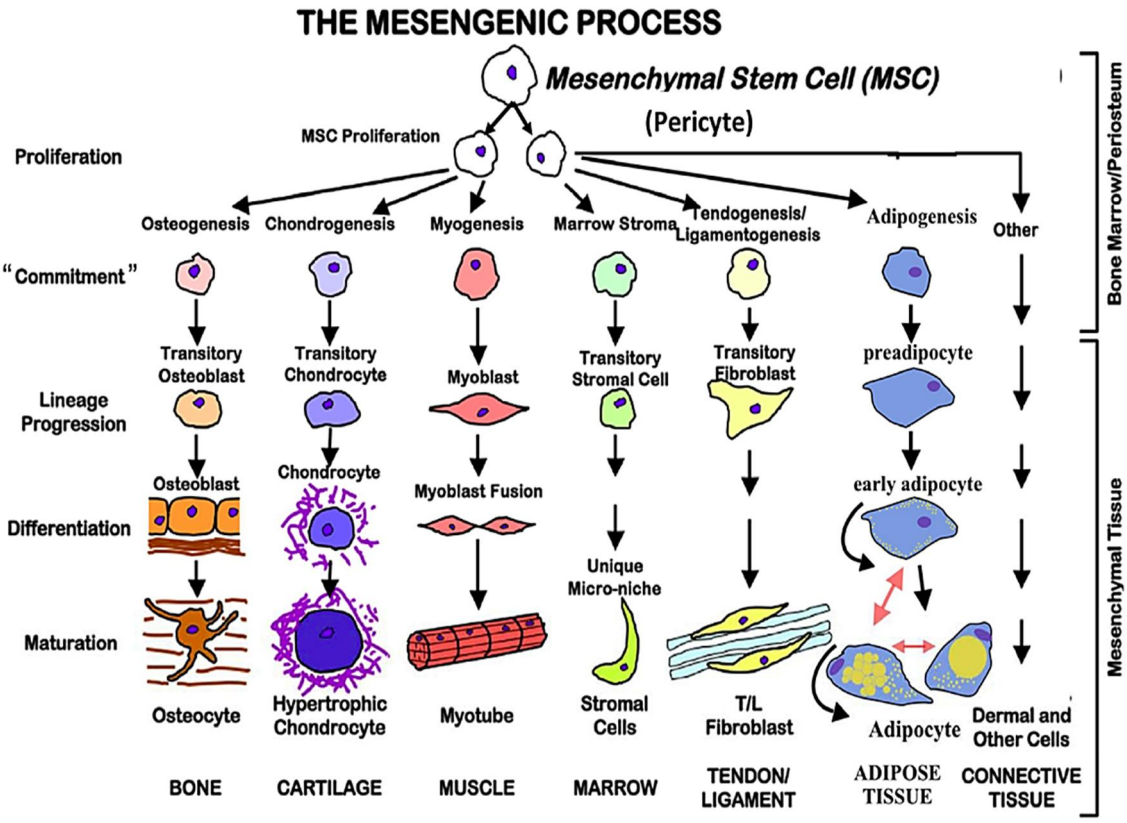



Figure 1.10. Mesenchymal stem cells have the potential to differentiate in multiple tissues. Taken from (Dimarino, Caplan et al. 2013)

MSCs in bone marrow are a source of osteoblast progenitors. They are part of the stromal population supporting hematopoiesis as well as contributing to the structural organization as an integral component of the sinusoidal walls (Sacchetti, Funari et al. 2007, Bianco, Robey et al. 2008).A minimal criteria for the identification of human MSCs has been established. First, MSC´s must be plastic-adherent. Second, MSCs must express specific surface antigen CD105, CD73 and CD90. Third, they must have multipotent differentiation potential (Dominici, Le Blanc et al. 2006). Table 1.1.

Summary of criteria to identify MSC

| | | | |
|---|---|---|---|
| 1 | Adherence to plastic in standard culture conditions | | |
| 2 | Phenotype | Positive (≥ 95% +) | Negative (≤ 2% +) |
| | | CD105 | CD45 |
| | | CD73 | CD34 |
| | | CD90 | CD14 or CD11b |
| | | | CD79α or CD19 |
| | | | HLA-DR |
| 3 | *In vitro* differentiation: osteoblasts, adipocytes, chondroblasts (demonstrated by staining of *in vitro* cell culture) | | |

Table 1.1.Minimal criteria to define human MSC´s. Taken from (Dominici, Le Blanc et al. 2006)

Skeletal development in the embryo, repair and remodeling in the adult require the direct contribution of mesenchymal stem cells. The gradual progression in the differentiation sequence into bone involves several overlapping stages. Figure 1.11. Progression from one stage to the next depends on the presence of specific local bioactive factors from surrounding cells (paracrine regulation), signals emitted by the cell itself (autocrine regulation) as well as other environmental cues (Caplan 1991, Bruder, Fink et al. 1994).The sum of these various intrinsic and extrinsic signals defines the developmental position of the cells (Caplan 1991).

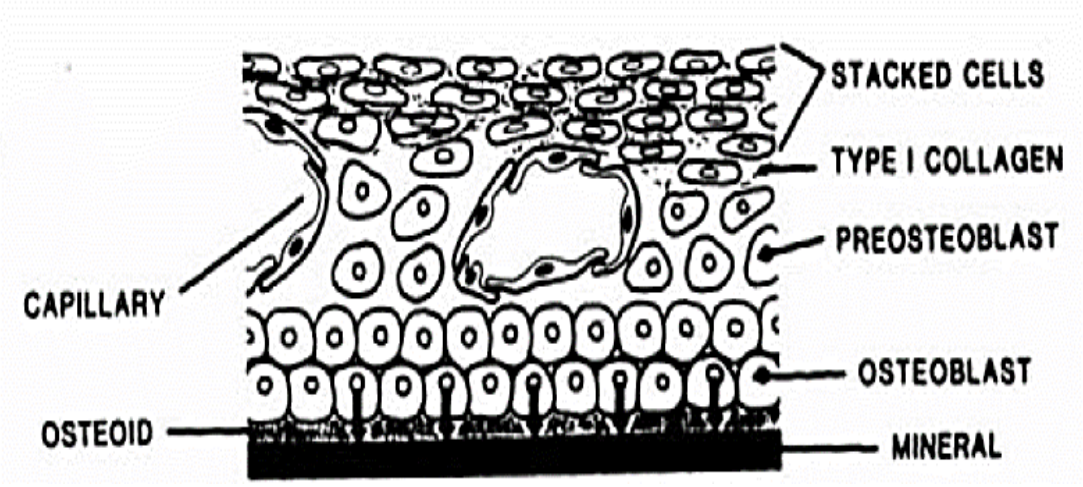



Figure 1.11. During skeletal development exists an intimate relationship between vasculature and newly forming bone. Taken from (Caplan 1991)

Adult MSCs *in vivo* function to supply replacement units for the differentiated cells that naturally expire or reservoirs for the regeneration of tissues after injury or disease (Caplan 2005, da Silva Meirelles, Chagastelles et al. 2006). This process of stem cell-generated replacement decreases with age after reaching its peak in the mid to late 20s in humans (Caplan 2005).Since mesenchymal stem cells are present in concentrations of less than 1 in 100,000-500,000 nucleated cells in bone marrow aspirates from adults, the MSCs must be culture expanded to obtain sufficient numbers for clinical use (Caplan 2005). In addition, MSC frequency seems to decline with age, from 1/10,000 nucleated cells in a newborn to about 1/1,000,000 nucleated marrow cells in an 80 year-old person (Caplan 1994). Figure 1.12.

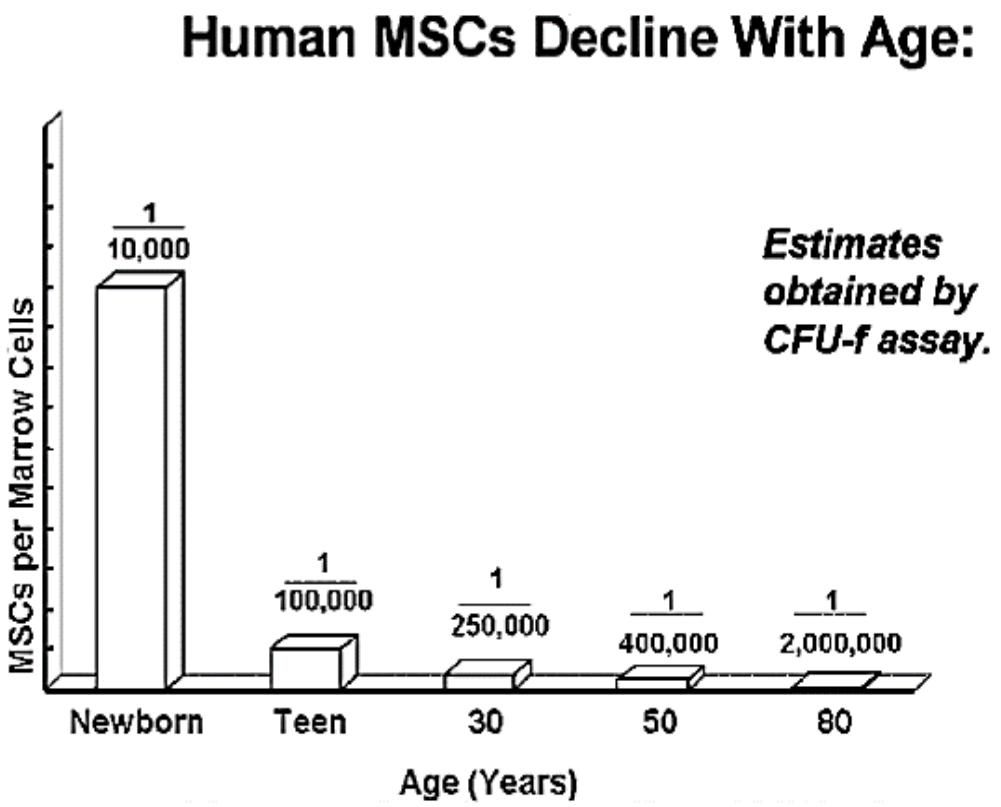



Figure 1.12. Decrease in MSC´s titers between newborn and 80 year old. Taken from (Caplan 2007)

According to these data, the regenerative ability of bone could be inverse to age in humans. In addition, as it has been mentioned above, a deficiency in the appropriate initial cell density could be detrimental in the sequence of bone formation. Although they can be managed safely during a standard *ex vivo* expansion period (6-8 weeks), human MSC can undergo spontaneous transformation following long-term *in vitro* culture (4-5 months) (Rubio, Garcia-Castro et al. 2005).This *ex vivo* amplification of MSCs may lead to malignant transformation in mice, where extensive passaging leads to cytogenetic aberrations (Charbord 2010).

### 1.3.2 Scaffold

A scaffold is a provisional three-dimensional structure that defines the area where osteogenic cells will produce bone. It has been suggested (Schieker, Seitz et al. 2006, Porter, Ruckh et al. 2009, Matassi, Nistri et al. 2011) that ideally, biomaterials used as scaffolds for bone tissue engineering should meet several design criteria:

-Scaffolds must favor cellular attachment, growth and differentiation.

-Deliver bioactive molecules to accelerate healing and prevent pathology.

-Scaffolds should be biocompatible, with lack of immunogenic response.

-Scaffolds should be biodegradable and eventually eliminated and produce non-toxic degradation products.

-Porosity should be high enough to provide sufficient space for cell adhesion and extracellular matrix regeneration. The porous architecture must allow vascularization and bone ingrowth.

-The material should be reproducibly processable into three dimensional structures. The material must be able to be sterilized without loss of bioactivity.

- It must provide temporary mechanical support. It is important to design a matrix that possesses mechanical properties similar to the tissue in the immediate surrounding area of the defect.

### 1.3.3 Signals

#### 1.3.3.1 Bone Morphogenetic Proteins (BMPs)

One of the earliest reports about the treatment of calvarial bone defects was made for Nicholas Seen in 1889 (Senn,1889). He used implantations of decalcified bone after trephining dog's skull. Decalcification and disinfection of bone was performed by keeping the prepared tissue in dilute muriatic acid (Hydrochloric Acid). Sixty five years later, Marshall Urist discovered that intramuscular implantation of demineralized bone matrix was able to produce bone and bone marrow as final product. Decalcified bone was replaced by new bone tissue very rapidly over a period of several weeks by a mechanism called bone formation by autoinduction (Urist 1965).

The osteogenic effect was associated with the organic component of bone. The active component was identified as being proteinaceous and named bone morphogenetic protein (BMP) (Wozney and Rosen 1998). In the1980s Sampath and Reddi made a fundamental contribution to the description of the mechanism of action of demineralized bone matrix. Approximately 3% of the proteins were solubilized from demineralized bone matrix and the remaining residue was mainly insoluble type I bone collagen. The soluble extract alone or the insoluble residue alone was incapable of new bone formation. However, the addition of the extract to the insoluble collagen and its subsequent implantation resulted in bone induction. Optimal osteogenic activity in this case was a collaboration between soluble extract and the insoluble collagenous substratum (Sampath and Reddi 1981, Reddi 2000, Reddi 2000).

BMP concentration in demineralized bone matrix was in the range of 1-2 μg. That is, a few micrograms of osteogenic proteins were isolated from over a ton of bovine bone (Reddi 2005). This limitation led to the cloning and recombination of BMPs in 1988 (Wozney and Rosen 1998). With this, large amounts of recombinant proteins were available for clinical application. Then, demineralized bone matrix was the original source for discovery of several key

growth factors that were subsequently cloned and recombinantly expressed to become FDA approved clinical therapeutic proteins (Gruskin, Doll et al. 2012).

BMPs are proteins produced by cells and secreted as ligands to act as autocrine or paracrine signals. Initially, they were identified as bone osteoinductive factors, but currently, we know they are involved in a myriad of biological activities. BMPs are a subclass of molecules of the TGFβ superfamily and as members of this family they initiate signalling by binding to type I and II Serine/Threonine Kinase receptors. To date, over 20 BMP family members have been isolated and characterized. BMP-1 through BMP-7 are expressed in skeletal tissue and BMP-2, -4 and -6 are the most readily detectable BMPs in osteoblast cultures (Gazzerro and Canalis 2006).

BMPs transduce signals through Smad and non-Smad signalling pathways(Miyazono, Kamiya et al. 2010). Figure 1.13. BMP target genes include a growing number of osteoblast-determining transcription factors such as *Runx2, Osterix and Dlx3/5 (Ulsamer, Ortuno et al. 2008, Ortuno, Ruiz-Gaspa et al. 2010)*. Smads are proteins that mediate the TGFβ and BMP responses. The Smads can be classified in 3 different groups: R-Smad, Co-Smad and I-Smad. The receptor mediated Smad proteins (R-Smad) include Smad1, Smad2, Smad3, Smad5 and Smad8. They function in ligand specific pathways and are phosphorylated at the C-terminus upon transmembrane receptor kinase activation (Chacko, Qin et al. 2001). Smad1, Smad5 and Smad8 transduce signalling from BMP ligands whereas Smad2 and Smad3 from TGFβ. C-terminal phosphorylation of R-Smad leads to the recruitment of Smad4 (Co-Smad) and the formation of active signalling complexes (Chacko, Qin et al. 2001).

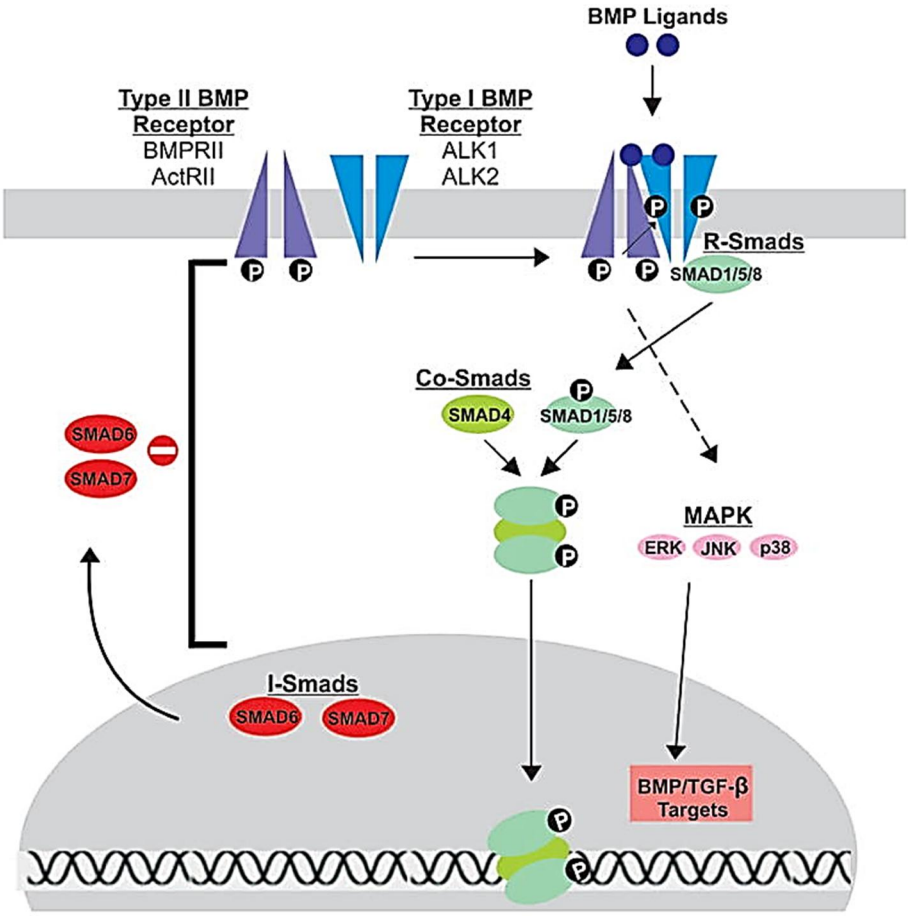



Figure 1.13. BMP´s activate Smad and non-Smad signalling pathways. Taken from (Beederman, Lamplot et al. 2013)

In addition to these positively acting Smads, the inhibitory Smads (I-Smads), Smad6 and Smad7, antagonize signalling by interacting with the receptor complex to prevent access and phosphorylation of R-Smad or by interfering with R-Smad/Smad4 complexing (Wrana and Attisano 2000). In addition, BMP effects can be regulated at different levels including:

-Inhibition of BMP-BMP receptor interaction by extracellular BMP binding proteins.

-Presence of dominant negative non-signalling membrane pseudoreceptors.

- Blocking of BMP signalling by inhibitory Smads.

-Blocking of BMP signalling by intracellular Smad binding proteins.

-Ubiquination and proteosomal degradation of BMP signalling effectors (Gazzerro and Canalis 2006).

This BMP/Smad signalling is one of the most prominent pathways promoting osteoblast differentiation. However, binding of BMPs also induces the activation of other signalling cascades. Smad independent pathways include ERK 1/2, p38, PI3K/AKT and β-catenin dependent signalling (Ghosh-Choudhury, Abboud et al. 2003, Lee, Lim et al. 2009, Sieber, Kopf et al. 2009, Gamez, Rodriguez-Carballo et al. 2016).

The efficacy of BMPs to regenerate bone in animal models and several applications in the clinical context are well known: bone fracture healing (Lane, Yasko et al. 1999, Vaccaro, Anderson et al. 2002, Axelrad and Einhorn 2009), alveolar cleft defects (Dickinson, Ashley et al. 2008), spinal fusion (Minamide, Yoshida et al. 2005) and defects in craniofacial bones (Yuan, Cao et al. 2012).

As a result, medical use of BMP-2 and BMP-7 was FDA approved for specific osteoinductive applications. However, most studies of bone regeneration in animal models make use of supra-physiological doses of BMPs. More importantly, BMP therapy in clinical practice also employs high amounts of BMPs, ranging between 1.5-3.3 mg (1.5 mg BMP-2, or 3.3mg BMP-7), even though in some cases, only minimal tissue regeneration is induced (Ripamonti 2010). Reports have demonstrated the safety of BMPs (White, Vaccaro et al. 2007). Nonetheless, some adverse effects have been documented with these

high doses of clinical BMP treatments(Carragee, Hurwitz et al. 2011). That is, BMPs when used in supraphysiological doses can stimulate bone resorption and turnover, eliciting a counterproductive response rather than achieving the intended objective of bone formation (Nakashima and Reddi 2003).

BMP is an expensive medication, adding anywhere from $5,000 to $15,000 to the cost of treatment, and its prescription is officially restricted to certain situations (Einhorn 2010, Courvoisier, Sailhan et al. 2014).This limited approval suggest that the FDA is only mildly impressed with its efficacy (Einhorn 2010).Then, an alternative to overcome the current shortcomings of high doses of BMPs is the combination of cellular and molecular approaches to enhance osteogenic differentiation and bone formation with lower amounts of growth factors.

#### 1.3.3.2 Wnt

Wnt are lipid modified glycoproteins secreted by cells as signalling molecules. They act primarily over short ranges to control stem cell behavior (Yang, Wang et al. 2016). In 1982 Roel Nusse reported the identification of Int-1 protooncogene now known as Wnt-1 (acronym from wingless and Int-1, Wnt-1) (Nusse and Varmus 1982, Niehrs 2012). Currently, 19 human Wnt proteins are known and research evidence highlights the relevance of wnt signalling in bone development and regeneration. Wnts have historically been classified as “canonical” Wnt-1, Wnt3a, Wnt-8 and Wnt-10b or as “non-canonical” Wnt-4, Wnt-5a and Wnt-11 but these simplistic classifications are now being challenged (Hoeppner, Secreto et al. 2009).

Mesenchymal stem cells (MSCs) have a self-renewal and multilineage differentiation potential (Sarugaser, Hanoun et al. 2009).Wnt signalling proteins modulate self-renewal (Reya, Duncan et al. 2003, Willert, Brown et al. 2003, Morrell, Leucht et al. 2008) and multipotential differentiation of these progenitor

cells. Binding of Wnt ligands to their cognate receptors in MSCs promotes osteoblast differentiation over the chondrogenic lineage (Day, Guo et al. 2005, Hill, Spater et al. 2005) and prevent the commitment to adipocytes by suppressing PPARγ (Bennett, Longo et al. 2005, Kang, Bennett et al. 2007). Figure 1.14.

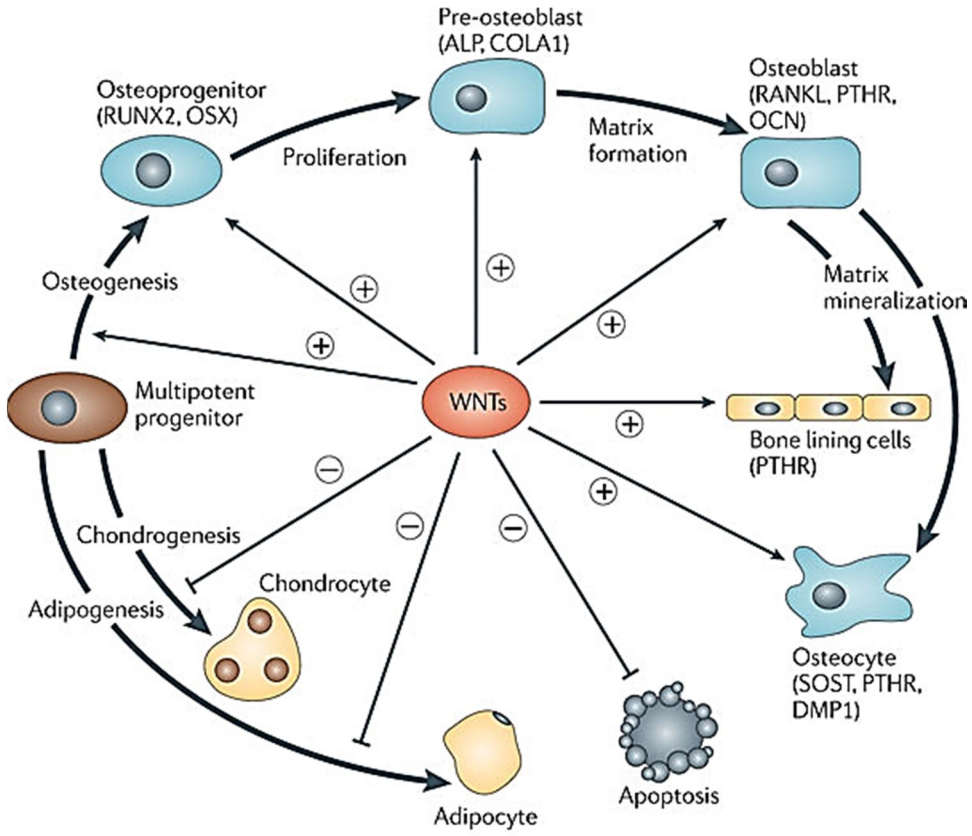



Figure 1.14.Wnt promotes osteoblast differentiation and prevent adipocyte or chondrogenic differentiation. Taken from (Kawai, Modder et al. 2011)

Wnt proteins and BMPs share the ability of activating the osteogenic transcription factors Runx2, Osterix and Dlx5 (Bennett, Longo et al. 2005, Kang,

Bennett et al. 2007, Ulsamer, Ortuno et al. 2008, Ortuno, Ruiz-Gaspa et al. 2010), but both Wnt and BMPs bind to different membrane receptors. In addition, as it was mentioned above, BMP-2 treatment induces endochondral ossification whereas the same injuries treated with liposomal Wnt heal via intramembranous ossification (Minear, Leucht et al. 2010). However, a cross-talk between both pathways has been reported extensively (Nakashima, Katagiri et al. 2005, Itasaki and Hoppler 2010, Rodriguez-Carballo, Ulsamer et al. 2011, Zhang, Oyajobi et al. 2013). Figure 1.15.

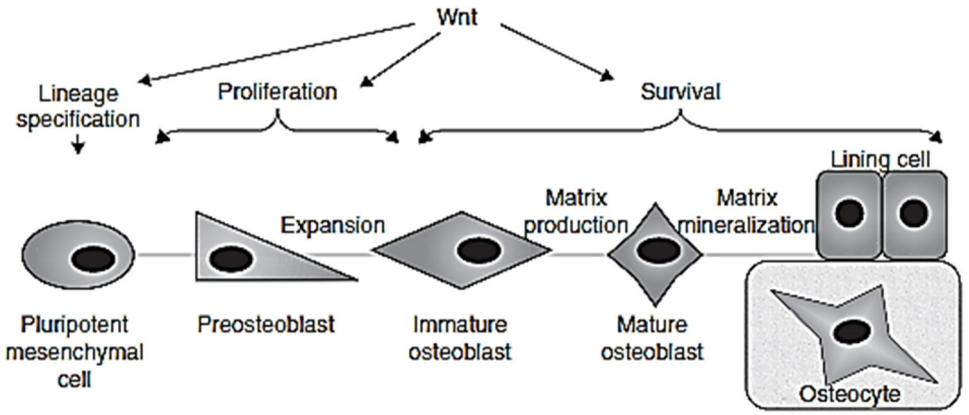



Figure 1.15.Wnt participates in multiple stages of osteoblast differentiation. Taken from (Hoeppner, Secreto et al. 2009)

Wnt ligands bind to Frizzled as well as LRP5/6 receptors. After Wnt receptors are activated three different pathways can be triggered, the canonical Wnt/β–catenin pathway, the Wnt/$Ca^{2+}$ pathway (both the most relevant Wnt pathways for osteoblast differentiation) and Planar Cell Polarity (PCP). Wnt-3a is a representative ligand that activates the β–catenin dependent pathway or canonical Wnt signalling (Komekado, Yamamoto et al. 2007). Cells constantly synthesize β–catenin but in the absence of Wnt ligand receptor activation it is degraded. This degradation is through the incorporation of β–catenin into the

destruction complex. The destruction complex is a multiprotein assembly, but its core components include, in addition to β−catenin itself, glycogen synthase kinase 3 (GSK3) and CK1, the scaffolding protein Axin, the adenomatous polyposis coli (APC) protein and β−TrCPand PP2A (Stamos and Weis 2013).

After activation of Wnt signalling an inhibition of β−catenin degradation is produced. Figure 1.16. This stabilization of β−catenin levels is either through the inactivation of the destruction complex by promoting the degradation of the scaffold protein Axin (Lee, Salic et al. 2003, Cselenyi, Jernigan et al. 2008) or by glycogen synthase kinase-3 β (GSK3 β) inhibition activity by Dishevelled (Malbon 2005).

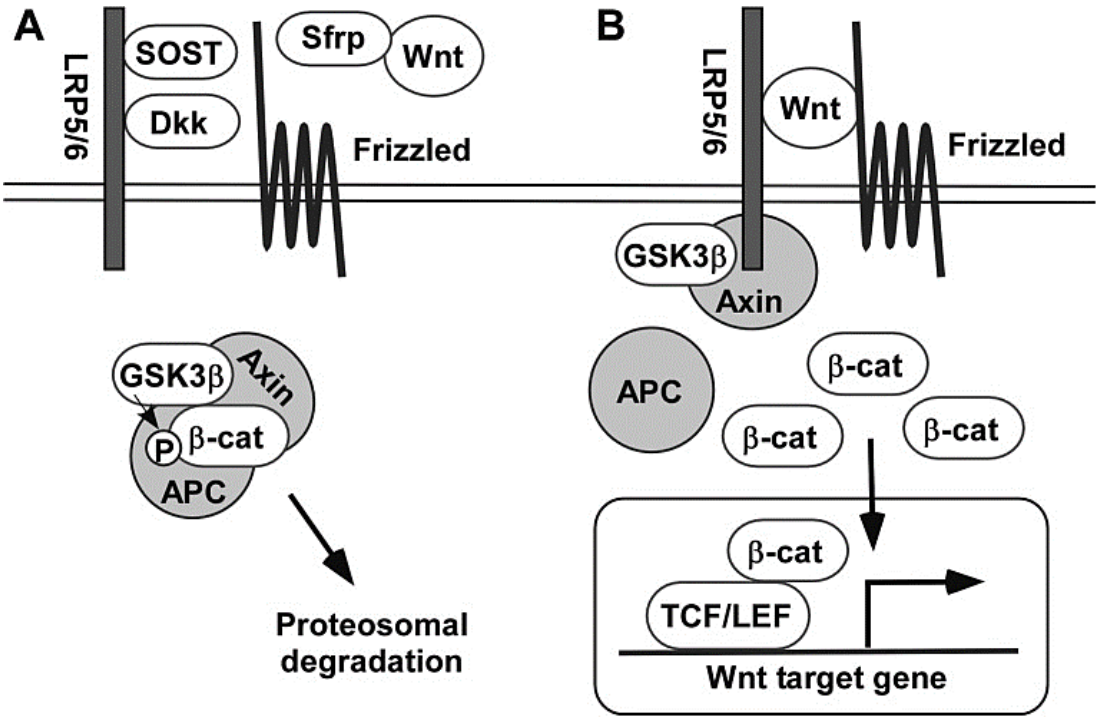



Figure 1.16. Without Wnt ligands β-catenin is degradated.
Taken from (Kubota, Michigami et al. 2010)

Dishevelled is required for Wnt dependent inhibition of the degradation complex (Yokoyama, Yin et al. 2007). Therefore, in the presence of the Wnt signalling the activated Dishevelled binds to Axin and inhibits β–catenin phosphorylation and the consequent degradation by GSK3 β.

Regarding non canonical Wnt pathways, a distinctive attribute of Wnt/$Ca^{2+}$ signalling is the release and elevation of intracellular calcium concentrations. Wnt ligands such as Wnt-5a and Wnt-11 trigger calcium release. However, Wnt-3a has been reported to activate both the β–catenin dependent canonical pathway and the $Ca^{2+}$/CaMKII noncanonical pathways (Nalesso, Sherwood et al. 2011).

Kestler and Kühl (2011) identified a concentration dependent activation of Wnt pathways. Figure 1.17.Wnt/ β–catenin signalling is activated by high concentrations of Wnt ligands. In contrast, Wnt/$Ca^{2+}$ signalling is favored by lower concentrations of Wnt ligands, mediating the activation of phospholipase Cβ that generates diacylglycerol (DAG) and inositol-3,4,5 trisphosphate ($IP_3$) and the consequent release of calcium ions from endoplasmic reticulum (ER). Both pathways reciprocally inhibit each other (Kestler and Kuhl 2011, Nalesso, Sherwood et al. 2011).

Wnt/PCP signalling controls tissue polarity and cell movement and is observed in an array of developmental processes involving tissue organization. Its disruption can lead to severe developmental defects (Katoh 2005, Bayly and Axelrod 2011). In addition, the non-canonical Wnt/PCP pathway plays a major role in neural crest migration (De Calisto, Araya et al. 2005, Mayor and Theveneau 2014).

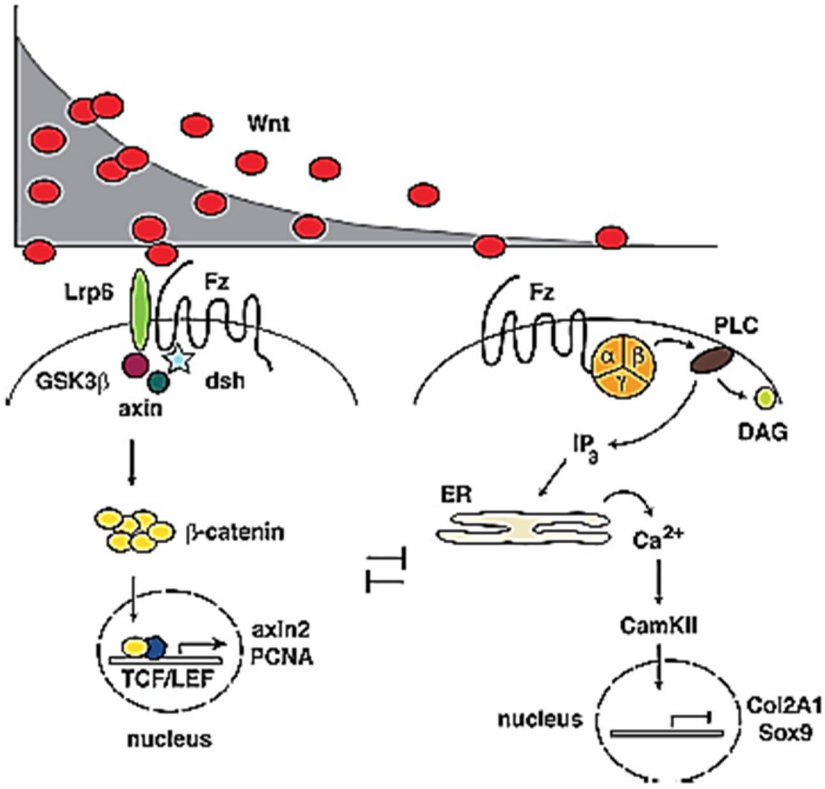



Figure 1.17.Concentration-dependent activation of Wnt pathways.
Taken from (Kestler and Kuhl 2011)

Currently, there is extensive research supporting the ability of BMPs to promote localized bone regeneration. However, BMPs cannot be delivered systemically and they are not available to treat osteoporosis (Hoeppner, Secreto et al. 2009). In contrast, Wnts for local bone regeneration have not been directly tested because of practical difficulties in purifying and administering the highly hydrophobic and insoluble Wnt proteins *in vivo* (Minear, Leucht et al. 2010). It has been shown that Wnt signalling increases osteoblast differentiation, bone formation and bone mass by the stabilization and accumulation of β–catenin. This increased bone mass by GSK3 inhibition results in an early temporal

amplification of MSCs which are driven to osteoblast differentiation at the expense of adipogenesis (Gambardella, Nagaraju et al. 2011).

Features of aging bone or osteoporosis are reduced osteoblast differentiation, increased osteoclastic resorption, decreased bone mineral density, as well as increased adipose tissue. Wnt signalling has been associated with aging effects on bone. In fact, decreased gene expression of various Wnt related proteins, as well as Wnt co-receptors and Wnt inhibitors, is downregulated in the bone tissue of aged mice (Rauner, Sipos et al. 2008). Figure 1.18.

In addition, decreased osteoblast differentiation and bone formation during aging and osteoporosis are the result of enhanced adipogenesis versus osteoblastogenesis from precursor cells (Justesen, Stenderup et al. 2001, Rauner, Sipos et al. 2008). With aging, there is a change in bone marrow composition increasing the volume of adipose tissue associated with an imbalance between augmented osteoclast activity and declined osteoblast bone formation, resulting in osteoporosis (Justesen, Stenderup et al. 2001, Verma, Rajaratnam et al. 2002, Rosen and Bouxsein 2006).

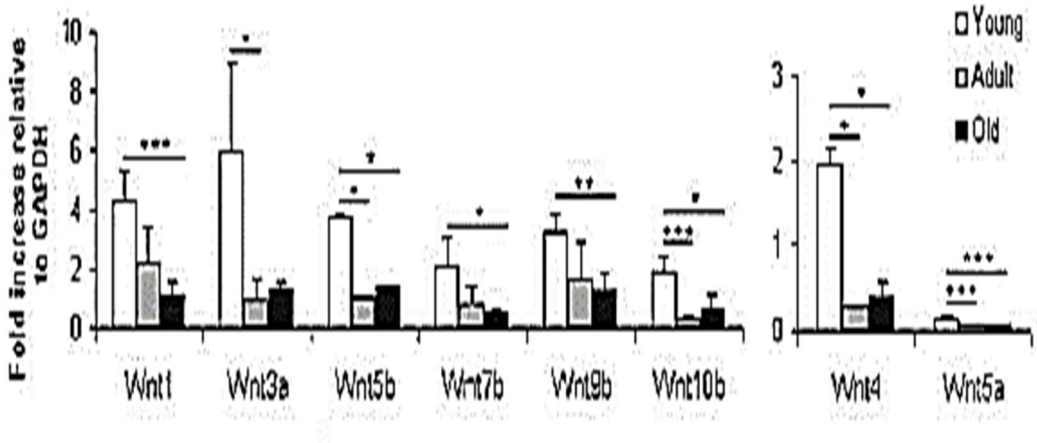



Figure 1.18. Expression of various Wnt related genes is downregulated with age in mice. Taken from (Rauner, Sipos et al. 2008).

Wnt regulation is a tightly controlled mechanism which is predominantly driven by two antagonists, Sclerostin (SOST) and DKK1 (Rossini, Gatti et al. 2013). Two basic therapeutic strategies for enhancing bone regeneration through Wnt signalling exist: adding agonists or blocking naturally occurring antagonists (Hoeppner, Secreto et al. 2009). Since Wnt receptors are expressed in multiple cell types, the use of Wnt agonists are considerably unspecific and more selective targets are needed. Sclerostin is an inhibitor of bone formation and is expressed by all three terminally differentiated cell types embedded within mineralized matrices: osteocytes, cementocytes and hypertrophic chondrocytes (van Bezooijen, Bronckers et al. 2009).

Because SOST is an inhibitor of osteoblast differentiation and Wnt signalling, antagonists of SOST have been considered as potential therapeutic targets to treat osteoporosis. In addition, they have also been considered in promoting bone regeneration by systemic administration. Pharmacologic inhibition of SOST using a Sclerostin neutralizing monoclonal antibody has been used after placement of titanium implants (Virdi, Irish et al. 2015), to reconstruct large defects due to periodontitis in rats (Taut, Jin et al. 2013), in healing of proximal tibial defects in ovariectomized rats (McDonald, Morse et al. 2012) and to treat osteoporosis using a rat model (Li, Niu et al. 2014). In every case, an enhancement of bone-implant contact and increased levels of bone formation were obtained.

#### 1.3.3.3 Calcium

The skeletal tissue is the largest reserve of minerals in the human body and almost all the calcium is stored in bones. After bone resorption by osteoclasts, the mineral component is dissolved and calcium ions are released first within the immediate remodeling microenvironment and then toward the circulation. This localized elevation in the extracellular calcium concentration

induces cellular and molecular effects on the responding osteoprogenitor cells. Therefore, there is an exchange of calcium ions between blood and bone and calcium can be transferred into and out of the bone by two independent mechanisms (requiring the activity of osteoclasts and osteoblasts) (Parfitt 1989).

In bone, extracellular ionic calcium concentration, a major extracellular factor in the bone microenvironment during bone remodeling, could potentially serve as an extracellular first messenger, acting via Calcium Sensing Receptor (CaR) (Brown and MacLeod 2001, Chattopadhyay, Yano et al. 2004). This Calcium Sensing Receptor (CaR) regulates four relevant cellular processes decisive for MSC´s fate: migration, proliferation, differentiation and apoptosis (Godwin and Soltoff 1997, Yamaguchi, Chattopadhyay et al. 1998, Yamaguchi, Chattopadhyay et al. 2000, Brown and MacLeod 2001, Chattopadhyay, Yano et al. 2004, Dvorak, Siddiqua et al. 2004, González-Vázquez, Planell et al 2014 et al 2014).

It has been reported that during *in vivo* resorption, the levels of extracellular ionized calcium are < 2mM over the non-resorbing surface of osteoclasts (Berger, Rathod et al. 2001).Thus, this localized elevation in extracellular calcium concentrations could serve as cues that initiate the sequence of osteoblast differentiation.

In 1993 Brown *et al.* reported the cloning of an extracellular calcium sensing receptor (CaR) from bovine parathyroid. The novel receptor features a large extracellular domain involved in calcium binding (Brown, Gamba et al. 1993).Figure 1.19. As a GPCR, the CaR is comprised of the three main structural features of this receptor family: an extracellular domain, a seven transmembrane domain and an intracellular tail (Magno, Ward et al. 2011). The function of the receptor could be divided by the plasma membrane into an extracellular component (“sensor”) and an intracellular component (“transmitter”) (Magno, Ward et al. 2011).

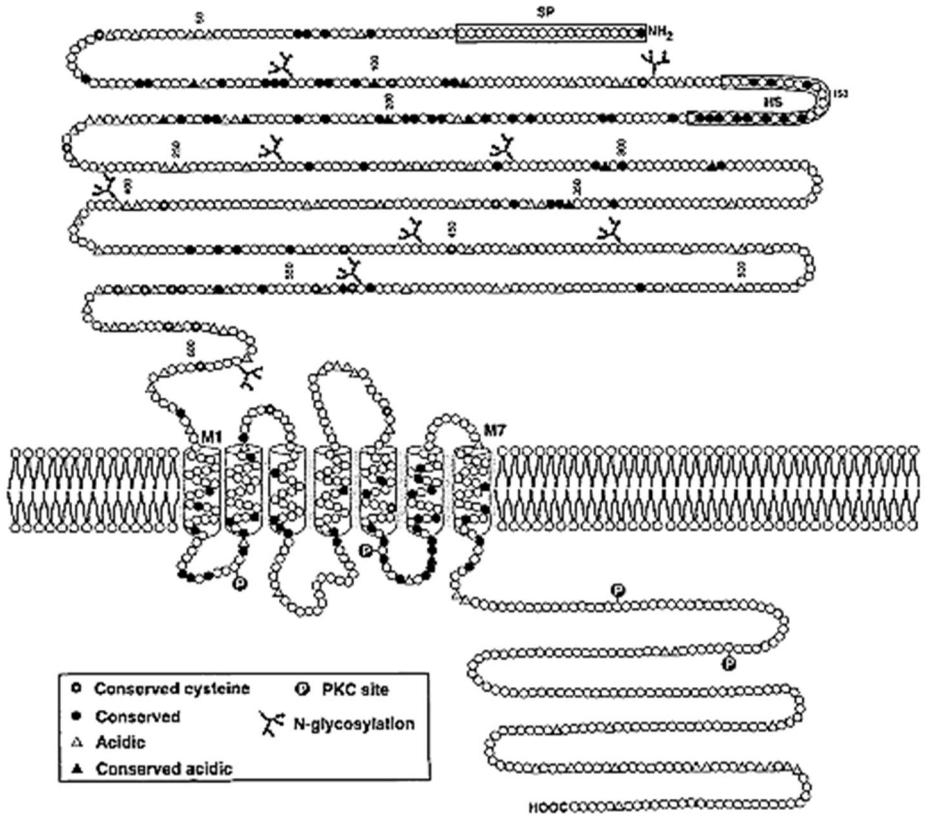



Figure 1.19. Structure of Calcium sensing Receptor (CaR). Taken from (Brown, Gamba et al. 1993)

The sensory aspect of the CaR relates to its ability to detect changes in the extracellular environment through binding its agonists, whereas the transmitter characteristics of the receptor relate to its ability to modulate intracellular signalling events (Magno, Ward et al. 2011). Figure 1.20. Because the extracellular domain of CaR contains highly concentrated regions of negative charge, a characteristic shared by many of the diverse agonists which activate CaR is a high concentration of positive charge (Breitwieser, Miedlich et al. 2004).

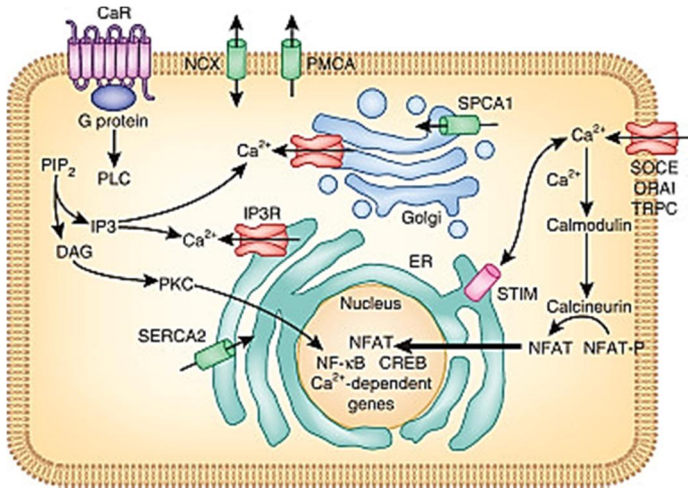



Figure 1.20. After $Ca^{2+}$binds to the extracellular domain $IP_3$ and DAG are activated by PLC. Taken from (Mascia, Denning et al. 2012)

Cells at rest have a $Ca^{2+}$ concentration of 100nM but are activated when this level rises to roughly 1000nM (Berridge, Lipp et al. 2000). This elevation in the cytosolic calcium concentration is observed after extracellular calcium and growth factors bind to their cognate receptors. BMP (Mandal, Das et al. 2016), Wnt (Slusarski, Yang-Snyder et al. 1997), VEGF (Hamdollah Zadeh, Glass et al. 2008), PDGF (Tucker, Chang et al. 1989), and EGF (Moolenaar, Aerts et al. 1986), mediate this rapid and transient intracellular $Ca^{2+}$ increment. Mobilization of ions from extracellular sources crossing the surface membrane and from the endoplasmic reticulum contribute to this increased cytosolic calcium levels.

The $Ca^{2+}$ signalling network can be divided into four functional units:

- Signalling is triggered by a stimulus that generates various $Ca^{2+}$ mobilizing signals.
- ON activation mechanisms that feed $Ca^{2+}$into the cytoplasm.
- $Ca^{2+}$functions as a messenger to stimulate numerous $Ca^{2+}$ sensitive processes. Figure 1.21.
- OFF mechanisms that removes $Ca^{2+}$ from the cytoplasm. (Berridge, Lipp et al. 2000).

Extracellular $Ca^{2+}$binding to CaR activates phospholipase Cβ (PLCβ) and produces inositol 1,4,5 trisphosphate ($IP_3$) and diacylglycerol (DAG). $IP_3$ promotes calcium release endoplasmic reticulum whereas DAG activates protein kinase C (PKC). This initiates an intricate intracellular network including but not limited to ERK 1/2, p38, Smad, PI3K/AKT and GSK3β (Dvorak and Riccardi 2004, Dvorak, Siddiqua et al. 2004, Leclerc, Neant et al. 2011).

In addition, Daub *et al* reported an activation of MAPK pathway by G protein coupled receptor (GPCR) through intracellular signal crosstalk, by a transactivation mechanism (Daub, Weiss et al. 1996). Therefore, extracellular calcium as ligand induces its own intracellular release and strengthens its signal by intermingling with other intracellular inputs.

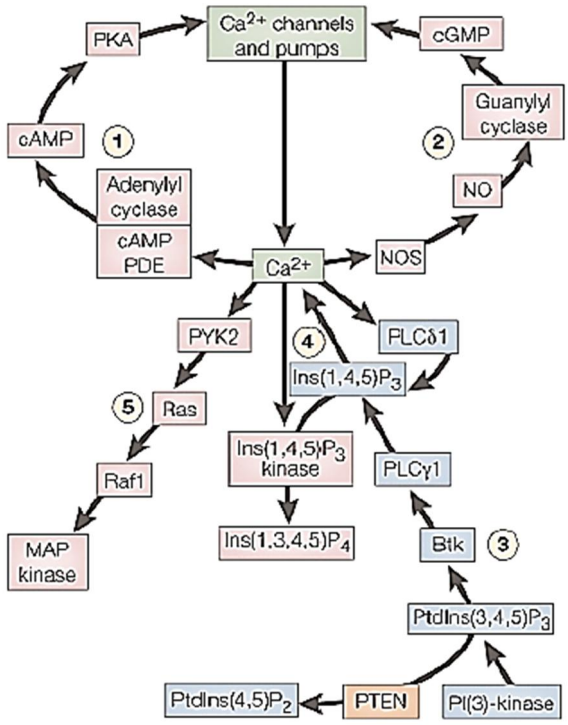



Figure 1.21. $Ca^{2+}$interact with many other signalling pathways. Taken from (Berridge, Lipp et al. 2000)

## 2. CHAPTER II: AIMS

**AIM 1:**

To select an optimized three dimensional scaffold by evaluating the effects of gelatin and $CaSO_4$ on MSC differentiation into osteoblasts using *in vitro* and *in vivo* models.

**AIM 2:**

To evaluate the interaction of BMP-2 and Wnt-3a on MSCs differentiation into osteoblasts *in vitro*, after 24 hours and after 10 days, and *in vivo* after five weeks using the selected three-dimensional (3D) scaffold.

**AIM 3:**

To identify the molecular mechanisms through which extracellular calcium and BMP-2 interact in the short- (24 hours) and long-term (10 days).

# 3. CHAPTER III: MATERIALS AND METHODS

***Bone Marrow Mesenchymal Stem Cells (BMMSC´s) isolation and culture***

BMMSCs were isolated from femurs of 6-8 week old BALB/c mice. Mice were euthanized by $CO_2$ and femurs were dissected. Next, the skin and muscle were cleaned and conserved in complete media (DMEM supplemented with 10% FBS, penicillin/streptomycin, 1mM pyruvate and 2mM glutamine). Femur ends were cut using a Rongeaur. Then, 1 milliliter syringe with a 26 gauge needle attached was filled with warmed Media. Next, the marrow was flushed and collected in a 50 ml Falcon. The cell solution was resuspended several times by pipetting and finally filtered with a 70 µm cell strainer (Falcon, USA).

The filtered cell solution was transferred to a 100 mm cell culture plate and incubated to 37º. Media was changed 24 hours later and then each 8 hours for 2-3 days to discard non- adherent cells. Approximately between 5-7 days the adherent cells reach 75-80% of confluence.

At this time, attached cells were washed three times with warmed PBS and tripsinized for 3 minutes at room temperature. Lifted cells were recultured and expanded for future experiments.

**Protocol for freezing cells (Cryopreservation)**

- Identify the cryotubs with the cell type, date of freezing, name of the researcher. - Eliminate the medium and wash twice with warm PBS. Eliminate PBS and add 1 ml of trypsin and wait between 3-5 minutes.
- Using a 15 ml Falcon with 3 ml of 10% DMEM, recover the trypsin/cells solution and centrifuge at 1200 rpm for 5 minutes.
- Eliminate the supernadant and keep the cell pellet. Resuspend the pellet with 900 µl with 10% DMEM gently.
- Transfer the cell solution to the labeled cryotube and add 100 µl of DMSO.
- Wrap the cryotube using a piece of paper towel and then with aluminium foil.

- Place the cryotube/s into the -80$^0$ C. After 24 hours transfer the cryotube/s into the liquid nitrogen tank.

***2D gelatin coated and 3D gelatin Scaffold Preparation***

To prepare 2D gelatin coated cell culture plates, 12 well plates surfaces were coated with a thin layer of 0.1% gelatin/PBS solution mixed with $CaSO_4$ dissolved in media at different concentrations ( 3, 5, 7.5, 10 mM). Plastic surfaces coated just with gelatin solution were used as controls. Plate wells were filled with 1.5 ml of the respective solution and were incubated overnight at 37$^o$ C. Then, media was removed carefully and treated wells were left for 1 hour inside a laminar flow hood to dry the treated surfaces and then stored until their use. In the case of the monolayer cell culture wells respective $CaSO_4$ concentrations were added without gelatin. Finally, the cells were seeded.

To prepare a 3D scaffold we used a 1mm$^3$ Gelatin sponge (Gelita, B. Braun). We cut the sponges to obtain slices of 1mm thickness using a surgical blade No. 11 attached to a scalpel handle. For the *in vivo* experiment 2mm slices were used. Next a new middle cut was made and of this way we acquired a 10x5x1mm scaffold. Before seeding under sterile conditions, these scaffolds were soaked using a 100 mm cell culture plate with 7 ml of complete media or PBS and incubated for 12-24 hours. At that time, each soaked gelatin scaffold was placed in the bottom of a 1.5 ml microcentrifuge tube and the excess of media was absorbed by pipetting. BMMSC´s were detached by trypsin and collected by centrifugation (1500-1700 rpm for 5 minutes). The cell pellet was resuspended with a final volume of complete media according to the number of scaffold/well replicates of each condition of study (calculate 20 µl of Media and 2x10$^5$ cells for each scaffold).

Finally, the volume of the cell solution was added (20 µl containing 2x10$^5$ cells) to the gelatin scaffold in the microcentrifuge tube and incubated in vertical

position for 4-6 hours at 37º C. With this individual seeding we allowed cellular adhesion and equality in the number of cells to each scaffold. Then, we carefully transferred each seeded scaffold to a well (24 well plate) and 200 µl of complete media were added. An important issue is to avoid that the seeded scaffold float by adding additional media at this point. After 24 hours, the media was removed carefully and the scaffold washed 3 times with warmed PBS. PBS was aspirated with care and 200 µl of the respective condition of study were added. We refreshed the cultured cells with 100 µl of the corresponding condition each 3 days.

***Extracellular calcium concentration effect on BM-MSC´s differentiation***

To assess the effect of extracellular calcium on BMMSC´s differentiation into osteoblasts, $CaSO_4$was used as source of calcium. Three culture models, a monolayer plastic surface, 2D gelatin coated and 3D gelatin scaffolds were used. $CaSO_4$was dissolved in complete media and filtered under sterile conditions and the solution was stored as 20mM stocks at 4ºC. Then, BMMSC´s were treated with different $CaSO_4$concentrations from 3mM, 5mM, 7.5mM and 10mM, using complete media as control. After 10 days, cell cultures were prepared for RNA isolation and RT PCR.

***Extracellular calcium effect inhibition by EDTA***

BMMSC´s seeded on 3D gelatin scaffolds were cultured in the presence of 7.5 mM concentration of $CaSO_4$ or $CaCl_2$ (Sigma-Aldrich) as source of calcium, $CaSO_4$+ EDTA or $CaCl_2$ + EDTA, using complete media as control. EDTA was used as extracellular calcium chelator with a final concentration of 7.5 mM from a stock of 20mM. After 10 days, cell cultures were prepared for RNA isolation and RT PCR.

**Western blot assay**

BM-MSCs seeded in 3D gelatin scaffolds were cultured for 24 hours or 10 days. Cells were lysed with 75 µl of lysis buffer (PBS, 1% Triton X-100, 100 mM PMSF, 1µg/ml pepstatin, 1 µg/ml leupeptin, 1 mM of sodium orthovanadate, 10 mMNaF and 10mM β-glycerophosphate) for one hour at 4ºC. Thirty micrograms of protein samples were subjected to SDS-PAGE and immunoblotting.

Membranes were incubated with different antibodies: pGSK3α/β Ser9/21 (9331S), pSMAD1/5/8 Ser465/467 (9511S)and pS6 Ser235/236 (2211) and pp38 Thr180/Tyr182 (9211S) from Cell Signalling Technology, pErk1/2 (M5670) from Sigma, β-catenin (610154) from BD TransductionLaboratories and α-tubulin (T6199) from Sigma, all diluted to a ratio of 1:1000.Horseradish peroxidase-conjugated secondary antibodies were used, followed by incubation with EZ-ECL reagent (Biological Industries). A chemiluminescent image of the immunoblots was captured with a Fujifilm LAS 3000 device.

**Separating gel 10%**

| | |
|---|---|
| Acrylamide 40% | 2.5 ml |
| Lower buffer 4x | 2.5 ml |
| $H_2O$ Mili Q | 5 ml |
| APS 10% | 100 µl |
| TEMED (add last) | 5 µl |

**Stacking gel**

| | |
|---|---|
| Acrylamide 40% | 0.525 ml |
| Upper buffer 4% | 1.25 ml |
| $H_2O$ Mili Q | 3.225 ml |
| APS 10% | 25 µl |
| TEMED (add last) | 5 µl |

**Calvarial critical-size bone defects and in vivo bone regeneration**

A surgical procedure was performed in 10-week old male BALB/c mice. Animals were housed individually and fed ad libitum. All procedures were performed in accordance with the protocols approved by the University of Barcelona Animal Research Ethics Committee and the Generalitat de Catalunya. Animals were anesthetized with isoflurane inhalation and an intraperitoneal injection of buprenorphine (0.05 mg/kg) was administered to provide intraoperative analgesia. To expose the parietal bones, a longitudinal midline incision was made and the tissues retracted. A circular critical-size bone defect with an outer diameter of 5 mm was carried out with a trephine bur on the left parietal. We cultured 4 x $10^5$ BM-MSCs per scaffold in accordance with the protocol described above for 3D gelatin scaffold preparation. After cells had been exposed to the respective conditions for 48 hours, a 1% final concentration solution of low melting agarose at 36ºC was added as a bonding agent. Scaffolds were implanted to fill the bone defects, depending on the respective experimental group. The incised tissues were sutured and the animals monitored daily during the recovery phase. Five weeks after surgery, the animals were euthanized and the calvariae dissected.

**μCT analysis**

Five weeks after surgery, the mice were euthanized. The heads were fixed with 4% paraformaldehyde for 24 h and stored in PBS/azide at 4C until scanning. Scanning was performed using a Skyscan 1076 High-Resolution scanner (Skyscan). All samples were placed horizontally in a holder, and the exposure parameters were 49 kV, 200 mA, with an exposure time of 500 ms and 180rotation. Data reconstruction was performed using the NRecon software. A Gaussian noise filter was applied and the three-dimensional models were performed with the CTAn software. Both programs were provided by the manufacturer.

A cylindrical region of interest (ROI) with a 5mm diameter was positioned manually to cover the bone defect area. For each sample, 375 slices were processed and analyzed. The ROIs were converted into volumes of interest that were used to quantify the extent of newly formed bone.

**Quantitative RT-PCR analysis**

The BMMSC RNA isolation was performed using Trisure (Bioline), according to the manufacturer's instructions. The RNA quantification was performed by spectrophotometric analysis (Nanodrop ND 1000; Thermo Scientific). The purified RNA (3 mg) was reverse transcribed using a High-Capacity Retrotranscription Kit (Applied Biosystems), and50 ng of cDNA per reaction was used in each quantitative RT-PCR (qRT-PCR), with two replicates per sample. qRT PCRs were carried out using the TaqMan 5´-nuclease probe method (Applied Biosystems). The relative transcript expression levels were normalized to Gapdh expression (endogenous control).

**Cell proliferation assays**

BMMSC proliferation was evaluated using 7-AAD and BrdU labeling (BD), following the manufacturer's protocol. Briefly, 1 x $10^5$ cells were seeded in 24-well plates or onto scaffolds (according to the experimental groups) and incubated at $37^0$Cfor 24 h. Then, BrdU (10 mM) was added to the medium for 45min. The cells were harvested with 0.04mg/mL of liberase (Roche) for 10min and analyzed by flow cytometry.

**Histology**

After μCT image acquisition and 3D reconstruction, the calvaria samples were dissected and the soft tissue was removed. The dissected calvariae were descalcified with Decalcifier II (Leica Biosystems) for 2-3 days. Samples were placed in a tissue processing cassette and then dehydrated, embedded in paraffin and sectioned. The slides with the 5 mm sections were deparaffinized, rehydrated and stained.

**Hematoxylin and Eosin staining**

Deparaffinization

- 30 minutes in dry oven at 60ºC
- Immerse the slides in xylene 2 minutes. 2X

Rehydration

- Ethanol 100% 2 min
- Ethanol 96% 2 min
- Ethanol 80% 2 min
- Ethanol 70% 2 min
- Destilled water 2 min

Staining

- Stain in hematoxylin 3 min (check the samples)
- Wash with running tap water (use a glass staining dish)
- Acid alcohol for 15 seconds
- Wash with running tap water
- Ammonia water 30 seconds
- Wash with running tap water
- Ethanol 70% seconds
- Ethanol 80% seconds
- Counterstain eosin 1.5 min
- Wash using distilled water
- Ethanol 96% seconds 2X
- Ethanol 100% seconds
- Ethanol 100% 3 min
- Ethanol-Xylene 50% 2 min
- Xylene 3 min 3X
- Mount using DPX leave to dry overnight

**Masson´s Trichrome**

-from deparaffinization and rehydration

- Stain in Weigert`s hematoxylin 30 seconds
- Wash with running tap water
- Differentiate solution A seconds
- Wash using distilled water 3 mins
- Ponceau solution 30 seconds
- Differentiate solution B seconds
- Wash using distilled water seconds
- Ethanol 96% seconds 2X
- Ethanol 100% 3 min

| | | |
|---|---|---|
| -Ethanol-Xylene 50% | 2 min | |
| - Xylene | | 3mins 3X |
| - Mount with DPX | | leave to dry overnight |

**Immunohistochemistry**

For immunohistochemistry, the tissue samples were boiled in citrate buffer, washed and blocked with serum. The primary antibodies against Osterix (OSX ab22522 Abcam) or GFP (ab290 Abcam) were incubated at 1:200 dilution on the sections overnight. After washing, the samples were incubated with a biotinylated secondary antibody (1:100) and streptavidin –horseradish peroxidase (1:400) for 1 hour. The sections were incubated with diaminobenzidine and $H_2O_2$and counterstained with hematoxylin.

**Statistical analyses**

The statistical analyses were performed using Student'st-test with the GraphPad Prism 5 software. The quantitative data are presented as the mean – standard error of the mean. The differences were considered significant at p-values of <0.05, with *p < 0.05, **p < 0.01, and ***p < 0.001.

# 4 CHAPTER IV: RESULTS

**AIM 1:**

**GELATIN/$CaSO_4$ SCAFFOLD AS THREE-DIMENSIONAL (3D) CELL CULTURE SYSTEM**

### *4.1.1 Extracellular calcium increases osteogenic gene expression in BM-MSCs cultured in 3D gelatin scaffolds*

In order to evaluate the influence of the culture system on the osteoblast differentiation of BM-MSCs, we compared three different culture models: untreated plastic surface, 2D gelatin-coated surface and 3D gelatin scaffold. Cells seeded on 3D gelatin scaffolds showed greater upregulation of all osteogenic markers evaluated (Alpl $p< 0.001$; Osteocalcin $p< 0.001$ and Osterix $p< 0.001$) than monolayers on plastic surfaces or 2D gelatin coated plates. Figure 4.1.1

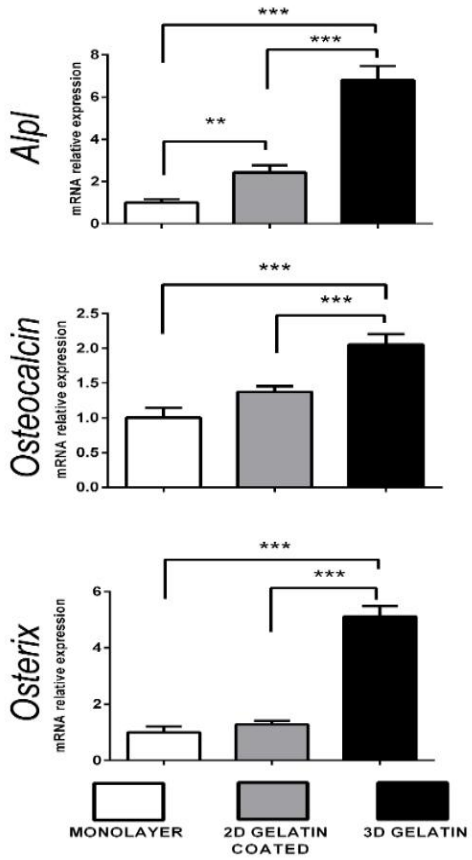



Figure 4.1.1 Three dimensional Gelatin/$CaSO_4$ scaffolds increase osteoblast gene expression.

This result suggests that 3D gelatin scaffolds promote higher osteoblast differentiation than plastic or 2D gelatin-coated surfaces. We then assessed whether extracellular $Ca^{2+}$ could have a beneficial effect on osteoblast differentiation. We evaluated the effects of different $Ca^{2+}$ concentrations on BM-MSCs using the three culture systems described above. Higher expressions of Alpl, Osteocalcin and Osterix were obtained using $Ca^{2+}$ concentrations from 3mM to 10mM. Figure 4.1.2

A concentration of 7.5mM was optimal for the late osteogenic differentiation markers Osteocalcin and Osterix. Taken together, these results suggest that extracellular $Ca^{2+}$ concentrations of between 3mM and 10mM produce a beneficial effect on the expression of Osteocalcin and Osterix, regardless of the culture model used. We confirmed the specificity of these effects by comparing $CaSO_4$ and $CaCl_2$ as calcium ion sources or by chelating $Ca^{2+}$ with EDTA. Both $CaSO_4$ and $CaCl_2$ stimulated the expression of osteogenic markers. Moreover, the addition of EDTA completely blocked the positive effects of $CaSO_4$ and $CaCl_2$ on gene expression. Figure 4.1.3

### *4.1.2 A composite gelatin/$CaSO_4$ scaffold increases osteogenic gene expression in vitro.*

In addition to growth factors, another challenge for bone regeneration is to find the optimal matrix for BMMSC transplantation and their osteogenic differentiation. We evaluated the influence of gelatin and biphasic $CaSO_4$ alone or in combination on BMMSC morphology, proliferation and osteogenic marker expression in vitro. Since the BMMSCs used in this study were obtained from transgenic GFP-expressing BALB/c mice, we could easily visualize these cells in the scaffolds.

Images revealed morphological differences between the evaluated seeding conditions, taking a monolayer plastic surface as control. Cells seeded on the control monolayer display a flattened morphology without intracellular vesicles whereas those adhered to $CaSO_4$ crystals in a 2D plastic surface revealed an increased number and size of intracellular vesicles. BMMSCs

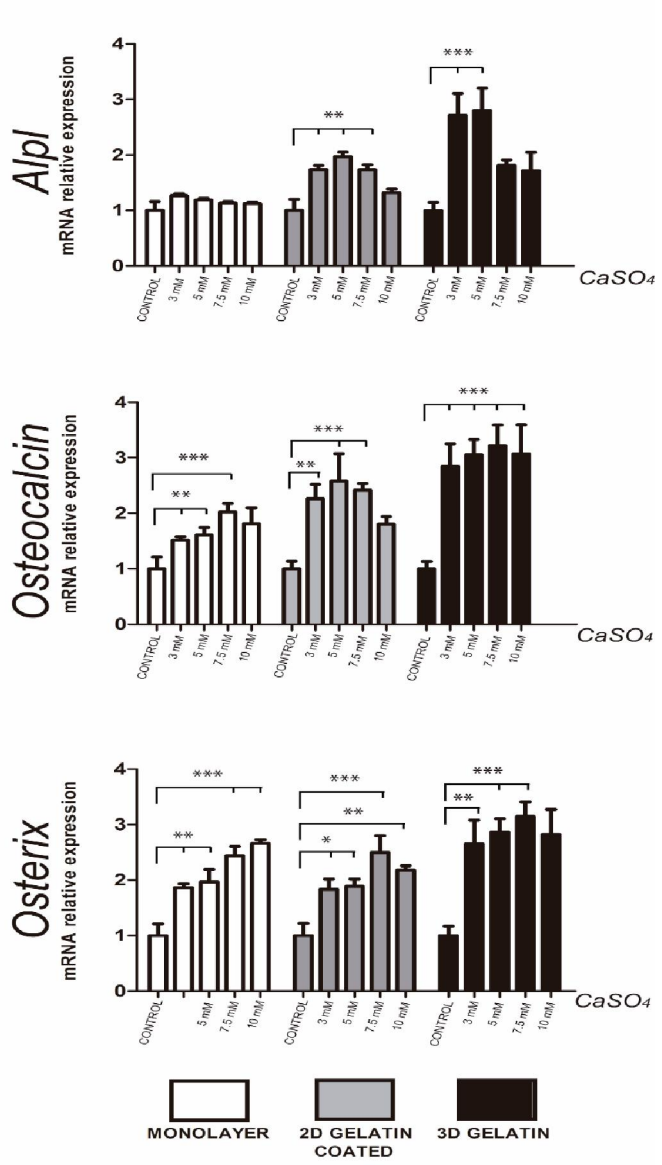



Figure 4.1.2 Extracellular $Ca^{2+}$induce osteoblast gene expression.

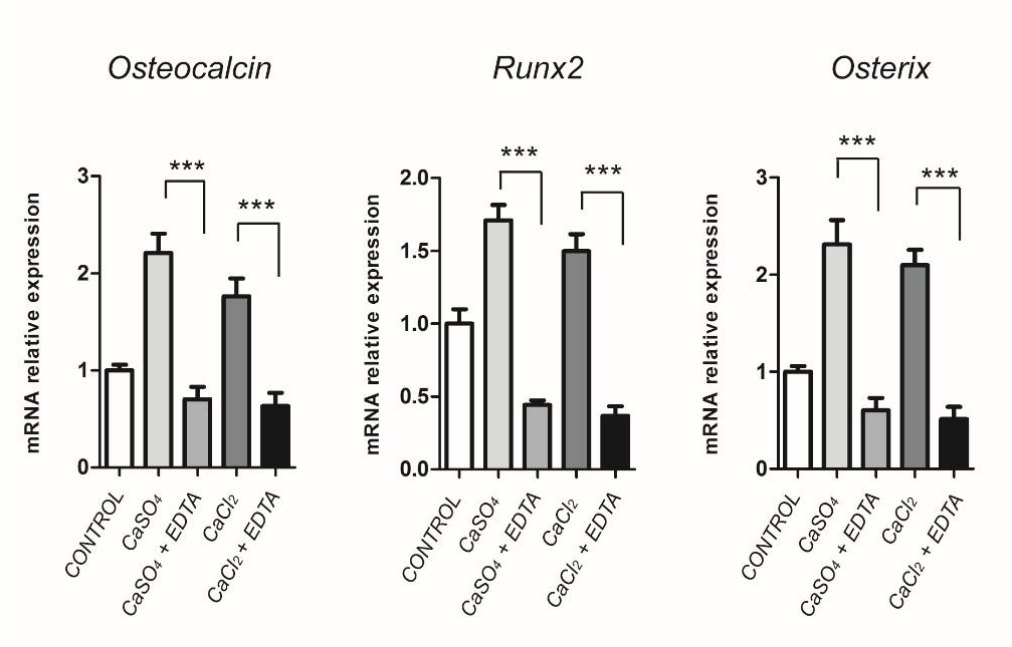



Figure 4.1.3 EDTA inhibits the osteogenic effect of extracellular $Ca^{2+}$.

grown on gelatin substrata were connected to each other by cytoplasmic prolongations in a marked reticular pattern according to the three dimensional structure of the sponge. Figure 4.1.4

Next, we examined the ability of these scaffolds to maintain the undifferentiated status of BMMSCs. Quantification of the gene expression of stemness markers *Oct4* and *Nanog* (Tsai, Su et al. 2012, Han, Han et al. 2014), demonstrate that gelatin sponge did not modify expression of *Nanog* or *Oct4* after 24 hours of culture. Interestingly, after long term culture on the scaffolds (10 days), expression of *Oct4* and *Nanog* significantly decreased when BMMSCs were cultured on the gelatin plus $CaSO_4$ scaffold. Addition of the osteogenic cytokine BMP-2 induced a slight decrease of stemness in most of the conditions analyzed. A parallel analysis of osteoblast-determining transcription factors showed no major differences in the expression of *Runx2* in either short or long term cultures.

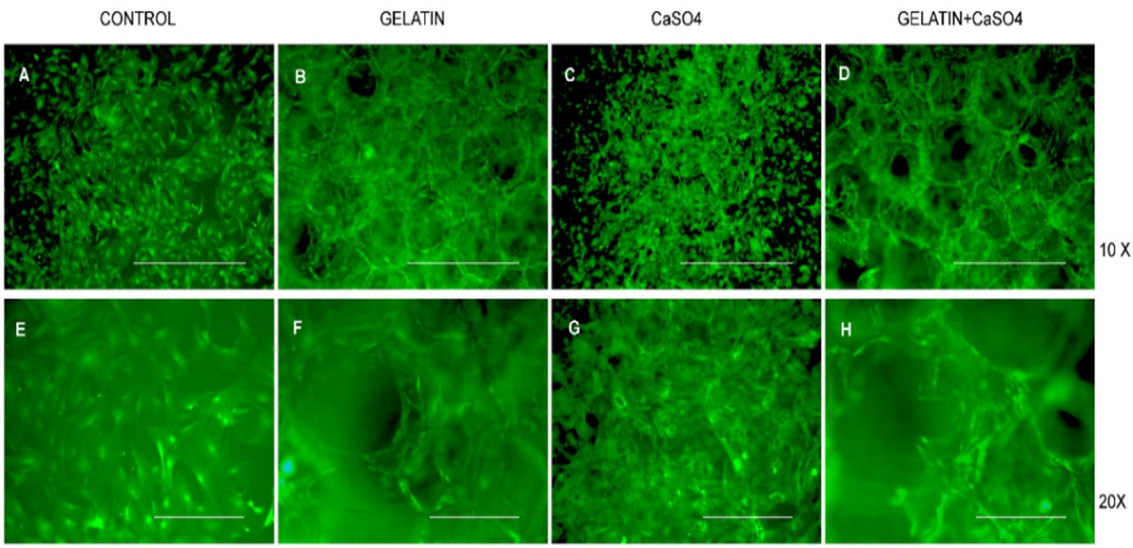



Figure 4.1.4 Green fluorescent protein (GFP) expressing MSCs cultured in different substrates for 24 hours.

Noteworthy, when cells were cultured in the gelatin plus $CaSO_4$ scaffolds, expression of *Osx* was significantly increased at both 24 hours and 10 days of culture. Figure 4.1.5. Addition of BMP-2 also induced increased expression of *Osx* after 10 days irrespective of the substrata employed.

We further analyzed proliferation of BMMSCs cultured in the different biomaterials for 24 hours by flow cytometry by labeling DNA with 7-AAD and BrdU incorporation. The results are in agreement with expression of the stemness markers, showing higher proliferative rate in gelatin or gelatin plus $CaSO_4$ scaffolds as measured by BrdU incorporation or percentage of cells in S or G2/M phases of the cell cycle. Figure 4.1.6. Altogether, these results suggest that this composite scaffold allows an early expansion of the BMMSCs at short times, which is followed by increased osteogenic differentiation after long-term culture.

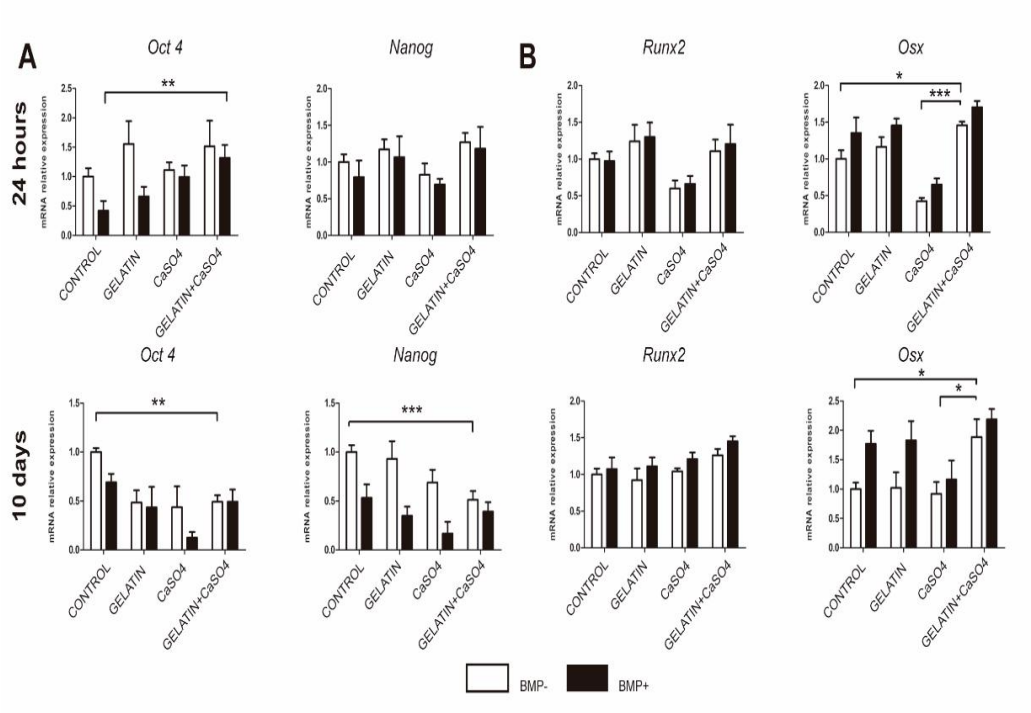



Figure 4.1.5 Effect of the gelatin/$CaSO_4$ scaffold on stemness and osteogenic marker expression in BMMSCs. Data presented as the mean of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

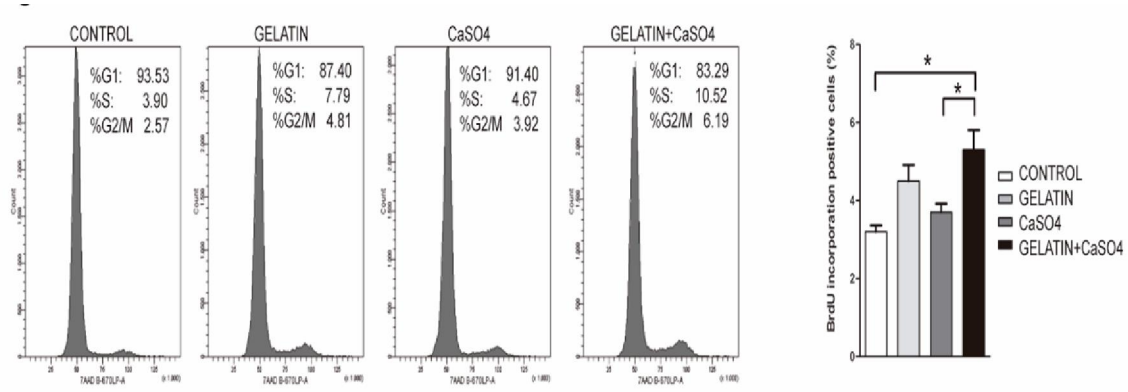



Figure 4.1.6 FACS analysis of 7-AAD labeling (left) and BrdU incorporation (right) of BMMSCs cultured in the indicated scaffold. Data presented as the mean of three independent experiments. *p < 0.05, **p < 0.01,***p < 0.001.

### *4.1.3 Gelatin/$CaSO_4$ scaffold improves bone formation in vivo.*

In order to verify these observations in bone regeneration we analyzed their ability to heal a criitical-size calvarial bone defect. We implanted the composite scaffold in the absence and presence of cells in a 5mm size calvarial defect (Cooper, Mooney et al. 2010). Defects of this diameter are unable to heal by themselves (Cooper, Mooney et al. 2010, Gomes and Fernandes 2011). Furthermore, we used a minimal irrigation during the surgical procedure for a more challenging cranial defect (Sawyer, Song et al. 2009). This was also proven in our model by the lack of bone formation in empty defects. Instead the defects were partially filled with a layer of fibrous tissue. After 5 weeks of scaffold implantation, bone formation was analyzed by μCT to evaluate new bone formation. Figure 4.1.7.

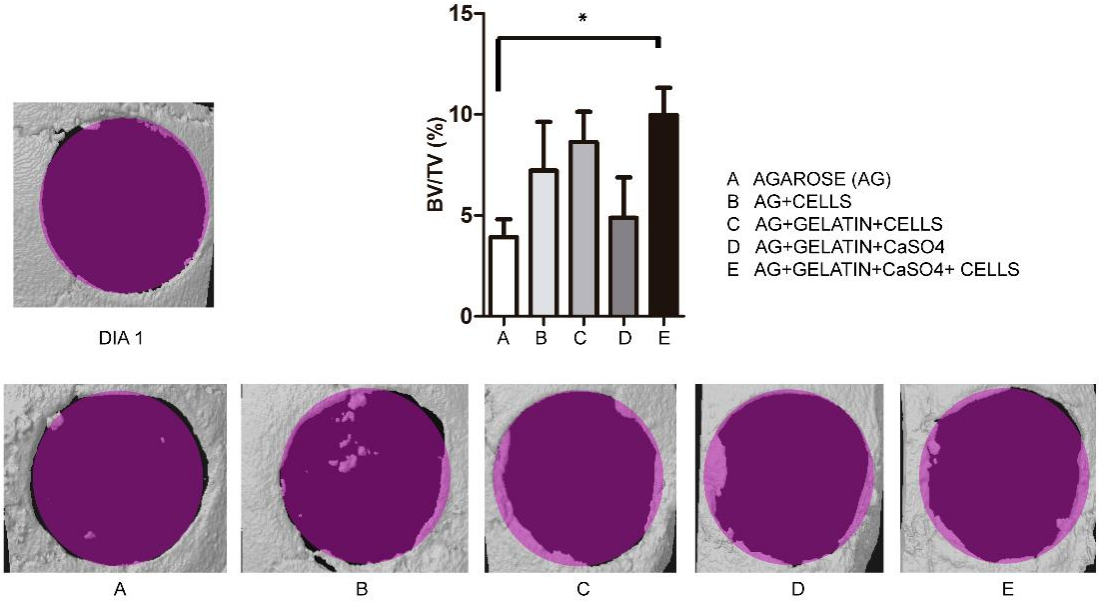



Figure 4.1.7 Microcomputed tomography quantification of bone regeneration of the gelatin/$CaSO_4$ scaffold implants.*$p < 0.05$.

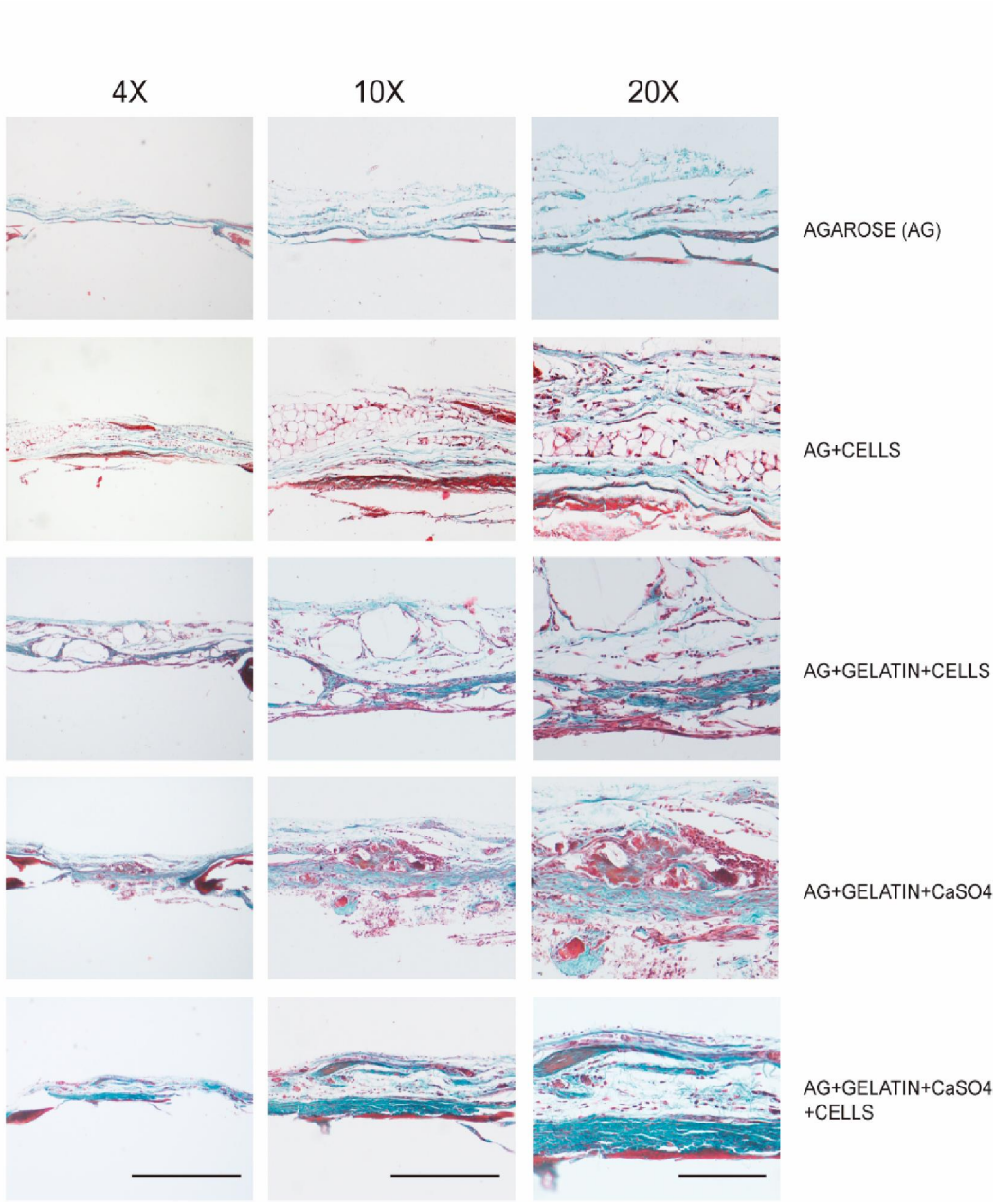



Figure 4.1.8 Masson's trichrome staining of representative calvarial sections of each group. 4X scale bar, 1000 μm. 10X scale bar,400 μm. 20X scale bar,200 μm.

The analysis of reconstructed images demonstrated that the gelatin/$CaSO_4$ scaffold seeded with BMMSCs had the greatest bone regeneration potential, as seen in the margins of the calvarial defects when compared to the other conditions assessed. Furthermore, these data shows that bone formation arises from the function of the implanted exogenous cells, since implantation of the scaffold by itself only promoted marginal effects on bone formation.

Histological analysis confirmed the findings obtained by μCT. Very limited bone formation was observed in control defects implanted only with agarose. Of note, when only the scaffold (gelatin plus $CaSO_4$) without cells was implanted, abundant endogenous cellular invasion was observed. However and more important, when BMMSCs in the composite scaffold were implanted, significant bone healing was obtained accompanied by a more mature structure. These data indicate that BMMSCs in combination with a composite gelatin/$CaSO_4$ scaffold are able to partially recover a defect that otherwise would not heal in these adult mice (Gomes and Fernandes 2011). Figure 4.1.8.

**AIM 2:**

**BMP-2 AND Wnt3A INCREASE OSTEOBLAST DIFFERENTIATION AND BONE FORMATION USING A GELATIN/$CaSO_4$ SCAFFOLD**

### *4.2.1 Wnt3a cooperates with BMP-2 increasing the expression of osteoblastic markers.*

Among the extra-cellular signals involved in the induction of the osteoblast phenotype, BMP and Wnt families of morphogens are essential for the commitment and differentiation of the osteoblast lineage (Tsumaki and Yoshikawa 2005, Hoeppner, Secreto et al. 2009, Regard, Zhong et al. 2012). Moreover, our group has shown that both signalling pathways have cooperative effects on the induction of osteogenesis (Rodriguez-Carballo, Ulsamer et al. 2011). We thus hypothesized that combination of the two osteogenic signals could lead to improved bone regeneration.

We cultured BMMSCs in the composite scaffold for 24 hours and then for further 24 hours or 10 days in the presence of BMP-2 and/or Wnt3a. We first assessed the expression of the stemness markers *Oct4* and *Nanog*. *Oct4* and *Nanog* expression were strongly activated by Wnt3a addition after 24 hours, whereas BMP-2 had no major effects. However, after 10 days, *Nanog* expression was strongly down-regulated when both BMP-2 and Wnt3a were added together.

In order to further assess the cooperative effect between Wnt3a and BMP-2, we also evaluated the expression of *Col1a1*, *Runx2* and *Osx*. Wnt3a alone or in combination with BMP-2 induced the expression of *Col1a1* at 24 hours. Figure 4.2.2. Moreover, stimulation with both cytokines conferred a strong and significant additive effect on the expression of *Runx2* and *Osx* after 24 hours. Interestingly synergic effects were maintained after 10 days of culture for *Col1a1* and *Osx* mRNA expression. Cell cycle and BrdU analysis of their proliferation rate of cells treated with these cytokines for 24 hours were also in agreement with expression of the stemness markers. Addition of Wnt3a increased growth rate in parallel to *Oct4* and *Nanog* expression, whereas BMP-

2 did not had major influences in proliferation or stemness marker expression. Figure 4.2.1. These results support the concept of collaboration between BMP and Wnt3a signalling in enhancing osteogenesis of BMMSCs in the cultured scaffold *ex-vivo*.

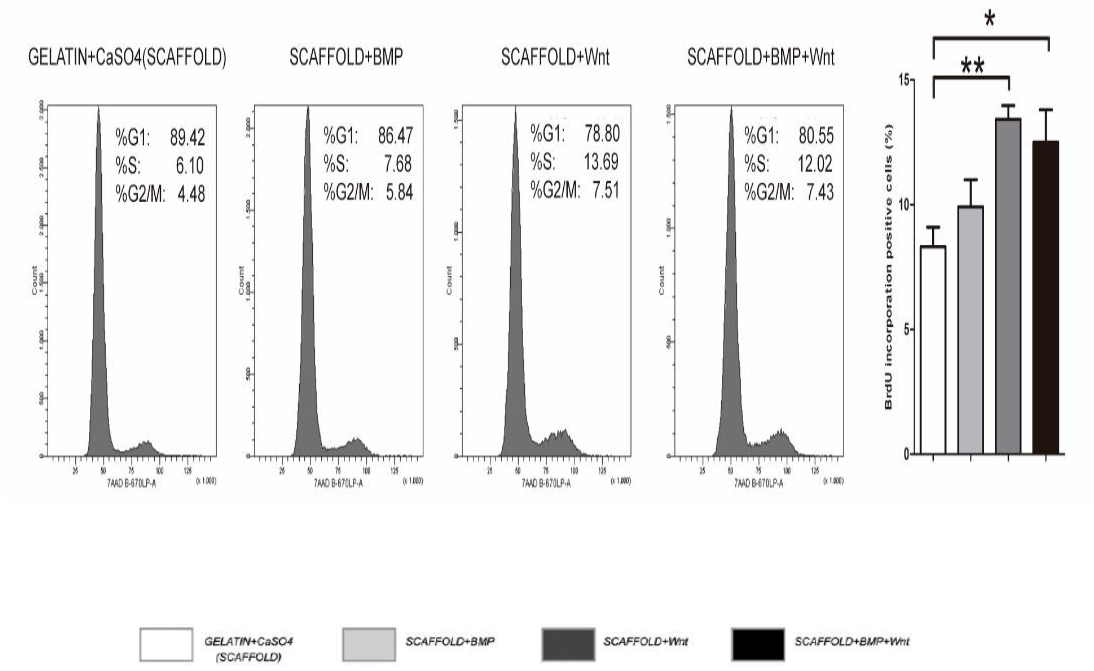



Figure 4.2.1 FACS analysis of 7-AAD labeling (left) and BrdU incorporation (right) of the BMMSCs cultured with the indicated cytokines for 24 h. Data presented as the mean ±standard error of the mean (SEM) of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

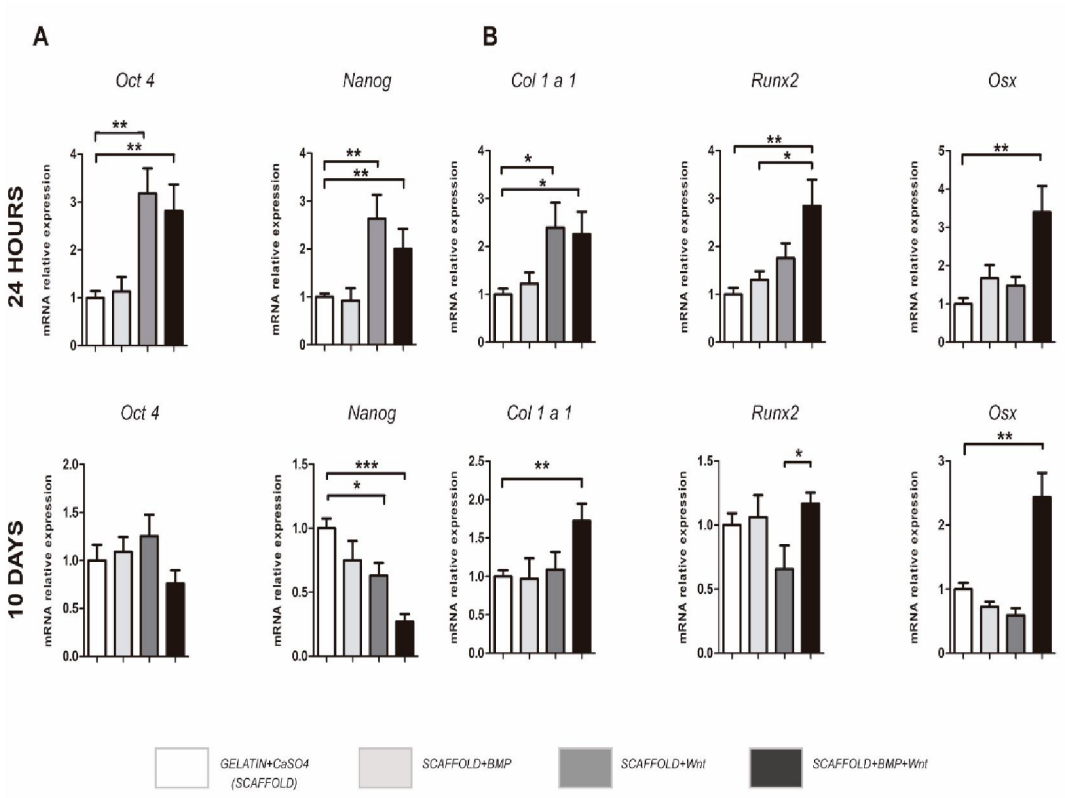



Figure 4.2.2 BMP-2 and Wnt3a addition increases osteoblast marker expression. (A) Expression levels of Oct4 and Nanog assessed at 24 h or 10 days of culture while being treated with the indicated cytokines. (B) Expression levels of Col1a1, Runx2, and Osx assessed at 24 h or 10 days of culture while being treated with the indicated cytokines. The mRNAs were normalized to Gapdh and four independent experiments were performed.

### *4.2.2 Wnt3a and BMP-2 cooperatively enhance bone regeneration in vivo.*

To assess whether combination of Wnt3a and BMP-2 increases bone regeneration in vivo, we implanted a gelatin/$CaSO_4$ scaffold seeded with BMMSCs pre-treated ex-vivo with BMP-2 and/or Wnt3a for 24 hours pre-implantation. Critical-size defects in calvarial bone were implanted with cultured

scaffolds using agarose as a gelling agent. After 5 weeks of the implantation, we evaluated the regenerated bone by a μCT scan and the outcomes presented as percentages (BV/TV) of the healed area. Representative images of the three-dimensional reconstructions are shown in Figure 4.2.3. Mineralized bone extending from the rims of the defect was significantly increased scaffolds were pre-treated with BMP-2 or BMP-2 and Wnt3a. Histological analysis revealed different characteristics in the regenerated bone. Figure 4.2.4. The volume of new bone formation was significantly greater in Wnt3a/BMP-2-treated implants. Moreover, bone structure was also more mature in Wnt3a/BMP-2 implants in comparison to the other treatment groups.

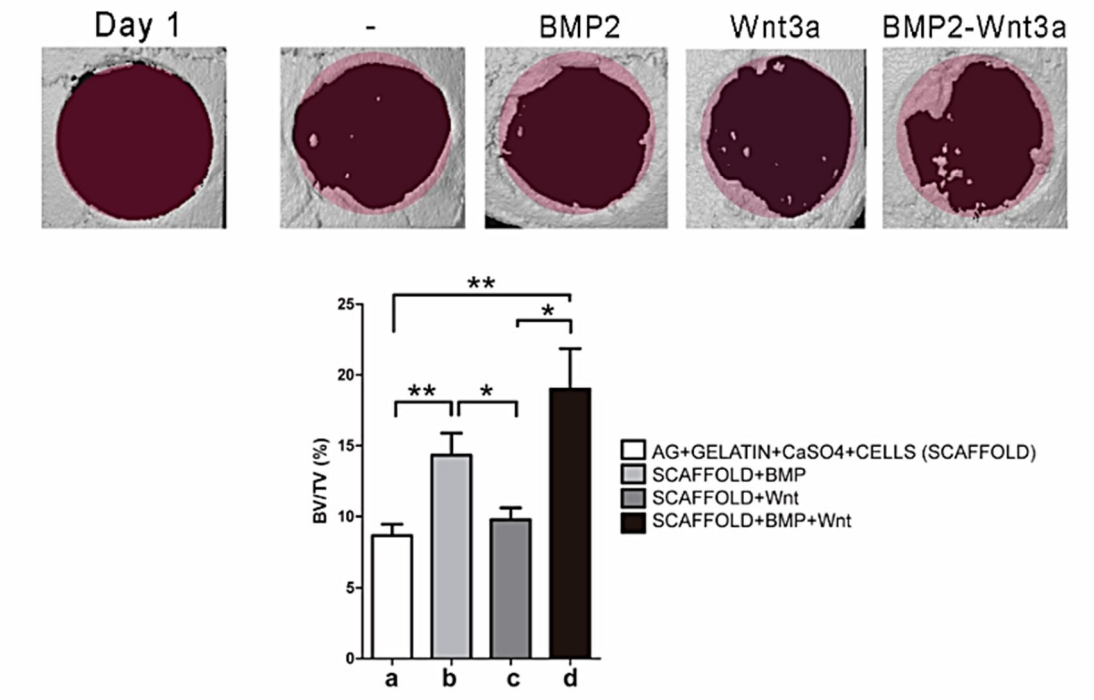



Figure 4.2.3 Representative images of reconstructions
of the calvarial defects treated with the described conditions.

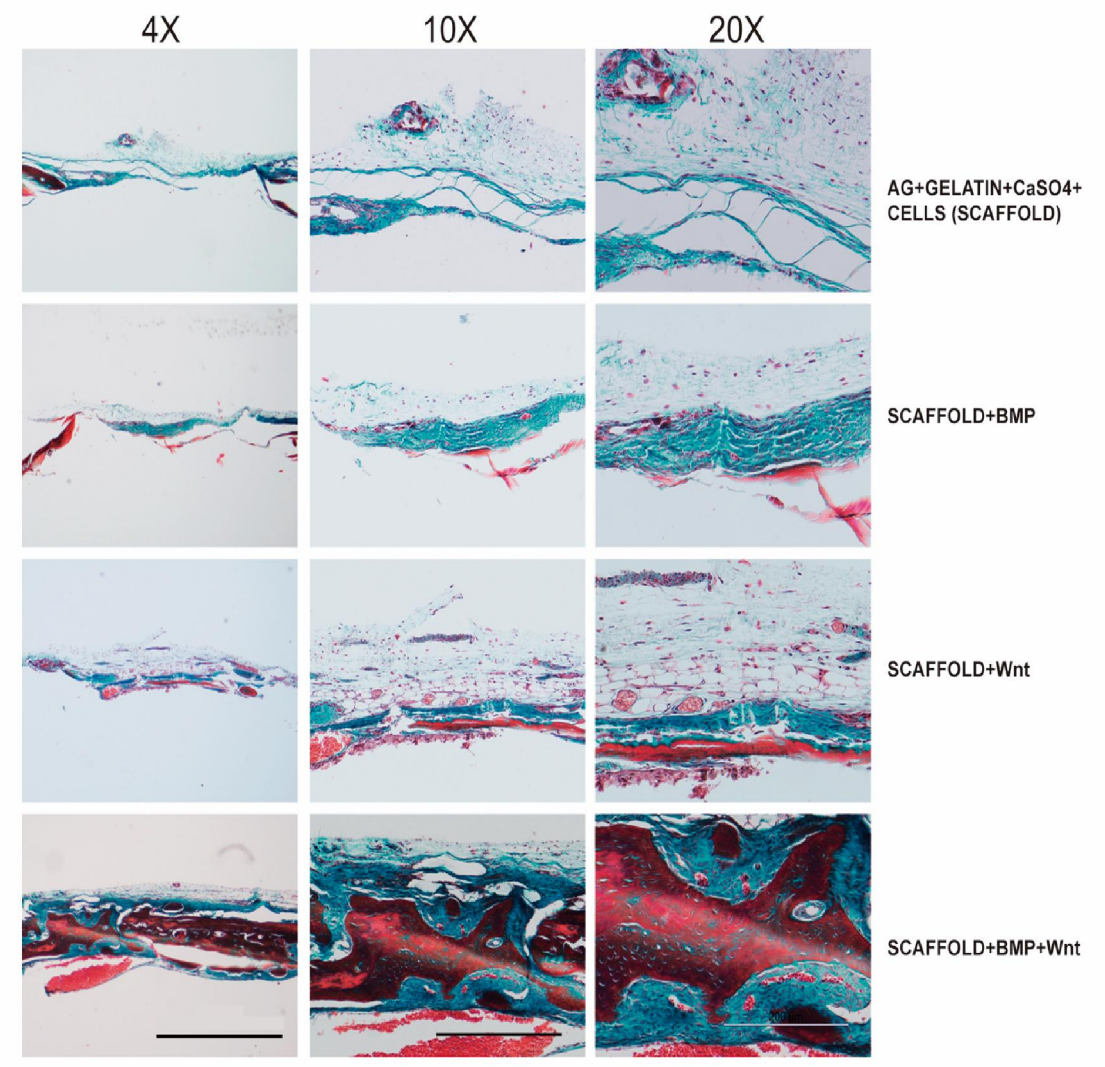



Figure 4.2.4 Masson's trichrome staining of representative calvarial sections of each group. 4X scale bar, 1000 μm. 10X scale bar, 400 μm. 20X scale bar, 200 μm.

We also wanted to confirm the relative contribution of implanted BMMSCs respective to endogenous recruitment of osteoprecursors in order to discriminate between a direct action of BMMSCs or a paracrine effect on endogenous cells. Since BMMSCs were isolated from a GFP-expressing transgenic mice strain, expression of GFP would discriminate between exogenous and endogenous cells. Thus, Wnt3a/BMP-2-treated implants were immunostained 5 weeks after implantation.

To assess the specificity of the labeling, another histological section from the same animal was used as negative control (without primary anti-GFP antibody) which resulted in total absence of signal. Figure 4.2.5. A significant number of cells, scattered through the new bone tissue, were positively stained for GFP suggesting that transplanted cells survived and partially contribute to new bone formation. However, additional paracrine effects on endogenous cells should not be discarded for their contribution to bone healing.

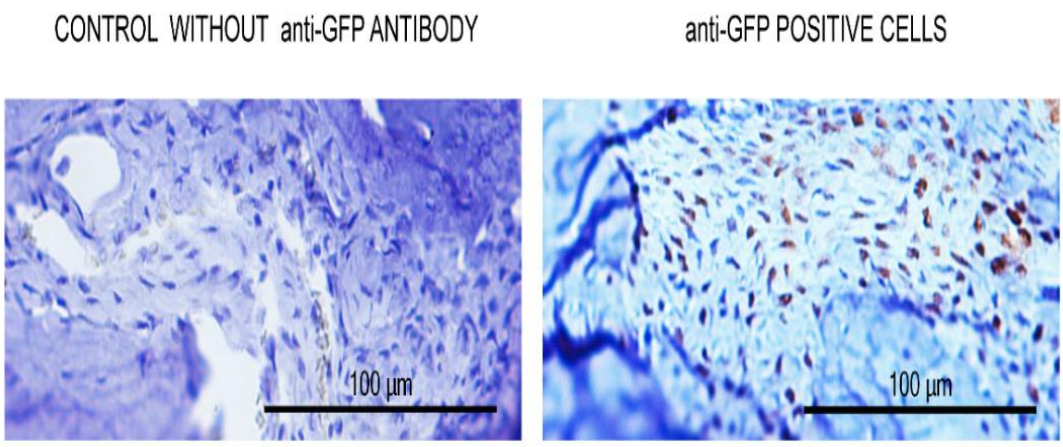



Figure 4.2.5 Transplanted BMMSCs revealed by anti-GFP immunohistochemistry 5 weeks after the implantation.

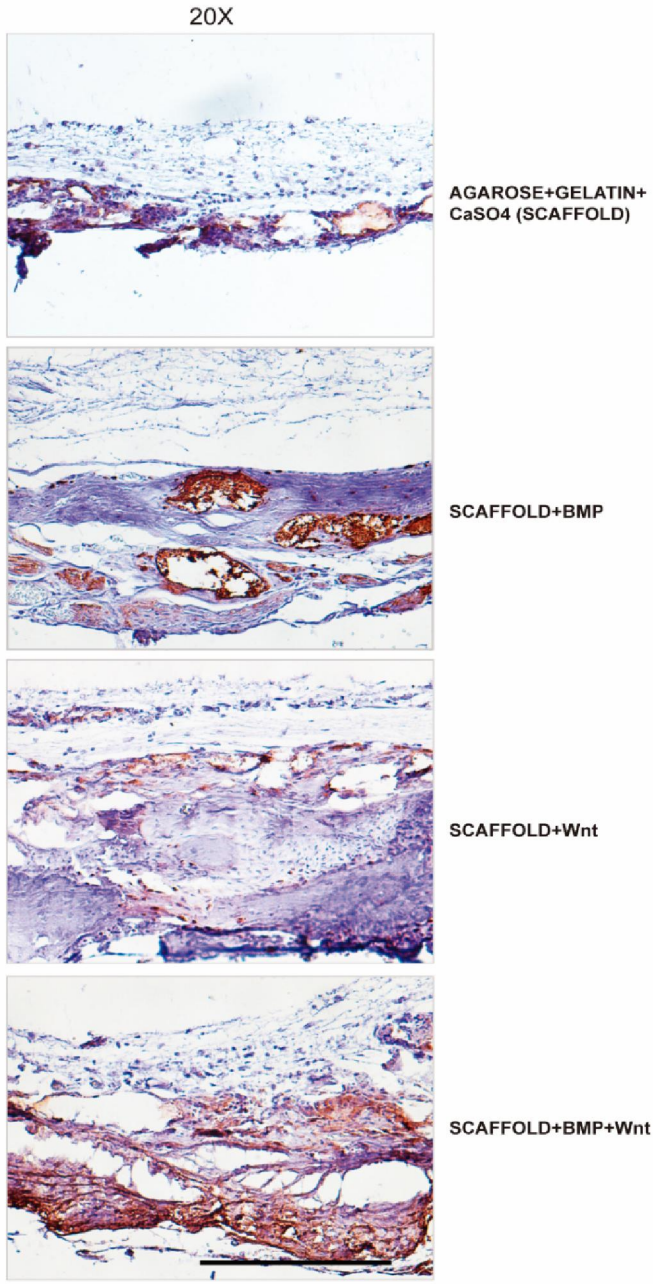



Figure 4.2.6 Osterix (OSX) immunohistochemistry identifies osteoblasts at the implanted sites. Histological sections from the calvarial defects implanted with a gelatin + $CaSO_4$ scaffold and cells pretreated with BMP-2 and/or Wnt3a.

Immunohistochemical analysis of OSX expression was also performed to assess whether cells that had survived after implantation also achieved osteogenic potential. Implants treated with Wnt3a alone displayed an increased cellularity but a sparse OSX staining which suggests an increased proliferative rate but not increased osteoblast differentiation. Figure 4.2.6.

BMP-2 treated scaffolds present higher expression of OSX that is also visible in the woven bone around the scaffold. More importantly, combination of BMP-2 and Wnt3a also led to an intense expression of OSX in a significant higher number of osteoblasts at the implant site. Altogether, these results suggest that *ex-vivo* treatment with considerably low amount of BMP-2 combined with Wnt3a cooperatively increases osteogenic potential *in vitro* and *in vivo.* This represents an improvement over current growth factor delivery strategies and highlights the importance of assessing combinations of osteogenic factors in bone tissue engineering.

**AIM 3:**

**EXTRACELLULAR CALCIUM PROMOTES OSTEOBLAST DIFFERENTIATION AND BONE FORMATION BY AMPLIFYING BMP-2 EFFECTS**

### *4.3.1 Cooperation of calcium and BMP-2 in osteoblast differentiation and bone regeneration in calvarial critical-size defects in mice.*

We further evaluated whether $Ca^{2+}$ would cooperate with osteoinductive cytokines such as BMPs. BM-MSCs were cultured in a 3D gelatin scaffold and stimulated with 7.5mM $CaSO_4$ and/or BMP-2 (2nM). Incubation for 10 days with $CaSO_4$ or BMP-2 alone promoted expression of all the bone markers analyzed. Figure 4.3.1. More importantly, although a combination of $Ca^{2+}$ and BMP-2 did not present any additive effects on the expression of the early osteogenic marker Alpl, they produced a significant additive effect on the expression of Osteocalcin, Runx2 and Osterix.

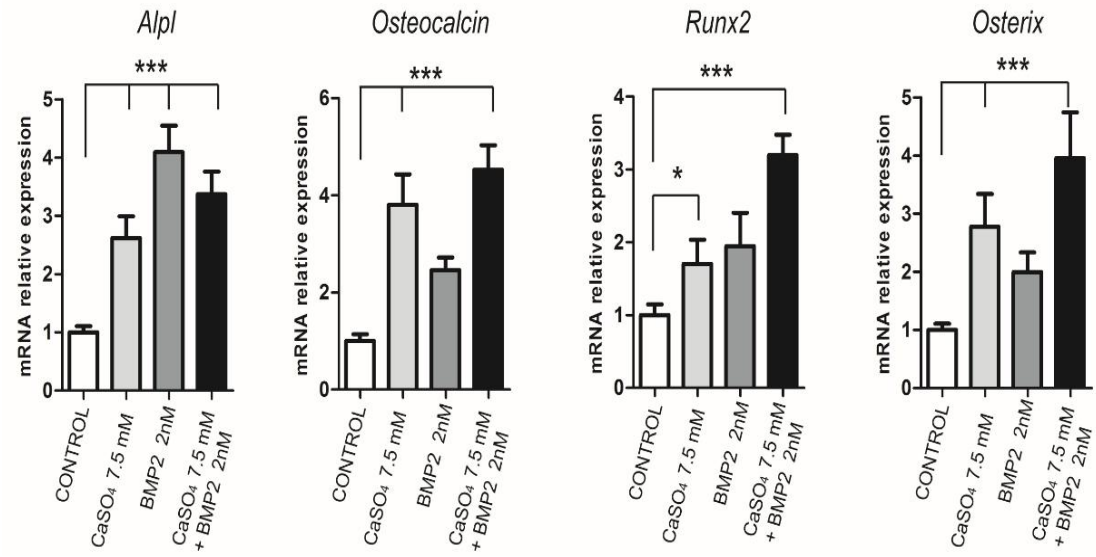



Figure 4.3.1 Extracellular calcium increases the effects of BMP-2 on osteogenic marker expression.

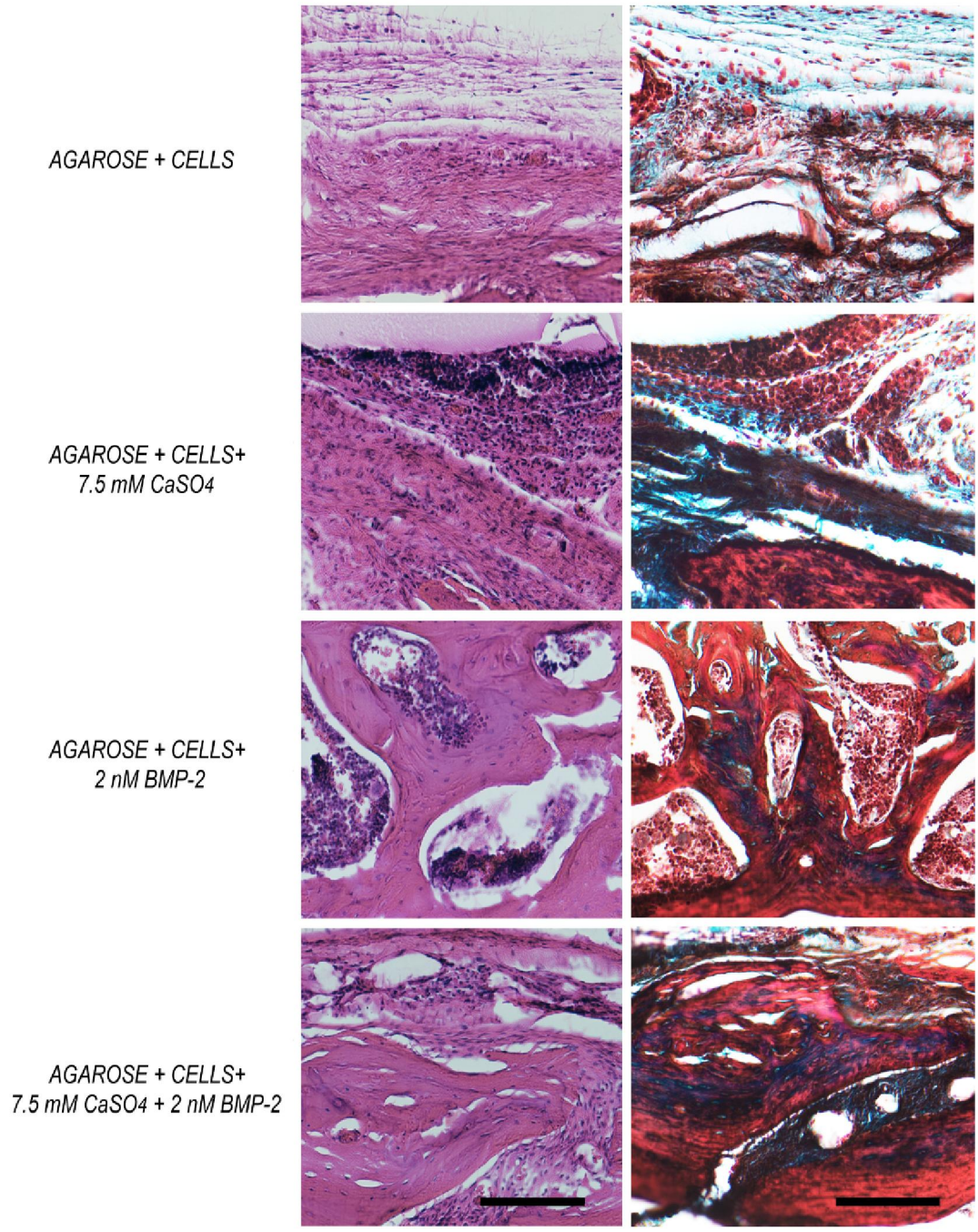



Figure 4.3.2 Extracellular calcium increases the effects of BMP-2 on bone regeneration *in vivo.*

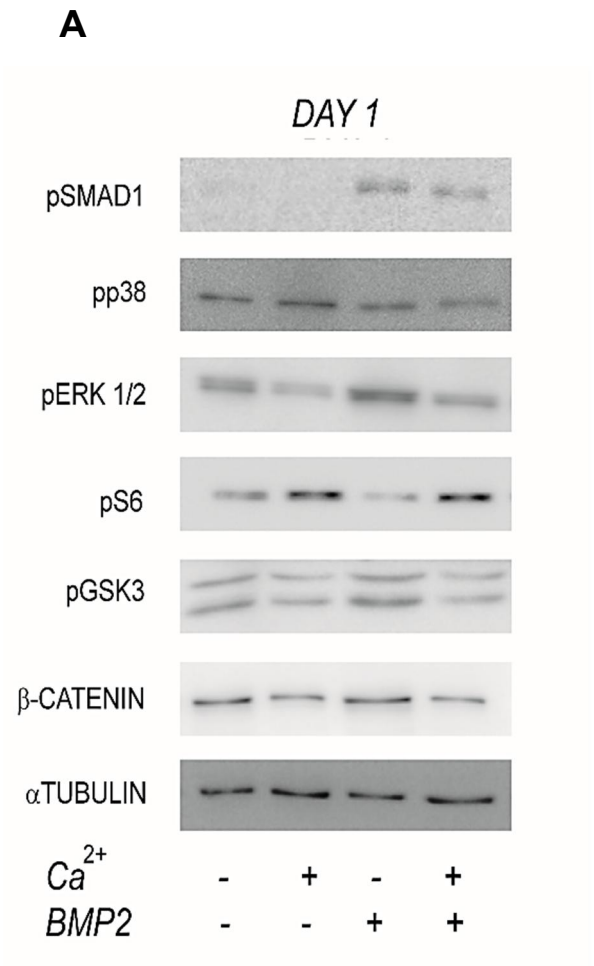
Thus, when added to culture media, $Ca^{2+}$ exerts a cooperative action with BMP-2 on late osteogenic marker expression. To extend our in vitro results on the cooperation between $Ca^{2+}$ and BMPs to an in vivo context, we analyzed bone formation in calvarial critical-size bone defects in mice. Five-millimeter defects were performed in parietal bones and further implanted with BM-MSCs previously seeded in 3D gelatin scaffolds and pre-treated for 48 hours with either 7.5mM $CaSO_4$ or 2nM BMP-2 alone or combined. After five weeks, skulls were retrieved and analyzed for bone formation in the defect. Hematoxylin/eosin and Masson's trichrome stains showed dense connective tissue but no major bone formation in the control group.

Higher levels of mineralization and bone maturation were found in those implants treated with $CaSO_4$, and even greater bone formation took place when implants were pre-treated in combination with BMP-2. Figure 4.3.2. Moreover, a combination of $CaSO_4$ and BMP-2 led to a new, more mature bone structure. Both osteoblast and osteocytes can be observed in these bone regeneration areas.

### *4.3.2 Signalling pathways involved in the cooperation of calcium and BMP-2 during osteogenesis of BM-MSCs*

To determine the mechanisms of cooperation between extracellular calcium and BMP-2 in BM-MSCs differentiation, we analyzed intracellular signalling triggered by both signalling molecules at early and late differentiation points. Early analysis was performed 24 hours after $CaSO_4$ and/or BMP-2 stimulation. As expected, BMP-2 promoted phosphorylation of SMAD1/5 and increased the levels of phosphorylated ERK1/2. Figure 4.3.3. By contrast, p38

and S6-kinase (S6K) signalling pathways were activated when cells were treated with $Ca^{2+}$ alone. It is worth noting that, at this initial differentiation stage, an antagonistic effect on each signalling pathways was obtained when $Ca^{2+}$ was added together with BMP-2.



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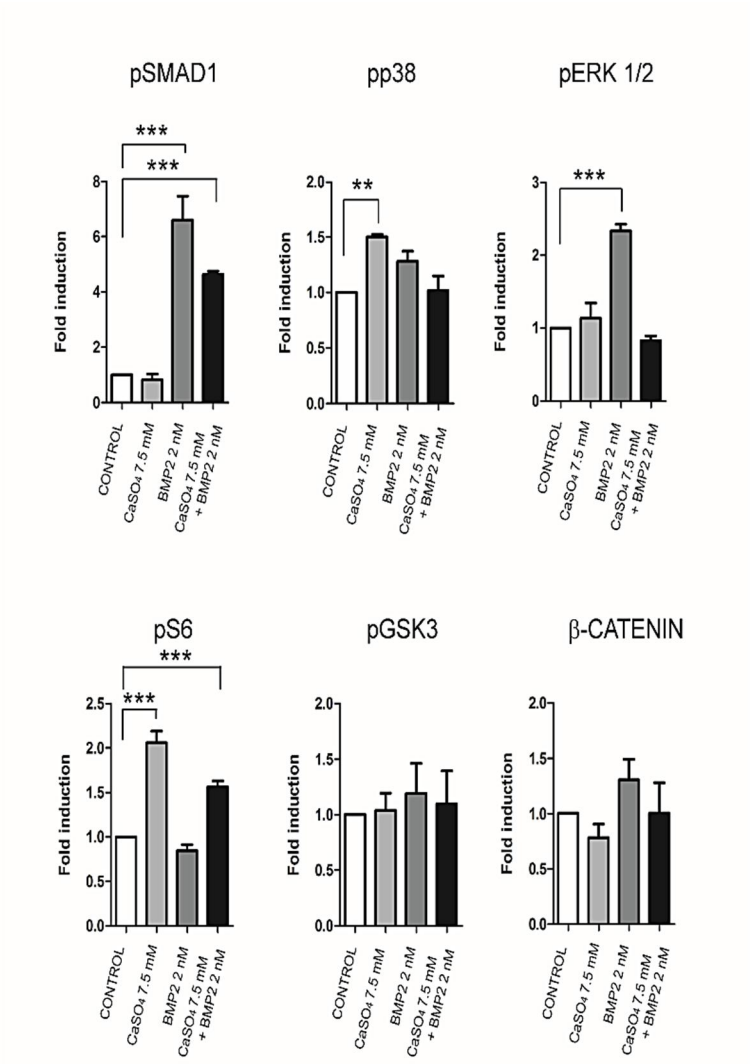
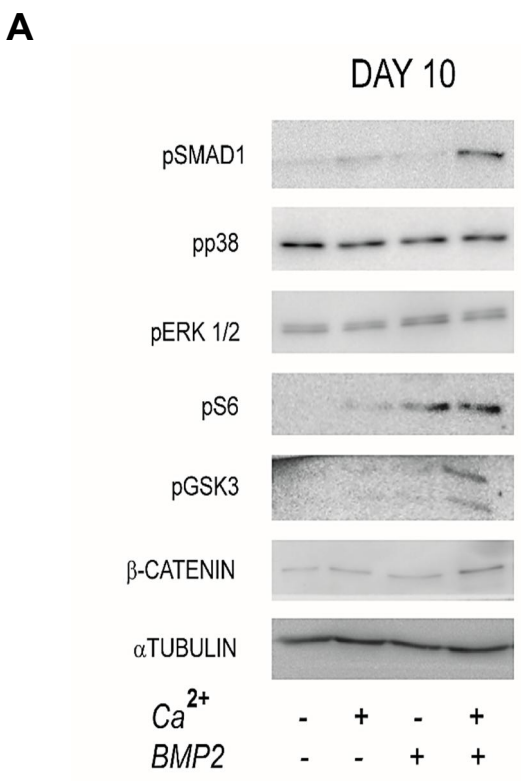



Figure 4.3.3 Early effects of extracellular calcium on cell signalling. **A**.The influence of extracellular calcium and/or BMP-2 on major signalling pathways was evaluated. Cells were cultured in 3D gelatin scaffolds with $Ca^{2+}$ (7.5mM) and/or BMP-2 (2nM) for 24 hours and extracts analyzed by Western blot. **B**. Data was quantified from three independent experiments.

The same intracellular components were subsequently assessed after treatment with a combination of $CaSO_4$ and/or BMP-2 for 10 days. A significant additive or cooperative effect between $Ca^{2+}$ and BMP-2 was observed on the phosphorylation levels of SMAD1/5 (Ser463-465), S6 (Ser235-236), GSK3 β (Ser9) and the total levels of β-CATENIN. Figure 4.3.4. Phosphorylation at Ser9 of GSK3 β is mediated by AKT and results in the inhibition of its β-CATENIN repression action.

Thus, these results suggest that extracellular calcium produces a differential time-dependent effect on BMP-2 and AKT signalling. A signalling network antagonistic to BMP-2 is activated early on, whereas $Ca^{2+}$ promotes a cooperative effect on several intracellular signalling events later on.



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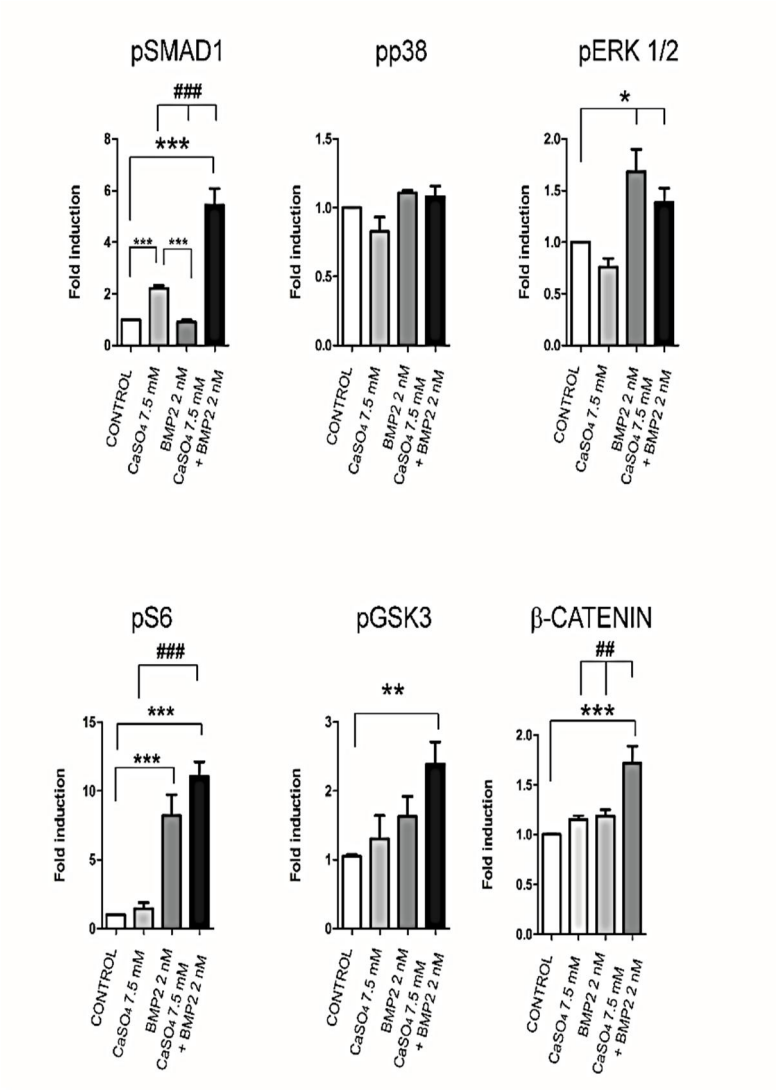



Figure 4.3.4 Late effects of extracellular calcium on cell signalling. **A**.The influence of extracellular calcium and/or BMP-2 on major signalling pathways at a later time point,10 days. **B**.Data from three independent experiments were quantified relative to the levels of α-TUBULIN. Quantifications are shown as means ± SEM. Differences were considered significant at p values: * $p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$ when compared to control and # $p < 0.05$, ## $p < 0.01$, and ###$p < 0.001$ when compared to cells treated with BMP-2 and $CaSO_4$.

### *4.3.3 Extracellular calcium promotes endogenous secreted BMP-2 and BMP-4 mRNA expression*

After 10 days of differentiation, BM-MSCs stimulated with calcium alone showed activation of the SMAD1/5 pathway. Figure 4.3.4. Since there is no evidence that calcium activates BMP receptors directly, it could be suggested that the increased availability of BMP receptor ligands was responsible. We therefore hypothesized that, once these cells are committed to the osteoblast lineage, $Ca^{2+}$ induces cells to secrete endogenous factors that reinforce differentiation through an autocrine/paracrine mechanism.

We assayed whether $Ca^{2+}$ induced BMP-2 or BMP-4 expression. BMMSCs cultured in 3D gelatin scaffolds were exposed to $CaSO_4$ concentrations (from 3mM to 10mM) for 10 days. An increase, which reached its maximum at 7.5mM, was obtained for both Bmp2 and Bmp4 mRNA expression. Figure 4.3.5. In addition, we also determined the mRNA levels of Fgf21 (a tyrosine kinase receptor ligand) that inhibits osteoblastogenesis (Wei, Dutchak et al., 2012) and Axin2 (a target of the Wnt/β-CATENIN pathway downstream of GSK3). A significant increase in Axin2 expression was found, in line with Bmp2 and Bmp4 mRNA expression. By contrast, Fgf21 mRNA expression was only slightly elevated, without any dose-response effect. Taken together, these results demonstrate that BM-MSCs stimulated with $Ca^{2+}$ secrete higher levels of multiple critical cytokines that amplify their osteoblastic differentiation response.

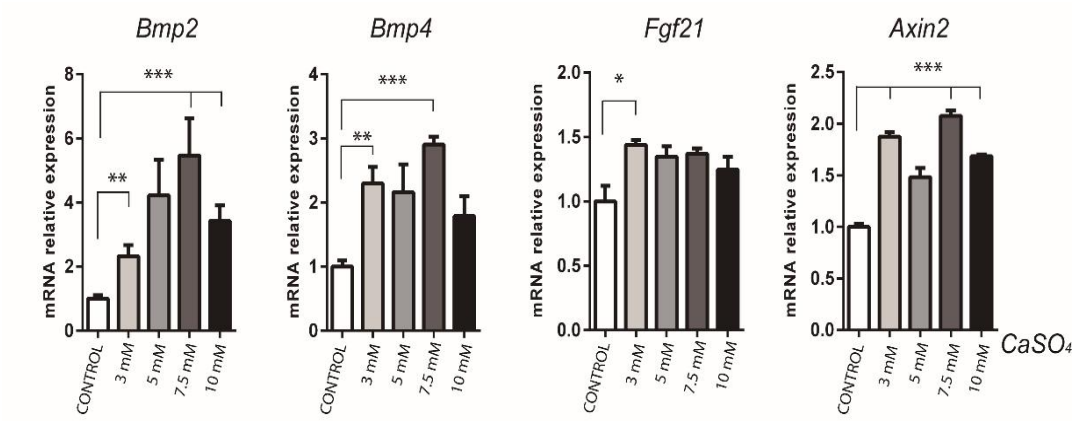



Figure 4.3.5 Extracellular calcium induces *Bmp2*, *Bmp4*, *Fgf21* and *Axin2* mRNA expression.

# 5. CHAPTER V: DISCUSSION

The clinical use of supra-physiological amounts of BMP-2 to induce bone formation can produce several side effects. In order to propose an alternative and to avoid these drawbacks, we considered that BMP-2 and Wnt3a, acting cooperatively, could reduce the required doses and produce similar therapeutic effects. In our study, an optimized gelatin/$CaSO_4$ scaffold was used to seed and expand BMMSCs that were pretreated ex-vivo with low doses of BMP-2 (2nM) and Wnt3a (50ng/ml). Based on osteogenic gene expression, μCT analysis, histological and immunohistochemistry data, we demonstrate that pretreatment of BMMSCs with a combination of BMP-2 and Wnt3a results in greater bone regeneration *in vivo* and increased osteogenic gene expression *in vitro*.

Efficient bone tissue engineering requires three components: biocompatible scaffolding materials, osteoblast progenitors and potent osteogenic cytokines. First, we directed our efforts to identify an optimal scaffold for BMMSC engraftment, expansion and further osteoblast specification. We took advantage of a composite scaffold made by a gelatin sponge with porous structure, incorporating biphasic $CaSO_4$. The Biosafety and biodegradation activity of gelatin has been demonstrated (Kohara and Tabata 2011). Moreover, biphasic $CaSO_4$ also has known osteoinductive activity with the rapid resorption time of only a few weeks (Grabowski and Cornett 2013). In turn, the released $Ca^{2+}$ ions stimulate osteoblast differentiation of the osteoprogenitors on the scaffold and are further converted into hydroxyapatite by osteogenic cells (Barrere, van Blitterswijk et al. 2006, Chai, Roberts et al. 2012). Our finding that the culture of BMMSCs in three dimensional gelatin scaffolds increase their stemness and expansion is in agreement with previous reports (Atari, Caballe-Serrano et al. 2012, Han, Zhao et al. 2012). Moreover, these effects, mediated by the gelatin substrata are improved in terms of growth rate as well as *Oct4* and *Nanog* expression by the addition of $CaSO_4$ in the composite biomaterial. Stemness maintenance during the steps of biomaterial engineering is essential for BMMSC expansion, self-renewal and ability to further differentiate into

osteoblasts later (Tsai, Su et al. 2012, Han, Han et al. 2014). A rationale exists for the addition of exogenous BM-MSC for bone regeneration. BMMSCs could contribute directly to the repair process by their differentiation into osteoblasts. We found that exogenous GFP-expressing cells survived and integrated into the healed bone area and were able to differentiate into OSX-expressing cells. Moreover, addition of cells, either in gelatin alone or in the composite scaffold, enhanced *in vivo* regeneration in higher extension than the composite scaffold alone.

Thus, it can be suggested that in our calvarial healing model exogenous transplanted cells directly contribute to new bone formation. Survival of transplanted cells is highly dependent on a correct nutrient and oxygen supply (Dupont, Sharma et al. 2010, Grayson, Bunnell et al. 2015). However, even in the case of lower survival rates for long periods in areas of low vascularization, addition of BMMSCs has proven to be beneficial. Transplanted cells support recruitment and activation of endogenous stem cells by paracrine effects and dampen the action of pro-inflammatory cytokines (Caplan and Dennis 2006, Liu, Wang et al. 2011, Gao, Usas et al. 2014). Our data show that the addition of BMMSCs also increases the recruitment of GFP-negative endogenous cells in the new bone tissue that collaborate in the healing process.

BMPs and Wnts seem to have a very relevant role in mesenchymal stem cell self-renewal and specification towards the osteogenic lineages (Chen, Zhao et al. 2004, Hoeppner, Secreto et al. 2009). During embryonic development, Wnts and BMPs are expressed, especially in the places of bone and cartilage formation. Also, the addition of high doses of BMPs constitutes an ongoing therapy in bone regeneration and bone-tissue engineering (Ripamonti 2010). In our study, we demonstrate that considerably low doses of BMP-2 (as low as 2nM) and Wnt3a (50ng/ml) display strong cooperation for osteogenic marker expression *in vitro* and bone regeneration *in vivo*.

This approach could be an alternative to the supra-physiologycal amounts of BMP-2 used clinically and at the same time an alternative to

autologous bone graft. These high doses display some off-target adverse effects and have been shown to also activate osteoclastic resorptive activity (Boerckel, Kolambkar et al. 2011, Kim, Oh et al. 2014). Moreover, our design based on *ex-vivo* pre-conditioning prior to implantation in the calvarial defect does not involve any kind of gene therapy, as reported in previous models of BMP or Wnt stimulation of BMMSCs (Shui, Zhang et al. 2014, Zhang, Wang et al. 2015). We hypothesized that pre-conditioning by BMP-2 and Wnt3a would be sufficient to trigger osteogenic responses based in several facts: (1) the systemic half-life of BMP-2 is of minutes and even when administered locally in collagen sponges, their effects soon vanished (Poynton and Lane 2002).(2) BMP2 activity is mostly required during the initial steps of fracture healing (Tsuji, Bandyopadhyay et al. 2006). (3)Wnt signalling and osteogenic capacity decline with age and the addition of exogenous Wnt3a restores osteogenic capacity (Jing, Smith et al. 2015).(4) the addition of BMP2 and Wnt3a to BMMSCs for 24 hours is sufficient to cooperatively induce the osteoblast-specific transcription factors (*Dlx3, Dlx5, Msx2, Runx2* and *Osx*) at high levels (Rodriguez-Carballo, Ulsamer et al. 2011).

Cultured scaffolds pre-treated with Wnt3a alone or in combination with BMP-2 resulted in an early increase in cell proliferation and *Oct4* and *Nanog* gene expression. However, as seen with the scaffold assay, the opposite effect in *Oct4* and *Noggin* gene expression was observed at 10 days. This apparently contradictory outcome is in agreement with the progression of BMMSC differentiation into the osteoblastic lineage. Proliferation is confined to the initial culture period. Once a sufficient population of progenitor cells has been generated, cells decelerate their proliferation and differentiate into osteoblast (Lin and Hankenson 2011). Minear *et al.* reported that Wnt3a stimulates skeletal stem cell proliferation and that the bone promoting effects of Wnt3a are achieved via this proliferative effect (Minear, Leucht et al. 2010). Moreover, this considerably low dose of BMP-2 (2nM) could also be mitogenic together with Wnt3a (Lysdahl, Baatrup et al. 2014).

This initial burst of BMP-2 and Wnt3a proliferation could increase the later osteoblast marker expression *in vitr*o and bone formation *in vivo*. The coordinated effect that we obtained in our model points to the existence of a close interrelationship between these pathways (Lin and Hankenson 2011). Canonical Wnt signalling is required for BMP-2 induced bone formation *in vivo*(Chen, Whetstone et al. 2007). Previous studies revealed that glycogen synthase kinase-3 (GSK3), the target of Wnt signalling, interferes with BMP signalling (Fuentealba, Eivers et al. 2007, Sapkota, Alarcon et al. 2007). GSK3 phosphorylation is essential for the SMAD1 polyubiquitinylation by the SMURF E3 ubiquitin ligases (Murakami, Watabe et al. 2003, Fuentealba, Eivers et al. 2007, Chong, Lin et al. 2010). Taking into account the central role of GSK3 in both BMP-2 and Wnt signalling and the fact that known specific inhibitors of that kinase are available (Krause, Harris et al. 2010), combination of BMP2 and these inhibitors could also be envisaged.

Different anatomical zones are used to obtain autogenous bone grafts in reconstructing bone defects. These sources include the iliac crest, cranial bone, mandibular symphysis, rib and tibia (Rawashdeh and Telfah, 2008). However, drawbacks include limited availability and morbidity at the donor site. To overcome these disadvantages, numerous tissue engineering approaches have been developed to take advantage of physiological osteoinductive signals (Henkel, Woodruff et al. 2013). Both $Ca^{2+}$ and BMP-2 are known to be co-released into the extracellular space by osteoclasts after bone matrix resorption. Our hypothesis was that extracellular $Ca^{2+}$signals interact with BMP and lead to higher osteoblast differentiation and bone formation from BM-MSCs. Here, early and late osteogenic marker expression and histological assessment of bone regeneration in a calvarial critical-size defect model demonstrated that extracellular $Ca^{2+}$ enhances the effects of BMP-2 on Osteocalcin, Runx2 and Osterix expression and promotes bone regeneration *in vivo*.

More importantly, mechanistically, both osteoinductors combined cooperate to increase long-term activation of SMAD and AKT signalling.

Osteogenic gene expression was significantly higher when extracellular $Ca^{2+}$ was added, regardless of the cell culture system used. MG63 osteoblastic cells, both in monolayer culture and 3D gelatin hydrogels, have been reported to show significant mineralization when cultured with 8mM $Ca^{2+}$ (Takagishi, Kawakami et al. 2006). Moreover, BMMSCs treated simultaneously with two different sources of $Ca^{2+}$, $CaSO_4$ and $CaCl_2$, with or without EDTA, demonstrated that the osteogenic effect was specific for $Ca^{2+}$. This outcome is consistent with the EGTA inhibition of osteocalcin secretion (Moreau, Aubin et al. 1997) and BAPTA, an intracellular $Ca^{2+}$ chelator, in the response of osteoblasts to extracellular calcium (Danciu, Adam et al. 2003).

Osteoinductive factors released from resident cells or after osteoclast bone resorption regulate the recruitment and differentiation of osteoblastic progenitor cells. The binding of BMPs to their cognate receptors triggers canonical Smad and Smad independent pathways, including ERK, p38 and PI3K/AKT signalling (Sieber, Kopf et al. 2009, Gamez, Rodriguez-Carballo et al. 2014). Several authors have reported that CaSR activation by extracellular $Ca^{2+}$ also activates these same pathways (Danciu et al., 2003; Dvorak and Riccardi, 2004; Riccardi, Finney et al., 2009).

We found that both calcium and BMP-2 induce activation of common signalling components, but in a differential time-dependent response. An early antagonistic effect between $Ca^{2+}$ and BMP-2 signalling was demonstrated. This contrasting effect is consistent with previous reports showing a crosstalk between calcium signalling and the BMP pathway in which high intracellular calcium inhibits BMP signalling (Leclerc, Neant et al. 2011). Furthermore, $Ca^{2+}$/calmodulin dependent kinase II (CAMKII), a primary transducer of calcium ions, directly interacts with Smads and antagonizes their function (Wicks, Lui et al. 2000). This reverse effect could be reinforced when we also consider that MAPK signalling inhibits BMP signals at the level of SMAD1 (Kretzschmar, Doody et al. 1999). Osteoblast differentiation is a multistep cascade of gene expression that initially supports proliferation and survival (Lefebvre and

Bhattaram 2010). The early prominent proliferative and pro-survival role of the MAPK and AKT/S6K network could induce the enlargement of the osteogenic progenitor pool (Dvorak and Riccardi, 2004).

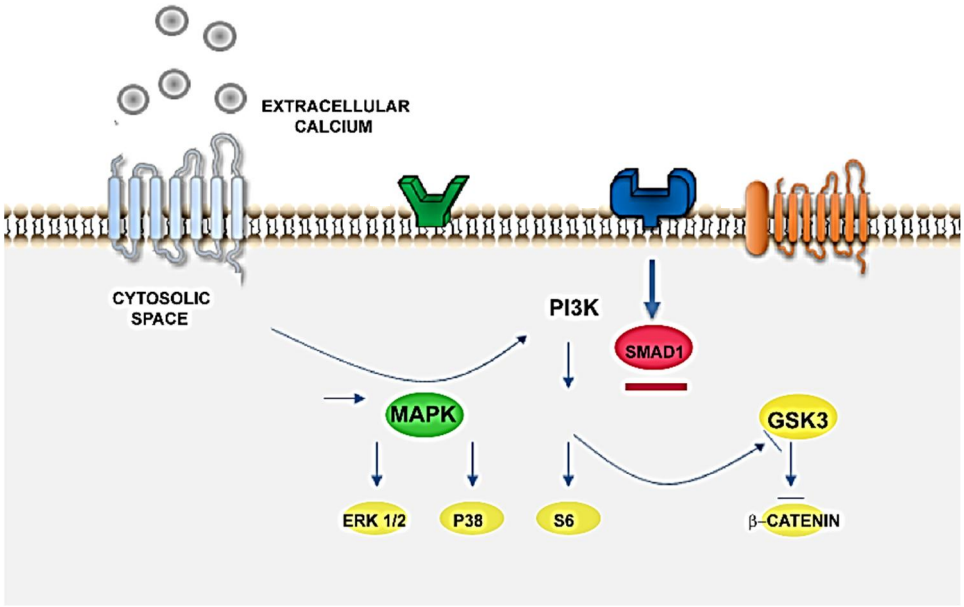



Figure 5.1.1 Model of the early $Ca^{2+}$ effect on BMP/Smad signalling. An intracellular network antagonistic to Smad1 is activated.

However, when late differentiation events were analyzed, $Ca^{2+}$ and BMP-2 were found to cooperatively stimulate osteoblast differentiation through the strengthening of specific osteogenic signalling pathways. Increases in the phosphorylation of SMAD1/5, S6, GSK3 β and expression of β-CATENIN were consistent with the significantly higher expression of Osteocalcin, Runx2 and Osterix and the greater bone formation in vivo (Rodriguez-Carballo, Ulsamer et al. 2011, Gamez, Rodriguez-Carballo et al. 2016). Cooperative crosstalk between $Ca^{2+}$ and BMP-2 in osteoblasts through the induction of the calcium-

dependent transcription factor NFATc1 by BMP-2 has also been described recently (Mandal, Das et al. 2016). NFAT transcription factors have proved necessary for osteoblast differentiation and bone formation (Koga, Matsui et al. 2005). Mechanistically, NFAT transcription factors activate osteogenesis through their interaction with OSX and their ability to stimulate Wnt/β-CATENIN signalling (Koga, Matsui et al. 2005, Fromigue, Hay et al. 2010). Unexpectedly, cell cultures exposed to $Ca^{2+}$ alone for 10 days displayed significantly higher SMAD signalling. This observation correlated with the increase in Bmp2, Bmp4 and Axin2 gene expression. BM-MSCs, periodontal ligament cells and dental pulp cells exposed to calcium-derived biomaterials have been reported to induce the upregulation of Bmp2 mRNA expression (Maeda, Nakano et al. 2010, Barradas, Fernandes et al. 2012, Tang, Peng et al. 2014). These studies implicated MAPK activity and AP-1 transcription factors in such effects (Tada, Nemoto et al. 2010, Barradas, Fernandes et al. 2012).

Thus, our data support the suggestion that calcium induces an autocrine/paracrine loop by endogenous BMP upregulation. Furthermore, Axin2 (a target gene of Wnt/ β-CATENIN signalling downstream of GSK3 β) was also expressed in long-term cell cultures. Our group recently showed that PI3K/AKT activity is relevant in bone formation *in vivo* and leads to the activation of SMAD1/5 and GSK3 β / β-CATENIN signalling (Gamez, Rodriguez et al., 2016). Several reports have demonstrated the synergistic interaction and the significance between BMP and Wnt during osteoblast differentiation and bone formation *in vitro* and *in vivo* (Rodriguez-Carballo, Ulsamer et al. 2011, Aquino-Martinez, Rodriguez-Carballo et al. 2016). Taken together, our results demonstrate a delayed calcium signalling effect that likely integrates and reinforces an osteogenic programme from multiple inputs.

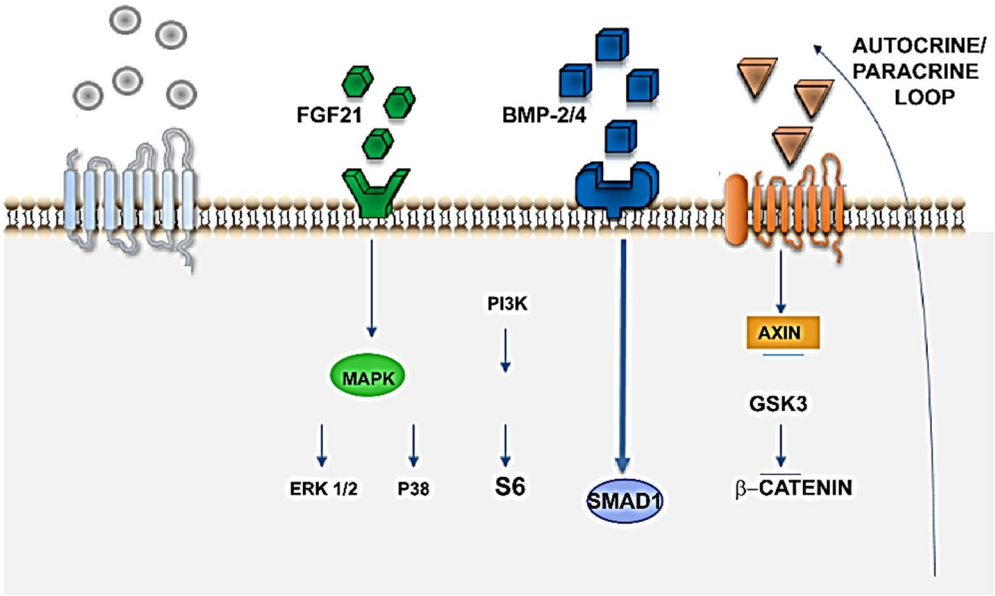



Figure 5.1.2 Model of the late $Ca^{2+}$ effect on BMP/Smad signalling. An autocrine/paracrine mechanism reinforce the initial BMP-2 effect.

# 6. CHAPTER VI: CONCLUSIONS

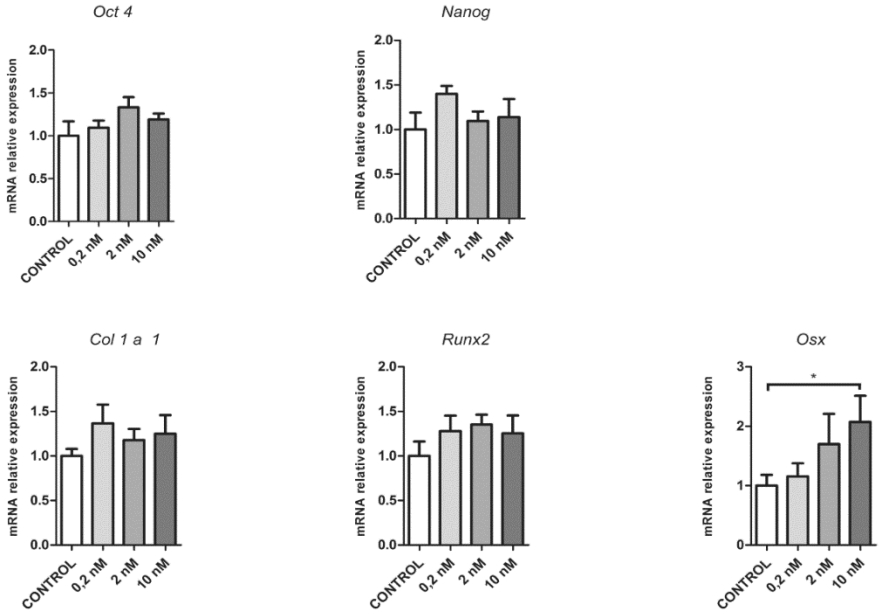
1- The combination of extracellular $Ca^{2+}$ signalling and a gelatin scaffold promotes higher osteoblast differentiation *in vitro* and bone formation *in vivo* compared to $Ca^{2+}$ or gelatin alone.

2- Low doses of BMP-2 and Wnt-3a additively increase osteoblast differentiation and bone regeneration using a gelatin/$CaSO_4$ scaffold as a 3D cell culture system.

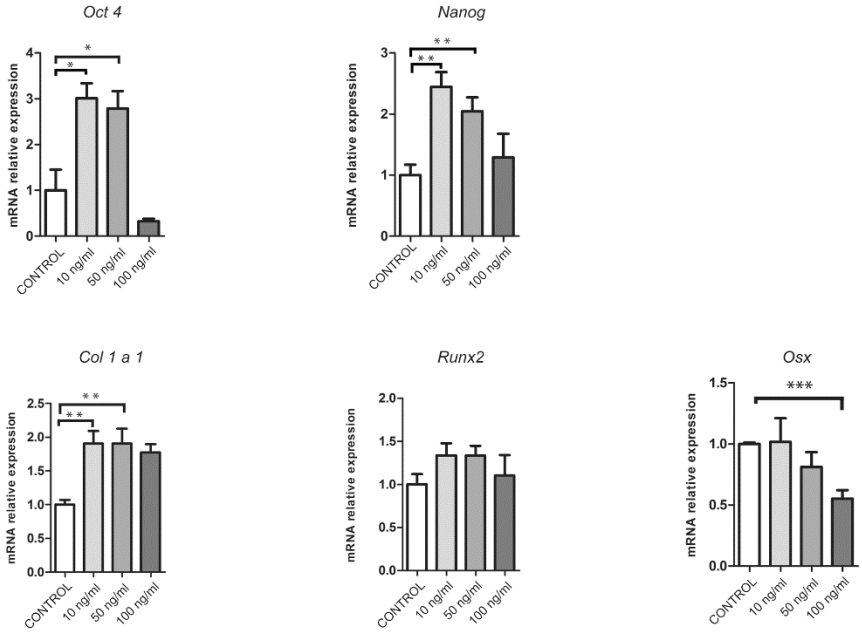
3- Extracellular $Ca^{2+}$ modulates the osteogenic effect of BMP-2. Early on, $Ca^{2+}$ activates an intracellular network that inhibits BMP/Smad signalling. In contrast, it later induces an autocrine/paracrine mechanism that reinforces the osteogenic output.

4- Mesenchymal stem cells seeded in a gelatin scaffold with $CaSO_4$ treated ex vivo with a combination of BMP-2 and Wnt3a could improve bone tissue engineering and $Ca^{2+}$ as a signal is an inexpensive and useful approach to regenerate bone defects.

**ANNEX**




**BMP-2 dose-response on stemness and osteogenic marker expression.**
Cell seeded on gelatin/$CaSO_4$ scaffolds were treated with different doses of BMP-2 (0.2nM, 2nM, 10nM). mRNA isolation was made 24 hours later. Stemness and osteogenic marker expression were evaluated by RT PCR and normalized to *Gapdh*.




**Wnt3a dose-response on stemness and osteogenic marker expression**
Cell seeded on gelatin/$CaSO_4$ scaffolds were treated with different dose of Wnt3a (10ng/ml, 50ng/ml, 100ng/ml) mRNA isolation was made 24 hours later. Stemness and osteogenic marker expression were evaluated by RT PCR and normalized to *Gapdh.*

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***ORIGINAL ARTICLE***

# Mesenchymal Stem Cells Within Gelatin/$CaSO_4$ Scaffolds Treated *Ex Vivo* with Low Doses of BMP-2 and Wnt3a Increase Bone Regeneration

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The delivery of osteogenic factors is a proven therapeutic strategy to promote bone regeneration. Bone morphogenetic proteins (BMPs) constitute a family of cytokines with well-known osteogenic and bone regenerative abilities. However, clinical uses of BMPs require high doses that have been associated with complications such as osteolysis, ectopic bone formation, or hematoma formation. In the present work, we sought to improve bone tissue engineering through an approach that combines the use of bone marrow-derived mesenchymal stem cells (BMMSCs), composite scaffolds, and osteoinductive agents. We employed a composite gelatin/$CaSO_4$ scaffold that allows for an early expansion of seeded BMMSCs, which is followed by an increased level of osteogenic differentiation after 10 days in culture. Furthermore, this scaffold enhanced bone formation by BMMSCs in a mouse model of critical-sized calvarial defect. More importantly, our results demonstrate that *ex vivo* pretreatment of BMMSCs with low amounts of BMP-2 (2 nM) and Wnt3a (50 ng/mL) for 24 h cooperatively increases the expression of osteogenic markers *in vitro* and bone regeneration in the critical-sized calvarial defect mouse model. These data provide a strong rationale for the development of an *ex vivo* cooperative use of BMP-2 and Wnt3a. Osteogenic factor cooperation might be applied to reduce the required amount of growth factors while obtaining higher therapeutic effects.

## Introduction

Understanding the basic principles of the cellular and molecular events regulating osteoblast differentiation is essential for the development of effective approaches to regenerate bones. Autologous bone grafting has been the gold standard for treating bone defects.[1] However, this approach is associated with numerous drawbacks, including the limited availability of grafting material and the morbidity associated with the collection of bone from a second surgery site. Bone tissue engineering has emerged as a potential alternative to overcome the inherent problems of autografts or allografts. The main components involved in tissue-engineered bone regeneration are stem or precursor cells, growth factors and/or cytokines, and appropriate bioactive carriers.[2]

Bioactive scaffolds provide an initial mechanical resistance and allow the attachment, propagation, and differentiation of the transplanted cells. The scaffolds for tissue engineering are classified as inorganic, such as calcium phosphate, or as organic materials, such as collagen or gelatin.[3] Osteoblastic cells show an increased expression of osteogenic markers when they are seeded on three-dimensional (3D) gelatin or collagen sponges compared with the 2D culture conditions.[4] In addition, integrin function has been proven to be essential for early osteoblast differentiation.[5] The calcium ions released from calcium-containing materials enhance osteogenesis because the extracellular calcium plays a critical role in the promotion of the differentiation and function of osteoblasts.[6]

The efficacy of bone morphogenetic proteins (BMPs) for regenerating bone is well known, both in animal models and in several clinical applications, such as bone fracture healing,[7] alveolar cleft defects,[8] spinal fusion,[9] and craniofacial bone defects.[10] As a result, the medical use of BMP-2 and BMP-7 was approved for specific osteoinductive applications. However, most bone regeneration studies using animal models entail supraphysiological doses of BMPs (e.g., 1–45 μg dose of BMP-2 in a femoral segmental defect in rat).[11–13] More importantly, BMP therapy in clinical practice also requires high amounts of BMPs, ranging between 1.5 and 3.3 mg (1.5 mg of BMP-2 or 3.3 mg of BMP-7).

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However, in some cases, only a minimal level of tissue regeneration is achieved.[14] Previous reports have demonstrated the safety of BMPs.[15] Nonetheless, some adverse effects such as osteolysis, bone cysts, retrograde ejaculation, or hematoma formation have been documented with the high BMP doses that are used for clinical treatments.[16,17] An alternative to overcome the current shortcomings associated with the requirement for high BMP doses is the use of stem and progenitor cells with scaffolds and combinations of osteoinductive agents to enhance the osteogenic differentiation and bone formation potential with lower amounts of growth factors.[2,12,18,19]

BMP signaling is transduced by the Smad family of transcription factors, which directly regulate gene expression as well as additional noncanonical pathways.[20] The BMP target genes include a growing number of osteoblast-determining transcription factors, such as *Runx2*, *Osterix*, and *Dlx3/5*.[21,22] Wnt signaling also plays important functions in osteoblastogenesis. We now know that an alteration at almost any step of the Wnt cascade significantly impacts bone physiology. For example, the loss of *Wnt10b*, *Lrp5/6*, or *β-catenin* causes osteopenia, whereas the loss of the β-catenin antagonist, *Apc* or *Axin2*, leads to increased bone mass.[23] Canonical Wnt signaling induces the expression of several osteoblast markers, such as *alkaline phosphatase* or *Runx2*. Interestingly, the synergism between BMP and Wnt signaling is critical for normal skeletal development and homeostasis. Wnt signaling is required for BMP-2-induced bone formation *in vivo*, and the inhibition of glycogen synthase kinase-3 (GSK3) activity, a Wnt signaling transducer, extends the duration of the BMP/Smad signal.[24] Moreover, recent work from our group demonstrates the cooperative effects of the BMP and Wnt canonical pathways on the expression of the master osteogenic genes *Dlx5*, *Msx2*, *Osx*, and *Runx2*.[25]

Therefore, we sought to improve bone tissue engineering through an approach that combines stem cells, bioactive scaffolds, and osteoinductive agents. Bone marrow-derived mesenchymal stem cells (BMMSCs) were induced to differentiate into osteogenic cells before *in vivo* implantation by combining distinct scaffolds and cytokine cocktails. We identified a method for enhancing the osteogenic potential of BMMSCs by culturing them in a gelatin/calcium sulfate ($CaSO_4$) scaffold and pretreating them with low doses of BMP-2 and Wnt3a, which increases their level of osteogenic gene expression and improves bone formation in a model of the critical size of calvarial bone defects *in vivo*.

## Materials and Methods

### *Isolation and culture of BMMSCs*

BMMSCs were isolated and cultured following the protocol described by Soleimani and Nadri.[26] Briefly, BMMSCs were isolated from the femur and tibia of 6- to 8-week-old green fluorescent protein (GFP) transgenic BALB/c mice. The cells were seeded in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, 1 mM pyruvate, and 2 mM glutamine. Once the stromal cells reached 70–80% confluence, the attached cells were trypsinized for 3 min at room temperature. The lifted cells were expanded and cultured for no more than seven passages before being used in subsequent experiments.

### *Scaffold preparation and cell culture on the scaffold*

Gelatin sponges (Gelita; BBraun) were cut into pieces that were 1 $cm^3$ with a surgical scalpel, resulting in 1-mm-thick slices. The slices were soaked for 24 h in phosphate-buffered saline (PBS). Then, the excess PBS was extracted and the soaked slices were partially air-dried for 15 min under the hood. A biphasic $CaSO_4$ solution (BondBone; MIS) in culture media was filtered with a 70-μm cell strainer (BD Falcon) and added (0.5 mg) in the corresponding experimental condition. For the *in vitro* experiments, $1.5 \times 10^5$ BMMSCs were seeded on each scaffold into multiwell plates for further culture in DMEM supplemented with 10% FBS, penicillin/streptomycin, 1 mM pyruvate, and 2 mM glutamine. Two-dimensional cultures were performed with gelatin or gelatin/$CaSO_4$-coated plates (coated with a thin layer of 0.1% gelatin solution for 1 h and air-dried for 1 h in the hood).

### *Experimental groups for BMMSC culture* in vitro

Four experimental conditions were evaluated for the *in vitro* experiments as follows: Group 1: cell monolayer grown on a plastic surface (control); Group 2: cells seeded on a gelatin sponge; Group 3: cell monolayer grown on a plastic surface and treated with 0.5 mg of $CaSO_4$; and Group 4: cells seeded on a gelatin sponge and treated with 0.5 mg of $CaSO_4$. Cells were analyzed for gene expression after 24 h or 10 days of culture, respectively. We also evaluated the effects of adding BMP-2 (2 nM) and/or Wnt3a (50 ng/mL) on gelatin/$CaSO_4$ scaffolds *in vitro*. The cells were cultured in serum-free DMEM containing 2 nM of BMP-2 and/or 50 ng/mL of Wnt3a for 24 h, and then analyzed for gene expression after 24 h or 10 days of culture, respectively.

### *Surgical procedure and calvarial defect model*

Male BALB/c mice (12-week-old) were anesthetized by isoflurane inhalation (Abbott) at a concentration of 5% for induction and 3% for maintenance. An intraperitoneal injection of buprenorphine (0.05 mg/kg) was administered for intraoperative analgesia. All of the surgical procedures were performed under aseptic conditions and on a thermal pad (37°C). The incision area was shaved and a subcutaneous injection of 0.25 mL of 3% mepivacaine was administered as local anesthesia. A full mid-longitudinal incision was made with a surgical scalpel. A circular bone defect was made at the center of the left parietal bone, taking care not to compromise the sagittal suture and to avoid damaging the dura. The bone defect was created with a 5-mm-diameter trephine mounted on a contra-angle handpiece using a dental implant motor (Satelec). Minimal irrigation was performed to heat damage the host bone on the edges to minimize spontaneous healing.[27] The bone disk was removed and the bone defect was covered and sealed with a randomly selected agarose-embedded scaffold according to the experimental group. The incisions were sutured and the animals were monitored daily during the recovery. Five weeks after surgery, the animals were euthanized by $CO_2$ inhalation and cervical dislocation. The heads were fixed in 4% paraformaldehyde for 24 h and prepared for micro-computed tomography (μCT) and histological analysis. All procedures were performed in accordance to the protocols approved by the Ethics Committee for Animal Experimentation of the University of Barcelona and by the Generalitat de Catalunya.

### *Experimental groups for* in vivo *BMMSC implantation into calvarial defects*

For the *in vivo* experiments, we seeded $3.5 \times 10^5$ BMMSCs per scaffold and cultured them in multiwell plates for 24 h. Each calvarial bone defect was filled with an agarose-embedded scaffold according to the following conditions: Group 1: agarose in growth media (control); Group 2: cells resuspended in growth media and agarose; Group 3: cells on a gelatin sponge and agarose; Group 4: gelatin sponge, $CaSO_4$, and agarose without cells; and Group 5: cells on a gelatin sponge, $CaSO_4$, and agarose. Therefore, only the animals of the Groups 2, 3, and 5 were implanted with scaffolds seeded with cells. After 24 h of culture, a 1% final concentration solution of low-melting agarose (Sigma-Aldrich) at 36°C was added as a gelling agent. The pieces of agarose containing the different scaffolds were trimmed and carefully implanted into the critical-sized bone defect.

We also evaluated the effects of adding BMP-2 (2 nM) and/or Wnt3a (50 ng/mL) on gelatin/$CaSO_4$ scaffolds cultured for 24 h before implantation. The cells were cultured in serum-free DMEM containing 2 nM of BMP-2 and/or 50 ng/mL of Wnt3a for 24 h, and then implanted into the critical-sized bone defect as described above.

### *Quantitative RT-PCR analysis*

The BMMSC RNA isolation was performed using Trisure (Bioline), according to the manufacturer's instructions. The RNA quantification was performed by spectrophotometric analysis (Nanodrop ND 1000; Thermo Scientific). The purified RNA (3 μg) was reverse transcribed using a High-Capacity Retrotranscription Kit (Applied Biosystems), and 50 ng of cDNA per reaction was used in each quantitative RT-PCR (qRT-PCR), with two replicates per sample. qRT-PCRs were carried out using the TaqMan 5′-nuclease probe method (Applied Biosystems). The relative transcript expression levels were normalized to *Gapdh* expression (endogenous control).

### *Cell proliferation assays*

BMMSC proliferation was evaluated using 7-AAD and BrdU labeling (BD), following the manufacturer's protocol. Briefly, $1 \times 10^5$ cells were seeded in 24-well plates or onto scaffolds (according to the experimental groups) and incubated at 37°C for 24 h. Then, BrdU (10 μM) was added to the medium for 45 min. The cells were harvested with 0.04 mg/mL of liberase (Roche) for 10 min and analyzed by flow cytometry.

### *μCT analysis*

Five weeks after surgery, the mice were euthanized. The heads were fixed with 4% paraformaldehyde for 24 h and stored in PBS/azide at 4°C until scanning. Scanning was performed using a Skyscan 1076 High-Resolution scanner (Skyscan). All samples were placed horizontally in a holder, and the exposure parameters were 49 kV, 200 μA, with an exposure time of 500 ms and a 180° rotation. Data reconstruction was performed using the NRecon software. A Gaussian noise filter was applied and the three-dimensional models were performed with the CTAn software. Both programs were provided by the manufacturer. A cylindrical region of interest (ROI) with a 5 mm diameter was positioned manually to cover the bone defect area. For each sample, 375 slices were processed and analyzed. The ROIs were converted into volumes of interest that were used to quantify the extent of newly formed bone.

### *Histology and immunohistochemistry*

After μCT image acquisition and 3D reconstruction, the calvaria samples were dissected and the soft tissue was removed. The dissected calvariae were decalcified with Decalcifier II (Leica Biosystems) for 2–3 days, dehydrated, embedded in paraffin, and sectioned. The slides with the 5-μm sections were deparaffinized, rehydrated, and stained with Masson's trichrome. For immunohistochemistry, the tissue samples were boiled in citrate buffer, washed, and blocked with serum. The primary antibodies against Osterix (OSX, ab22552; Abcam) or GFP (ab290; Abcam) were incubated at 1:200 dilution on the sections overnight. After washing, the samples were incubated with a biotinylated secondary antibody (1:100) and streptavidin–horseradish peroxidase (1:400) for 1 h. The sections were incubated with diaminobenzidine and $H_2O_2$ and counterstained with hematoxylin.

### *Statistical analyses*

The statistical analyses were performed using Student's *t*-test with the GraphPad Prism 5 software. The quantitative data are presented as the mean ± standard error of the mean. The differences were considered significant at *p*-values of <0.05, with $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

## Results

### *A composite gelatin/$CaSO_4$ scaffold increases the expression of osteogenic genes* in vitro

In addition to the growth factors, another challenge for bone regeneration is to determine a suitable matrix for BMMSC transplantation and osteogenic differentiation *in vivo*. Collagen plays a major role in determining cell behavior, adhesion, and mineralization and has been extensively employed in bone regeneration with excellent biocompatibility.[28] Moreover, tridimensional gelatin sponges have been described as effective for osteoinduction *in vitro* and *in vivo*.[18,29] Calcium sulfate, tricalcium phosphate, and hydroxyapatite are also well-known, commercial, synthetic, ceramic bone grafts. They exhibit resorption periods of 1–2, 6–12, and 12–36 months, respectively.[30,31] We hypothesized that the resorption period of $CaSO_4$ would parallel the healing period of a calvarial defect in mice. Moreover, the release of calcium ions from $CaSO_4$ might enhance osteogenesis because the extracellular calcium promotes the differentiation and function of osteoblasts.[6] Thus, we evaluated the influence of gelatin and biphasic $CaSO_4$, alone or in combination, on the morphology, proliferation, and osteogenic marker expression of BMMSCs *in vitro*. Because the BMMSCs used in this study were obtained from transgenic GFP-expressing BALB/c mice, we were able to easily visualize these cells within the scaffolds. The images revealed morphological differences between the tested seeding conditions and the control, which was a monolayer grown on a plastic surface. The BMMSCs grown on the gelatin substrate were connected to each other by cytoplasmic prolongations in a marked reticular pattern according to the three-dimensional structure of the sponge (Fig. 1B).

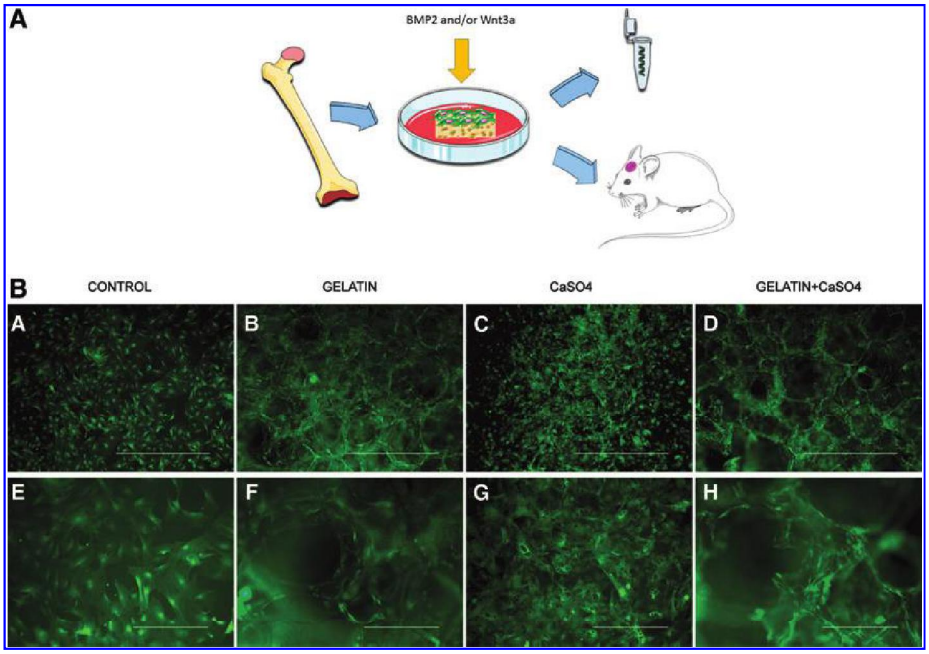


**FIG. 1.** Green fluorescent protein (GFP)-expressing bone marrow-derived mesenchymal stem cells (BMMSCs) cultured for 24 h in different scaffolds. **(A)** Scheme of the general strategy of the study. **(B)** Images showing the different morphological features of the culture conditions, namely a monolayer on a plastic surface (as control), gelatin scaffold, $CaSO_4$-treated monolayer, and gelatin + $CaSO_4$ scaffold. (A–D) A higher magnification shows the characteristic flattened morphology of mesenchymal stem cells. (E–H) The cells cultured on $CaSO_4$ crystals show a thicker cytoplasmic structure with abundant vacuoles. In gelatin conditions, the cultured cells aligned throughout the three-dimensional structure of the scaffold. Color images available online at www.liebertpub.com/tea

Next, we examined the ability of these scaffolds to maintain the undifferentiated status of the BMMSCs. The gene expression quantification of the stemness markers, *Oct4* and *Nanog*,[32] demonstrated that the gelatin sponge did not modify their expression after 24 h of culture (Fig. 2A). Interestingly, after long-term culture on the scaffolds (10 days), the expression of *Oct4* and *Nanog* was significantly decreased when the BMMSCs were cultured on the gelatin plus $CaSO_4$ scaffold ($p = 0.0001$ and $p = 0.001$ for *Oct4* and *Nanog*, respectively). A parallel analysis of osteoblast-determining transcription factors showed no differences in the expression of *Runx2* ($p > 0.05$) regardless of the culture time (24 h or 10 days) (Fig. 2B). Notably, when the cells were cultured in the gelatin plus $CaSO_4$ scaffolds, the expression of *Osx* was significantly increased at both 24 h and 10 days of culture ($p = 0.012$ and $p = 0.0019$, respectively) (Fig. 2B). We also determined whether the effects of gelatin and/or $CaSO_4$ on stemness and osteogenic markers were dependent on 3D culture. Cells seeded in monolayers of gelatin and/or CaSO4-coated plates expressed levels of *Nanog* similar to their respective 3D scaffold (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). However, the enhancement on the expression of *Osx* by the gelatin/$CaSO_4$ scaffold in 3D was not observed in cells cultured in the gelatin/$CaSO_4$ monolayer (Supplementary Fig. S1). Next, we analyzed the proliferation of BMMSCs cultured in different biomaterials for 24 h by flow cytometry after DNA labeling with 7-AAD and BrdU incorporation. The results were in agreement with the expression of the stemness markers, showing a higher proliferative rate in the gelatin plus $CaSO_4$ scaffolds, as shown by BrdU incorporation or by the percentage of cells in the S or G2/M phases of the cell cycle ($p = 0.0164$) (Fig. 2C). Altogether, these results suggest that this composite 3D scaffold allows an early expansion of the BMMSCs at shorter culture times, which is followed by an increased osteogenic differentiation after 10 days in culture.

### *Wnt3a cooperates with BMP-2 to increase the expression of osteoblastic markers*

Among the extracellular signals involved in the induction of the osteoblast phenotype, the BMP and Wnt families of morphogens are essential for the commitment and differentiation of the osteoblast lineage.[33–35] Moreover, our group has shown that both signaling pathways exert cooperative effects on the induction of osteogenesis.[25] Therefore, we hypothesized that the combination of the two osteogenic signals could improve bone regeneration. Cooperative effects between BMP-2 (at 2 nM) and Wnt3a (at either 30 or 100 ng/mL) were previously seen in C2C12 and BMMSCs.[25] We cultured BMMSCs in the composite scaffolds for 24 h and then for an additional 24 h in the presence of increasing amounts of BMP-2 or Wnt3a. Addition of BMP-2 resulted in a progressive increase in the expression of Osx that became

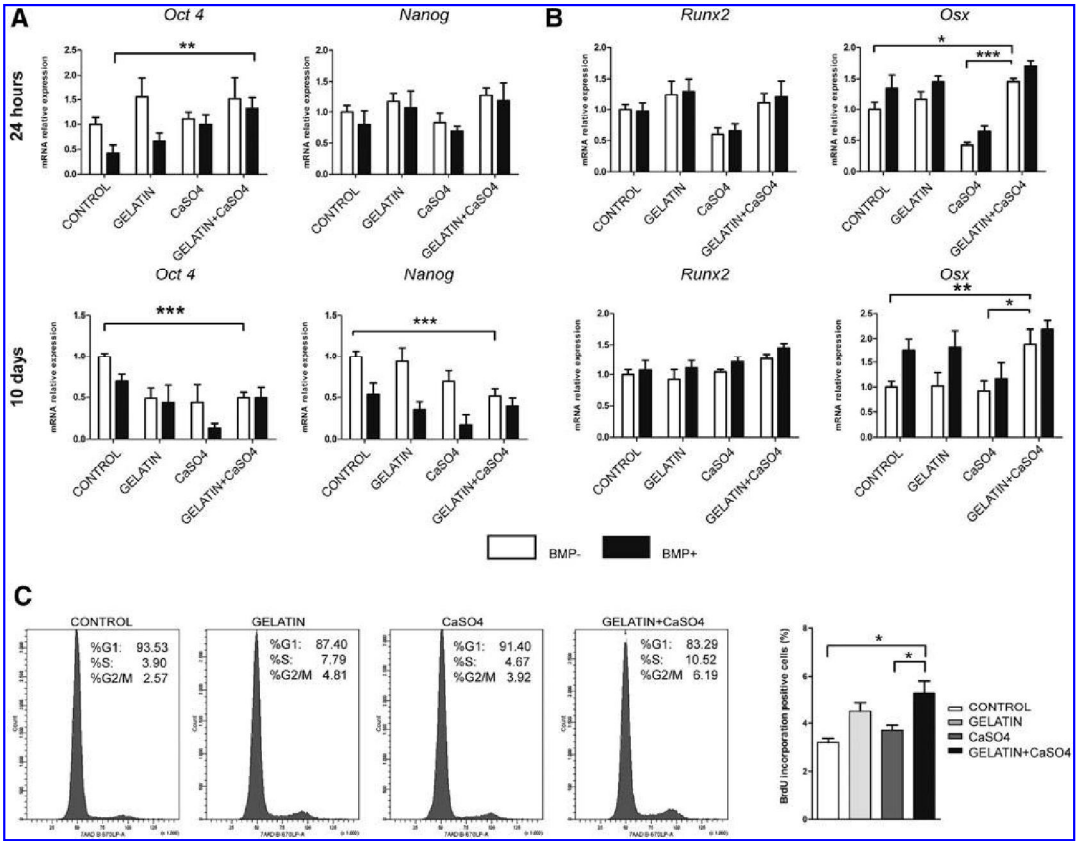


**FIG. 2.** Effect of the gelatin/$CaSO_4$ scaffold on stemness and osteogenic marker expression in BMMSCs. **(A)** Expression levels of *Oct4* and *Nanog* assessed at 24 h or 10 days of culture in the indicated scaffold in the presence (*black box*) or absence (*white box*) of bone morphogenetic protein (BMP)-2 ($n=8$). **(B)** Expression levels of *Runx2* and *Osx* assessed at 24 h or 10 days of culture in the indicated scaffold in the presence (*black box*) or absence (*white box*) of BMP-2. The indicated markers were normalized to *Gapdh* and four independent experiments were performed ($n=8$). **(C)** FACS analysis of 7-AAD labeling (*left*) and BrdU incorporation (*right*) of BMMSCs cultured in the indicated scaffold. Data presented as the mean of three independent experiments. $*p<0.05$, $**p<0.01$, $***p<0.001$.

significant at 10 nM ($p=0.0384$) (Supplementary Fig. S2). Furthermore, addition of 10 and 50 ng/mL of Wnt3a increased expression of the stemness markers, *Oct4* and *Nanog* ($p=0.0227$ and $p=0.0385$ for *Oct4*, and $p=0.0191$ and $p=0.0061$ for *Nanog*) (Supplementary Fig. S3). Moreover, Wnt3a addition led to induction of *Col1a1* expression at 10 and 50 ng/mL ($p=0.019$ and $p=0.0061$, respectively), whereas the higher concentration tested (100 ng/mL) led to reduction in Osx expression ($p=0.0009$) (Supplementary Fig. S3). The osteogenic effects of these doses of BMP-2 and Wnt3a correlate with previous evidence of transcriptional effects of BMPs and Wnts at this range of concentrations.[25,36,37] We cultured BMMSCs in the composite scaffolds for 24 h and then for an additional 24 h or 10 days in the presence of BMP-2 (2 nM) and/or Wnt3a (50 ng/mL). We assessed the expression of the stemness markers, *Oct4* and *Nanog*. *Oct4* and *Nanog* were upregulated by Wnt3a addition after 24 h ($p=0.001$ and $p=0.0059$ for *Oct4* and $p=0.0039$ and $p=0.279$ for *Nanog* in Wnt3a and BMP-2 + Wnt3a treatments, respectively), whereas BMP-2 had no effect on their expression (Fig. 3A). However, after 10 days, *Nanog* expression was downregulated when both BMP-2 and Wnt3a were added together ($p=0.0003$).

To further assess the cooperative effect between Wnt3a and BMP-2, we also evaluated the expression of *Col1a1*, *Runx2*, and *Osx*. Wnt3a alone or in combination with BMP-2 induced the expression of *Col1a1* at 24 h ($p=0.0169$ and $p=0.157$, respectively) (Fig. 3B). Moreover, stimulation with both cytokines conferred a significant additive effect on the expression of *Runx2* and *Osx* at 24 h ($p=0.0052$ and $p=0.0035$, respectively) (Fig. 3B). Interestingly, the synergic effects were maintained after 10 days of culture for the *Col1a1* and *Osx* mRNA expression ($p=0.0389$ and $p=0.0041$, respectively) (Fig. 3B). The cell cycle and BrdU incorporation analyses to assess the proliferation rate of the cells treated with these cytokines for 24 h yielded results that were in agreement with the

expression of the stemness markers. The addition of Wnt3a increased the growth rate of the cells in parallel to *Oct4* and *Nanog* expression ($p = 0.0017$ for Wnt3a alone and $p = 0.0327$ for BMP-2 + Wnt3a), whereas BMP-2 did not significantly influence the proliferation or stemness marker expression (Fig. 3C). These results support the concept of collaboration between BMP and Wnt3a signaling in enhancing the osteogenic potential of BMMSCs cultured in the scaffold *ex vivo*.

### *Gelatin/$CaSO_4$ scaffolds improve bone formation* in vivo

To verify these observations in bone regeneration, we analyzed their ability to heal a critical-sized calvarial bone defect *in vivo*. We implanted the composite scaffold in the absence and presence of cells in a 5-mm size calvarial defect.[38] Defects of this diameter are unable to heal by themselves.[38,39] Furthermore, we used a minimal irrigation during the surgical procedure to generate a more challenging cranial defect.[27] This was supported in our model by the lack of bone formation in the empty defects, which were partially filled with a layer of fibrous tissue instead of newly formed bone (Fig. 4). Five weeks after scaffold implantation, the defects were analyzed by μCT to evaluate new bone formation. The analysis of the reconstructed images demonstrated that the gelatin/$CaSO_4$ scaffold seeded with BMMSCs possessed the greatest bone regeneration potential, as observed by comparing the margins of the calvarial defects with the other

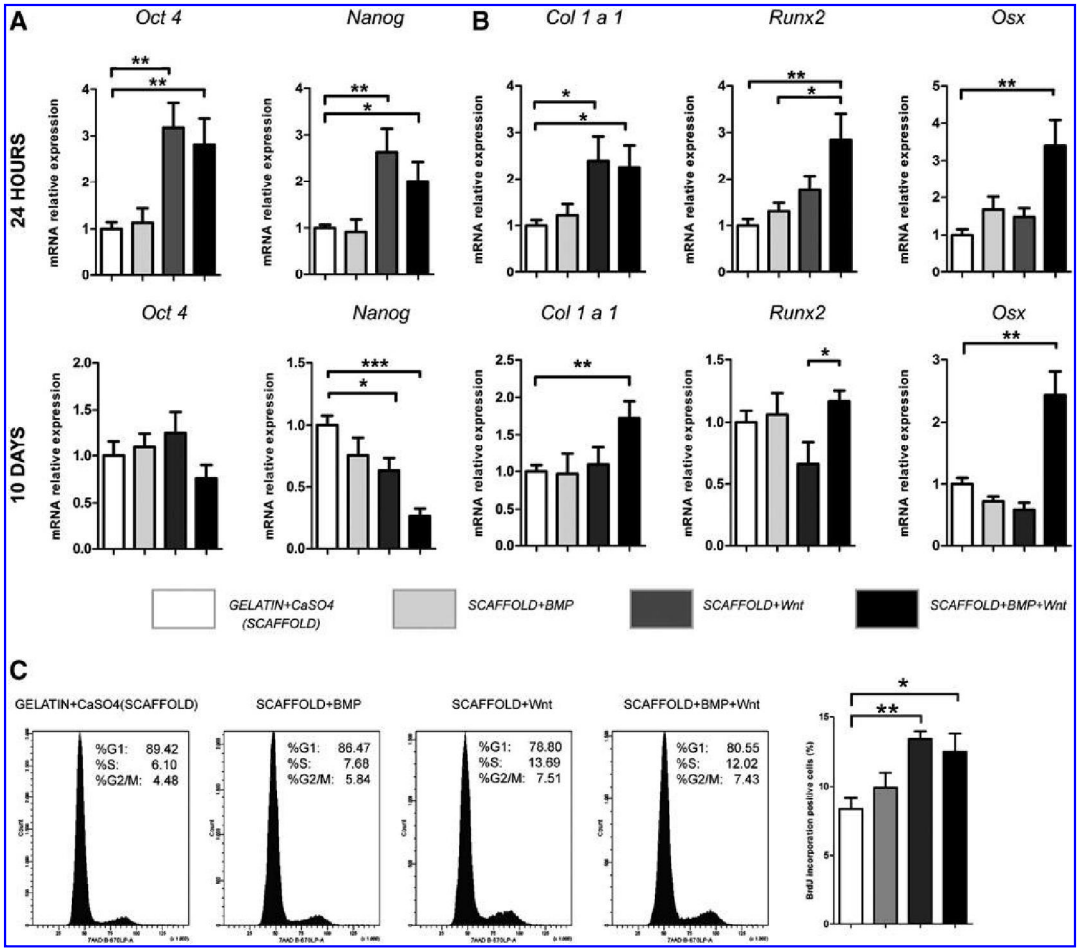


**FIG. 3.** BMP-2 and Wnt3a addition increases osteoblast marker expression. **(A)** Expression levels of *Oct4* and *Nanog* assessed at 24 h or 10 days of culture while being treated with the indicated cytokines. **(B)** Expression levels of *Col1a1*, *Runx2,* and *Osx* assessed at 24 h or 10 days of culture while being treated with the indicated cytokines. The mRNAs were normalized to *Gapdh* and four independent experiments were performed. **(C)** FACS analysis of 7-AAD labeling (*left*) and BrdU incorporation (*right*) of the BMMSCs cultured with the indicated cytokines for 24 h. Data presented as the mean ± standard error of the mean (SEM) of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

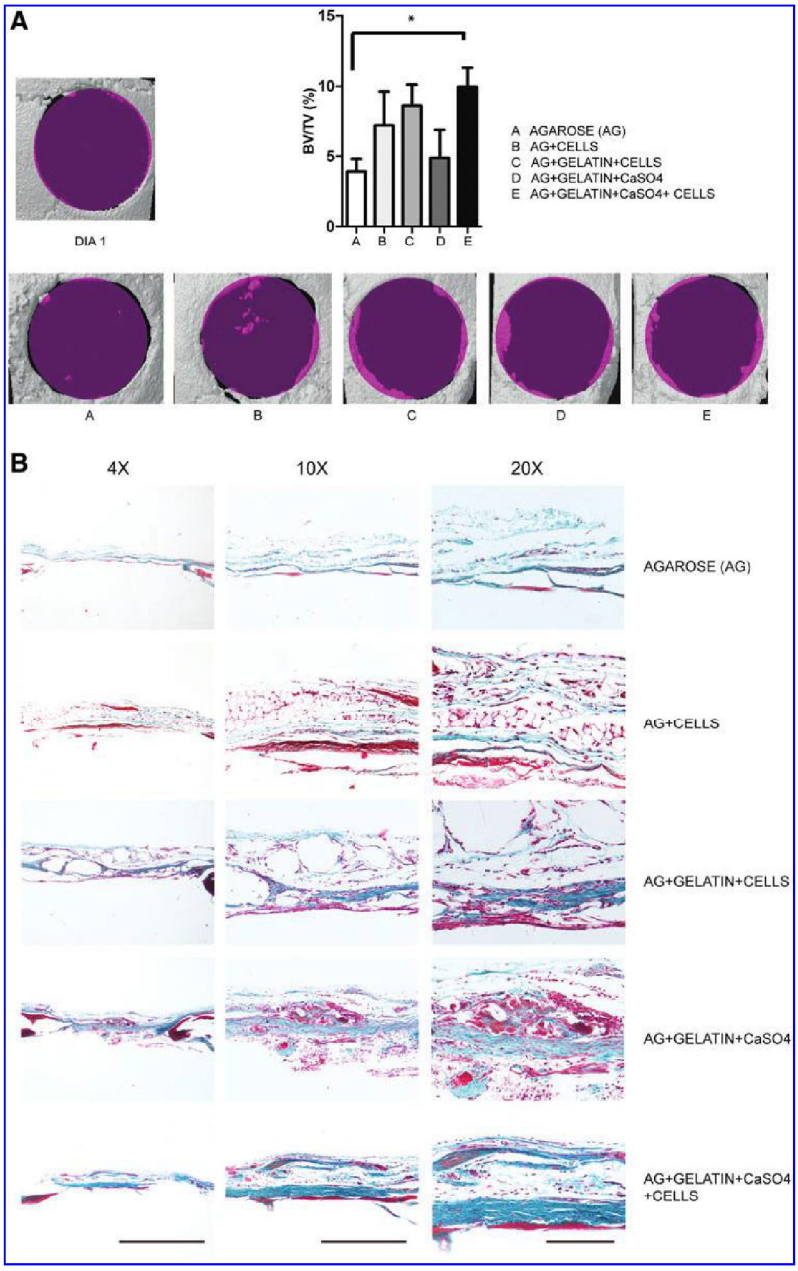


**FIG. 4.** Microcomputed tomography quantification of bone regeneration of the gelatin/$CaSO_4$ scaffold implants. **(A)** Representative images of a three-dimensional reconstruction of the calvarial defects implanted with agarose (AG) (A), AG + cells (B), AG + gelatin + cells (C), AG + gelatin + $CaSO_4$ (D), and AG + gelatin + $CaSO_4$ + cells (E) are shown. Quantification of new bone formation (BV/TV) is also presented. The results are shown as the mean ± SEM of five animals per group. An image at day 1 shows the original 5 mm diameter of the calvarial defect. **(B)** Masson's trichrome staining of representative calvarial sections of each group. 4× scale bar, 1000 μm. 10× scale bar, 400 μm. 20× scale bar, 200 μm. *$p < 0.05$.

conditions assessed ($p = 0.0121$) (Fig. 4A). Furthermore, these data show that bone formation arises from the function of the implanted exogenous cells because the implantation of the scaffold without cells only promoted a marginal effect on bone formation. The histological analyses confirmed the findings obtained by μCT. Very limited bone formation was observed in the control defects implanted with the agarose-only scaffold. Notably, an abundant endogenous cellular invasion was observed when the gelatin/$CaSO_4$ scaffold without cells was implanted. However, and more importantly, when the BMMSCs in the composite scaffold were implanted, significant bone healing was obtained and accompanied by a more mature structure (Fig. 4B). These data indicate that the BMMSCs in combination with a composite gelatin/$CaSO_4$

scaffold are able to partially repair a defect that otherwise would not heal in these adult mice.[39]

### *Wnt3a and BMP-2 cooperatively enhance bone regeneration* in vivo

To assess whether the combination of Wnt3a and BMP-2 increases bone regeneration *in vivo*, we implanted a gelatin/$CaSO_4$ scaffold seeded with BMMSCs that were pretreated *ex vivo* with BMP-2 and/or Wnt3a for 24 h before the implantation. The critical-sized calvarial bone defects were implanted with cultured scaffolds using agarose as a gelling agent. Five weeks after implantation, we evaluated bone regeneration through μCT scanning and presented the outcome as percentages (BV/TV) of the healed area. The mineralized bone extensions from the edges of the defects were significantly increased when the scaffolds were pretreated with BMP-2 alone or with BMP-2 and Wnt3a ($p=0.0085$ and $p=0.030$, respectively) (Fig. 5A). However, the histological analyses revealed different characteristics in the regenerated bone. The volume of new bone formation was greater in the Wnt3a/BMP-2-treated implants (Fig. 5B). Moreover, the bone structure was also more mature in Wnt3a/BMP-2 implants compared with the other treatment groups.

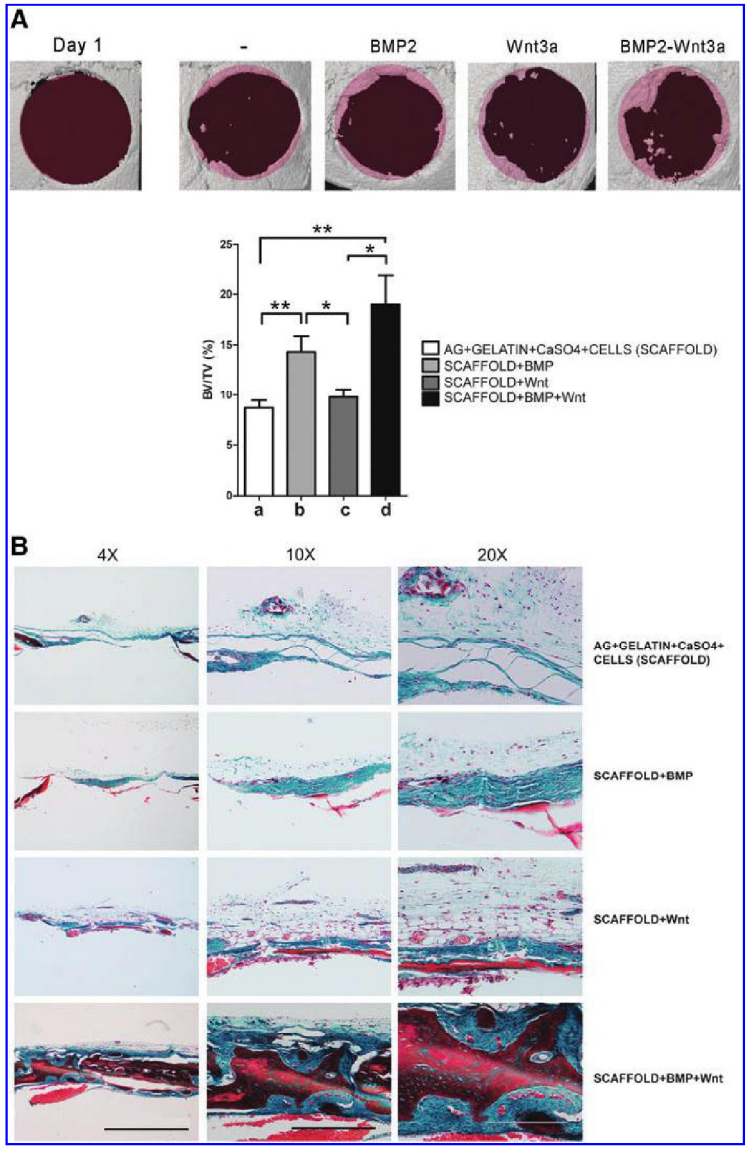


**FIG. 5.** BMP-2 (2 nM) and Wnt3a (50 ng/mL) increase bone formation *in vivo*. **(A)** Representative images of reconstructions of the calvarial defects treated with AG + gelatin + $CaSO_4$ + cells (scaffold) (a) and different cytokine treatments: BMP-2 (b), Wnt3a (c), and BMP-2 + Wnt3a (d). Quantification of new bone formation (BV/TV) is also indicated. The results are plotted as the mean ± SEM of six animals per group. An image at day 1 shows the original 5 mm diameter of the calvarial defect. **(B)** Masson's trichrome staining of representative calvarial sections of each group. 4× scale bar, 1000 μm. 10× scale bar, 400 μm. 20× scale bar, 200 μm. $*p<0.05$, $**p<0.01$.

We also wanted to confirm the relative contribution of the implanted BMMSCs in the recruitment of endogenous osteoprecursors to discriminate between a direct action of the BMMSCs or a paracrine effect on endogenous cells. Because the BMMSCs were isolated from a GFP-expressing transgenic mouse strain, GFP expression distinguished the exogenously implanted cells from the endogenous cells. Therefore, the Wnt3a/BMP-2-treated implants were immunostained 5 weeks after implantation. To assess the specificity of the labeling, another histological section from the same animal was used as negative control (without primary anti-GFP antibody), which resulted in the total absence of signal (Fig. 6A). A significant number of GFP-positive cells were scattered through the new bone tissue, suggesting that the transplanted cells survived and partially contributed to new bone formation. However, an additional paracrine effect on endogenous cells should not be excluded for their contribution to bone healing. The immunohistochemical analysis for OSX expression was also performed to assess whether the cells that had survived after the implantation also achieved their osteogenic potential. The implants treated with Wnt3a alone displayed an increased cellularity, but a sparse OSX staining, which suggests an increased proliferative rate, but not increased osteoblast differentiation (Fig. 6B). The BMP-2-treated scaffolds presented a higher OSX expression level that was also visible in the woven bone around the scaffold. More importantly, the combination of BMP-2 and Wnt3a also led to an intense expression of OSX in a significantly higher number of osteoblasts at the implanted site. Altogether, these results suggest that the *ex vivo* pretreatment with considerably low amounts of BMP-2 combined with Wnt3a cooperatively increased the osteogenic potential *in vitro* and *in vivo*. This represents an improvement over the current growth factor delivery strategies and highlights the importance of assessing the effects of osteogenic factor cocktails in bone tissue engineering.

## Discussion

The clinical use of supraphysiological amounts of BMP-2 to induce bone formation can produce several complications such as osteolysis, ectopic bone formation, or hematoma formation. As an alternative to avoid these drawbacks, we hypothesized that BMP-2 and Wnt3a, if acting cooperatively, could reduce the required doses and produce similar therapeutic effects. In our study, we developed a gelatin/$CaSO_4$ scaffold to seed and expand the BMMSCs that were pretreated *ex vivo* with low doses of BMP-2 (2 nM) and Wnt3a (50 ng/mL). Based on the expression of osteogenic genes and μCT analysis, as well as histological and immunohistochemical data, we demonstrated that the pretreatment of BMMSCs with a combination of BMP-2 and Wnt3a enhances bone regeneration *in vivo* and increases the expression of osteogenic genes *in vitro*.

Efficient bone tissue engineering requires three components: biocompatible scaffolding materials, osteoblast progenitors, and potent osteogenic cytokines. First, we directed our efforts to identify a suitable scaffold for the engraftment, expansion, and osteogenic differentiation of BMMSCs. We took advantage of a composite scaffold comprising a gelatin sponge with a porous structure incorporating a biphasic

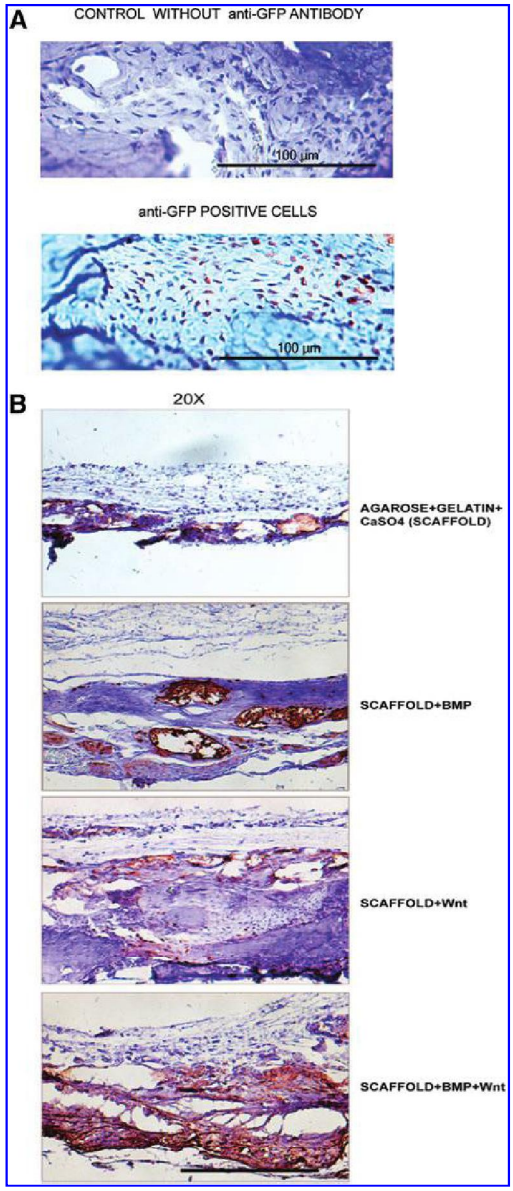


**FIG. 6.** **(A)** Transplanted BMMSCs revealed by anti-GFP immunohistochemistry 5 weeks after the implantation. Histological sections from the calvarial defects implanted with a gelatin + $CaSO_4$ scaffold with cells pretreated with BMP-2 and Wnt3a. Immunohistochemistry with an anti-GFP antibody and a control without a primary antibody are shown. **(B)** Osterix (OSX) immunohistochemistry identifies osteoblasts at the implanted sites. Histological sections from the calvarial defects implanted with a gelatin + $CaSO_4$ scaffold and cells pretreated with BMP-2 and/or Wnt3a. Immunohistochemistry with an anti-OSX antibody shows OSX expression in cells at the implantation sites (stained *brown*). *Arrows* indicate some positive cells.

$CaSO_4$ solution. The biosafety and biodegradability of gelatin have been proven.[18] Moreover, biphasic $CaSO_4$ is also known for its osteoinductive activity and its rapid resorption time within a few weeks.[30] Indeed, the released $Ca^{2+}$ ions stimulate the osteogenic differentiation of the osteoprogenitors on the scaffold and are converted into hydroxyapatite by the osteogenic cells.[40] Therefore, our finding that the BMMSCs cultured in three-dimensional gelatin scaffolds display an increased stemness and expansion potential is in agreement with previous reports.[41] Moreover, the effects mediated by the gelatin substrata are improved by the addition of $CaSO_4$ in the composite biomaterial, which increases the growth rate of the cells and promotes *Oct4* and *Nanog* expression. Stemness maintenance during the steps of biomaterial engineering is essential for the expansion of BMMSCs, their self-renewal capacity, and their ability to specifically differentiate into osteoblasts later.[32,42] A rationale exists behind the addition of exogenous BMMSCs for bone regeneration. Indeed, the BMMSCs contributed directly to the repair process by their differentiation into osteoblasts. We found that the exogenously implanted GFP-expressing cells survived and integrated into the healed bone area. Furthermore, they were able to differentiate into OSX-expressing cells. Moreover, the addition of cells, either into gelatin alone or in the gelatin/$CaSO_4$ composite scaffold, enhanced bone regeneration *in vivo* to a greater extent than the composite scaffold alone. Therefore, it can be suggested that in our calvarial healing model, the exogenously transplanted cells directly contributed to new bone formation. The survival of transplanted cells is highly dependent on the correct nutrient and oxygen supply.[2,43] However, even in the case where long-term survival is decreased because of the low vascularization of the defect area, the addition of BMMSCs has been proven to be beneficial. The transplanted cells support the recruitment and activation of endogenous stem cells through paracrine effects and reduce the proinflammatory effects of cytokines.[44–46] Our data show that the addition of BMMSCs increases the recruitment of GFP-negative endogenous cells in the new bone tissue, which contributes to the healing process.

The BMP and Wnt pathways seem to have a very relevant role on mesenchymal self-renewal and specification of stem cells toward the osteogenic lineage.[35,47] During embryonic development, the Wnts and BMPs are expressed in the areas of bone and cartilage formation. Furthermore, the addition of high doses of BMPs is current therapy in bone regeneration and bone tissue engineering.[14] In our study, we demonstrate that considerably low doses of BMP-2 (as low as 2 nM) and Wnt3a (50 ng/mL) exert a strong cooperation for the expression of osteogenic markers *in vitro* and bone regeneration *in vivo*. This approach offers an alternative to the high amounts of BMP-2 used clinically and, at the same time, provides an alternative to autologous bone graft. These high doses display some off-target adverse effects and have been shown to also activate the osteoclast resorptive activity.[12,48] Moreover, our design based on *ex vivo* preconditioning before implantation in the calvarial defect does not involve any type of gene therapy, as reported in previous models of BMP- or Wnt-mediated BMMSC stimulation.[49,50] We hypothesized that preconditioning the implants with BMP-2 and Wnt3a could be sufficient to trigger osteogenic responses based on several premises. First, the systemic half-life of BMP-2 is of minutes, and even when administered locally in collagen sponges, its effects shortly vanish.[51] Second, BMP-2 activity is mostly required at the initiation steps of fracture healing.[52] Third, both Wnt signaling and the osteogenic capacity decline with age, and the addition of exogenous Wnt3a restores the osteogenic capacity.[53] Fourth, the addition of BMP-2 and Wnt3a to BMMSCs for 24 h is sufficient to concurrently induce the expression of several osteoblast-specific transcription factors (*Dlx3*, *Dlx5*, *Msx2*, *Runx2*, and *Osx*) at high levels.[25]

The cultured scaffolds pretreated with Wnt3a alone or in combination with BMP-2 produced an early increase in cell proliferation and promoted *Oct4* and *Nanog* gene expression. However, as observed with the scaffold assay, the opposite effect on *Oct4* and *Noggin* gene expression was observed at 10 days. This apparently contradictory outcome is in agreement with the progression of BMMSC differentiation into the osteoblastic lineage. Indeed, cell proliferation is confined to the initial culture period. Once a sufficient population of progenitor cells has been generated, the cells decrease their proliferation rate and differentiate into osteoblasts.[54] Minear *et al.* reported that Wnt3a stimulates the proliferation of skeletal stem cells and that the bone formation-promoting effects of Wnt3a are achieved through this proliferative effect.[55] Moreover, this considerably low dose of BMP-2 (2 nM) could also be mitogenic together with Wnt3a.[56] This initial burst of BMP-2- and Wnt3a-mediated proliferation could increase the later expression of osteoblast markers *in vitro* and bone formation *in vivo*. The coordinated effect that we obtained in our model parallels the existence of a close inter-relationship between these pathways.[54] Indeed, canonical Wnt signaling is required for BMP-2-induced bone formation *in vivo*.[57] Previous studies revealed that GSK3, the target of Wnt signaling, interferes with BMP signaling.[24,58] Taking into account the central role of GSK3 in both BMP-2 and Wnt signaling and the fact that known specific inhibitors of that kinase are available,[59] the combination of BMP-2 with these inhibitors could also be envisaged.

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## Disclosure Statement

No competing financial interests exist.

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# EXTRACELLULAR CALCIUM PROMOTES BONE FORMATION FROM BONE MARROW MESENCHYMAL STEM CELLS BY AMPLIFYING THE EFFECTS OF BMP-2 ON SMAD SIGNALLING

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**ABSTRACT**

Understanding the molecular events that regulate osteoblast differentiation is essential for the development of effective approaches to bone regeneration. In this study, we analysed the osteoinductive properties of extracellular calcium in bone marrow-derived mesenchymal stem cell (BM-MSC) differentiation. We cultured BM-MSCs in 3D gelatin scaffolds with $Ca^{2+}$ and BMP-2 as osteoinductive agents. Early and late osteogenic gene expression and bone regeneration in a calvarial critical-size defect model demonstrate that extracellular $Ca^{2+}$ enhances the effects of BMP-2 on *Osteocalcin*, *Runx2* and *Osterix* expression and promotes bone regeneration *in vivo*. Moreover, we analyzed the molecular mechanisms involved and observed an antagonistic effect between $Ca^{2+}$ and BMP-2 on SMAD1/5, ERK and S6K signalling after24 hours. More importantly, a cooperative effect between $Ca^{2+}$ and BMP-2 on the phosphorylation of SMAD1/5, S6, GSK3 and total levels of β-CATENIN was observed at a later differentiation time (10 days). Furthermore, $Ca^{2+}$ alone favoured the phosphorylation of SMAD1, which correlates with the induction of *Bmp2* and *Bmp4* gene expression. These data suggest that $Ca^{2+}$ and BMP-2 cooperate and promote an autocrine/paracrine osteogenic feed-forward loop. On the whole, these results demonstrate the usefulness of calcium-based bone grafts or the addition of exogenous $Ca^{2+}$ in bone tissue engineering.

## INTRODUCTION

The combination of calcium-containing biomaterials and mesenchymal progenitors is regarded as a potential bone graft substitute in tissue engineering. In fact, bone is a heterogeneous biocomposite tissue that consists of an inorganic phase (essentially hydroxyapatite), an organic phase (mainly type I collagen) and cells [1] Physiological bone remodelling occurs when osteoblast precursors attach, differentiate and replace the bone previously resorbed by osteoclasts. Thus, osteoblast progenitors differentiate after exposure to multiple degradation products released by osteoclasts. The bone components released presented high extracellular calcium concentrations, collagen fragments and growth factors.

Functional type I collagen has a unique triple helical structure that uncoils during degradation to generate gelatin. This uncoiling exposes its RGD sequences, which promote integrin binding and cell attachment [2]. Unlike collagen, gelatin does not present antigenicity in physiological conditions [3]. These properties make gelatin an appropriate scaffold for use in 3D cell cultures [3]

$Ca^{2+}$ levels differ significantly in different bone niches. Calcium from bone matrix is released in its ionic form into the remodelling microenvironment [4]. For instance, in the hemivacuole, studies have reported values in the range of 8-40 mM, in contrast to the non-resorbing surface of the osteoclast, where values were < 2 mM during resorption [5]. This local increment of $Ca^{2+}$ concentration has a strong impact on the chemotaxis, proliferation and differentiation of osteoblasts [6]. Mechanistically, extracellular $Ca^{2+}$ activates calcium-sensing receptors (CaSR). CaSRs are G protein-coupled receptors that are expressed in mesenchymal stem cells and osteoblasts [7], although their functional role in bone homeostasis remains unclear. Furthermore, voltage-gated $Ca^{2+}$ channels increase intracellular $Ca^{2+}$ levels in osteoblasts [8]. The

maximum effects of extracellular $Ca^{2+}$ on osteoprogenitor migration and differentiation are achieved at concentrations in the range of 2-10 mM [6]. However, although the osteoconductive or osteoinductive properties of $Ca^{2+}$-containing scaffolds have been thoroughly exploited, little is known about their intracellular signal transduction in osteoblasts.

Osteogenic growth factors might be secreted by bone resident cells or released from bone matrix during bone remodelling. These factors that are released after resorption include several members of the TGFβ/BMP superfamily of osteogenic signals. TGFβ/BMP signals at the cell surface are transduced by the Smad family of transcription factors, which directly regulate gene expression and additional non-canonical pathways such as p38 MAP-kinase and phosphoinositide 3-kinase (PI3K)/AKT [9]. BMP target genes include a growing number of osteoblast-determining transcription factors such as *Dlx5*, *Runx2* and *Osterix* [10,11]. More importantly, the efficacy of BMPs in bone regeneration is well known in both animal models and clinical applications such as bone fracture healing [12], alveolar cleft defects [13], spinal fusion [14] and craniofacial bone defects [15,16]. As a result, BMP-2 and BMP-7 have been approved for medical use in specific osteoinductive applications.

Tissue engineered for bone regeneration should lead to the attachment, propagation, and differentiation of the transplanted cells. We combined bone marrow-derived mesenchymal stem cells (BM-MSCs) and 3D gelatin scaffolds with $Ca^{2+}$ and BMP-2 as osteoinductive agents. In an attempt to define the mechanisms of osteoinduction by $Ca^{2+}$, we first assessed the potential effects of $Ca^{2+}$ on osteoblast differentiation and bone formation and its cooperation with BMP-2. More importantly, we identified the molecular mechanisms involved in this cooperative osteogenic interaction. We propose that knowledge of these signalling events induced by $Ca^{2+}$

could benefit the field of bone tissue engineering.

## RESULTS

### Extracellular calcium increases osteogenic gene expression in BM-MSCs cultured in 3D gelatin scaffolds

In order to evaluate the influence of the culture system on the osteoblast differentiation of BM-MSCs, we compared three different culture models: untreated plastic surface, 2D gelatin-coated surface and 3D gelatin scaffold. Cells seeded on 3D gelatin scaffolds showed greater upregulation of all osteogenic markers evaluated (*Alpl* $p< 0.001$; *Osteocalcin* $p< 0.001$ and *Osterix* $p< 0.001$) than monolayers on plastic surfaces or 2D gelatin coated plates (Suppl. Fig. 1). This result suggests that 3D gelatin scaffolds promote higher osteoblast differentiation than plastic or 2D gelatin-coated surfaces.

We then assessed whether extracellular $Ca^{2+}$ could have a beneficial effect on osteoblast differentiation. We evaluated the effects of different $Ca^{2+}$ concentrations on BM-MSCs using the three culture systems described above. Higher expressions of *Alpl*, *Osteocalcin* and *Osterix* were obtained using $Ca^{2+}$ concentrations from 3 mM to 10 mM (Fig. 1). A concentration of 7.5 mM was optimal for the late osteogenic differentiation markers *Osteocalcin* and *Osterix*. Taken together, these results suggest that extracellular $Ca^{2+}$ concentrations of between 3 mM and 10 mM produce a beneficial effect on the expression of *Osteocalcin* and *Osterix*, regardless of the culture model used. We confirmed the specificity of these effects by comparing $CaSO_4$ and $CaCl_2$ as calcium ion sources or by chelating $Ca^{2+}$ with EDTA. Both $CaSO_4$ and $CaCl_2$ stimulated the expression of osteogenic markers. Moreover, the addition of EDTA completely blocked the positive effects of $CaSO_4$ and $CaCl_2$ on gene expression (Suppl. Fig. 2).

**Cooperation of calcium and BMP-2 in osteoblast differentiation and bone regeneration of calvarial critical-size defects in mice.**

We further evaluated whether $Ca^{2+}$ would cooperate with osteoinductive cytokines such as BMPs. BM-MSCs were cultured in a 3D gelatin scaffold and stimulated with 7.5 mM $CaSO_4$ and/or BMP-2 (2 nM). Incubation for 10 days with $CaSO_4$ or BMP-2 alone promoted expression of all the bone markers analysed (Fig. 2). More importantly, although a combination of $Ca^{2+}$ and BMP-2 did not present any additive effects on the expression of the early osteogenic marker *Alpl*, they produced a significant additive effect on the expression of *Osteocalcin*, *Runx2* and *Osterix* (Fig. 2). Thus, when added to culture media, $Ca^{2+}$ exerts a cooperative action with BMP-2 on late osteogenic marker expression.

To extend our *in vitro* results on the cooperation between $Ca^{2+}$ and BMPs to an *in vivo* context, we analysed bone formation in calvarial critical-size bone defects in mice. Five-millimetre defects were performed in parietal bones and further implanted with BM-MSCs previously seeded in 3D gelatin scaffolds and pre-treated for 48 hours with either 7.5 mM $CaSO_4$ or 2 nM BMP-2 alone or combined. After five weeks, skulls were retrieved and analysed for bone formation in the defect. Hematoxylin/eosin and Masson's trichrome stains showed dense connective tissue but no major bone formation in the control group. Higher levels of mineralisation and bone maturation were found in those implants treated with $CaSO_4$, and even greater bone formation took place when implants were pre-treated in combination with BMP-2 (Fig. 3). Moreover, a combination of $CaSO_4$ and BMP-2 led to a new, more mature bone structure. Both osteoblast and osteocytes can be observed in these bone regeneration areas (Fig. 3).

**Signalling pathways involved in the cooperation of calcium and BMP-2 during osteogenesis of BM-MSCs**

To determine the mechanisms of cooperation between extracellular calcium and BMP-2 in BM-MSCs differentiation, we analysed intracellular signalling triggered by both signalling molecules at early and late differentiation points. Early analysis was performed 24 hours after $CaSO_4$ and/or BMP-2 stimulation. As expected, BMP-2 promoted phosphorylation of SMAD1/5 and increased the levels of phosphorylated ERK1/2 (Fig. 4). By contrast, p38 and S6-kinase (S6K) signalling pathways were activated when cells were treated with $Ca^{2+}$ alone. It is worth noting that, at this initial differentiation stage, an antagonistic effect on each signalling pathways was obtained when $Ca^{2+}$ was added together with BMP-2 (Fig. 4).

The same intracellular components were subsequently assessed after treatment with a combination of $CaSO_4$ and/or BMP-2 for 10 days. A significant additive or cooperative effect between $Ca^{2+}$ and BMP-2 was observed on the phosphorylation levels of SMAD1/5 (Ser463-465), S6 (Ser235-236), GSK3β (Ser9) and the total levels of β-CATENIN (Fig. 5). Phosphorylation at Ser9 of GSK3β is mediated by AKT and results in the inhibition of its β-CATENIN repression action. Thus, these results suggest that extracellular calcium produces a differential time-dependent effect on BMP-2 and AKT signalling. A signalling network antagonistic to BMP-2 is activated early on, whereas $Ca^{2+}$ promotes a cooperative effect on several intracellular signalling events later on.

**Extracellular calcium promotes endogenous secreted BMP-2 and BMP-4 mRNA expression**

After 10 days of differentiation, BM-MSCs stimulated with calcium alone showed activation of the SMAD1/5 pathway (Fig. 5). Since there is no evidence that calcium activates BMP receptors directly, it could be suggested that the increased

availability of BMP receptor ligands was responsible. We therefore hypothesised that, once these cells are committed to the osteoblast lineage, $Ca^{2+}$ induces cells to secrete endogenous factors that reinforce differentiation through an autocrine/paracrine mechanism. We assayed whether $Ca^{2+}$ induced BMP-2 or BMP-4 expression. BM-MSCs cultured in 3D gelatin scaffolds were exposed to $CaSO_4$ concentrations (from 3 mM to 10 mM) for 10 days. An increase, which reached its maximum at 7.5 mM, was obtained for both *Bmp2* and *Bmp4* mRNA expression (Fig. 6). In addition, we also determined the mRNA levels of *Fgf21* (a tyrosine kinase receptor ligand that inhibits osteoblastogenesis [17]) and *Axin2* (a target of the Wnt/β-CATENIN pathway downstream of GSK3). A significant increase in *Axin2* expression was found, in line with *Bmp2* and *Bmp4* mRNA expression. By contrast, *Fgf21* mRNA expression was only slightly elevated, without any dose-response effect (Fig. 6). Taken together, these results demonstrate that BM-MSCs stimulated with $Ca^{2+}$ secrete higher levels of multiple critical cytokines that amplify their osteoblastic differentiation response.

## DISCUSSION

Different anatomical zones are used to obtain autogenous bone grafts to reconstruct bone defects. These sources include the iliac crest, cranial bone, mandibular symphysis, rib and tibia [18]. However, drawbacks include limited availability and morbidity at the donor site. To overcome these disadvantages, numerous tissue engineering approaches have been developed to take advantage of physiological osteoinductive signals [19]. Both $Ca^{2+}$ and BMP-2 are known to be co-released into the extracellular space by osteoclasts after bone matrix resorption. Our hypothesis was that extracellular $Ca^{2+}$ signals interact with BMP and lead to higher osteoblast differentiation and bone formation from BM-MSCs. Here, early and late osteogenic marker expression and histological assessment of bone regeneration in a calvarial critical-size defect model demonstrated that extracellular $Ca^{2+}$ enhances the effects of BMP-2 on *Osteocalcin*, *Runx2* and *Osterix* expression and promotes bone regeneration *in vivo*. More importantly, mechanistically, both osteoinductors combined cooperate to increase long-term activation of SMAD and AKT signalling.

Osteogenic gene expression was significantly higher when extracellular $Ca^{2+}$ was added, regardless of the cell culture system used. MG63 osteoblastic cells, both in monolayer culture and 3D gelatin hydrogels, have been reported to show significant mineralisation when cultured with 8 mM $Ca^{2+}$ [20]. Moreover, BM-MSCs treated simultaneously with two different sources of $Ca^{2+}$, $CaSO_4$ and $CaCl_2$, with or without EDTA, demonstrated that the osteogenic effect was specific for $Ca^{2+}$. This outcome is consistent with the EGTA inhibition of osteocalcin secretion [21] and BAPTA, an intracellular $Ca^{2+}$ chelator, in the response of osteoblasts to extracellular calcium [22].

Osteoinductive factors released from resident cells or after osteoclast bone resorption regulate the recruitment and differentiation of osteoblastic progenitor cells.

The binding of BMPs to their cognate receptors triggers canonical Smad and Smad-independent pathways, including ERK, p38 and PI3K/AKT signalling [9,23]. Several authors have reported that CaSR activation by extracellular $Ca^{2+}$ also activates these same pathways [4,22,24]. We found that both calcium and BMP-2 induce activation of common signalling components, but in a differential time-dependent response. An early antagonistic effect between $Ca^{2+}$ and BMP-2 signalling was demonstrated. This contrasting effect is consistent with previous reports showing a crosstalk between calcium signalling and BMP pathway in which high intracellular calcium inhibits BMP signalling [25]. Furthermore, $Ca^{2+}$/calmodulin dependent kinase II (CAMKII), a primary transducer of calcium ions, directly interacts with SMADs and antagonises their function [26]. This reverse effect could be reinforced when we also consider that MAPK signalling inhibits BMP signals at the level of SMAD1 [27]. Osteoblast differentiation is a multistep cascade of gene expression that initially supports proliferation and survival [28]. The early prominent proliferative and pro-survival role of the MAPK and AKT/S6K network could induce the enlargement of the osteogenic progenitor pool [4].

However, when late differentiation events were analysed, $Ca^{2+}$ and BMP-2 were found to cooperatively stimulate osteoblast differentiation through the strengthening of specific osteogenic signalling pathways. Increases in the phosphorylation of SMAD1/5, S6, GSK3β and expression of β-CATENIN were consistent with the significantly higher expression of *Osteocalcin*, *Runx2* and *Osterix* and the greater bone formation *in vivo* [29,30]. Cooperative crosstalk between $Ca^{2+}$ and BMP-2 in osteoblasts through the induction of the calcium-dependent transcription factor NFATc1 by BMP-2 has also been described recently [31]). NFAT transcription factors have proved necessary for osteoblast differentiation and bone formation [32]. Mechanistically, NFAT transcription factors activate osteogenesis through their interaction with OSX and their ability to

stimulate Wnt/β-CATENIN signalling [32,33]. Unexpectedly, cell cultures exposed to $Ca^{2+}$ alone for 10 days displayed significantly higher SMAD signalling. This observation correlated with the increase in *Bmp2*, *Bmp4* and *Axin2* gene expression. BM-MSCs, periodontal ligament cells and dental pulp cells exposed to calcium-derived biomaterials have been reported to induce the upregulation of *Bmp2* mRNA expression [8,34,35]. These studies implicated MAPK activity and AP-1 transcription factors in such effects [8,36]. Thus, our data support the suggestion that calcium induces an autocrine/paracrine loop by endogenous BMP upregulation. Furthermore, *Axin2* (a target gene of Wnt/β-CATENIN signalling downstream of GSK3β) was also expressed in long-term cell cultures. Our group recently showed that PI3K/AKT activity is relevant in bone formation *in vivo* and leads to the activation of SMAD1/5 and GSK3β/β-CATENIN signalling [30]. Several reports have demonstrated the synergistic interaction and the significance between BMP and Wnt during osteoblast differentiation and bone formation *in vitro* and *in viv*o [29,37]. Taken together, our results demonstrate a delayed calcium signalling effect that likely integrates and reinforces an osteogenic programme from multiple inputs.

## MATERIALS AND METHODS

### Isolation and culture of bone marrow-derived mesenchymal stem cells (BM-MSCs)

BM-MSCs were isolated from the femurs of 6-8 week old BALB/c mice, as previously described [37,38]. Mice were euthanised and the femurs dissected. Next, the soft tissues were cleaned and the femurs kept in complete media (DMEM supplemented with 10% FBS, penicillin/streptomycin, 1 mM pyruvate and 2 mM glutamine). The femur ends were cut using a rongeur and the bone marrow was flushed and collected. Cell suspension was filtered with a 70 μm cell strainer (Falcon, USA), transferred to a 100 mm cell culture plate and incubated at 37ºC. The media was changed after 24 hours and then every eight hours for two to three days to discard non-adherent cells. After five to seven days, when the adherent cells reached 75%-80% of confluence, the cells were washed three times with warmed PBS and trypsinised for three minutes at room temperature. The lifted cells were cultured and expanded for future experiments.

### 2D gelatin coating and 3D gelatin scaffold preparation

Twelve-well plate surfaces were coated with a thin layer of 0.1% gelatin/PBS solution. Plates were left for one hour inside a laminar flow hood to dry the treated surfaces and then stored until use. To prepare 3D scaffolds, a 1 $mm^3$ gelatin sponge (Gelita, B. Braun) was used. Sponges were cut into 1 mm thick slices for *in vitro* assays, whereas 2 mm slices were used for the *in vivo* experiments. Scaffolds were soaked in complete media and incubated for 12-24 hours at 37ºC. The BM-MSCs (two $10^5$ cells in 20 μl of media per scaffold) were then seeded under sterile conditions into the gelatin scaffold, and everything was introduced into a microcentrifuge tube and incubated in a vertical position for four to six hours at 37ºC. This individual seeding allowed equal cell attachment to be achieved in each scaffold. To assess the effect of extracellular calcium

on the osteoblast differentiation of BM-MSCs, $CaSO_4$ or $CaCl_2$ (Sigma-Aldrich) was used as source of $Ca^{2+}$. BM-MSCs seeded on 3D gelatin scaffolds were cultured in the presence of different $CaCl_2$ or $CaSO_4$ concentrations, which ranged from 3 mM to 10 mM. EDTA was used as a calcium chelator with a final concentration of 7.5 mM. Fresh media (100 µl) was added to the corresponding condition every three days.

**Gene expression analysis by RT-qPCR**

RNA isolation was performed using Trisure (Bioline Reagents, UK) in accordance with the manufacturer's protocol. The purified RNA was quantified using a spectrophotometer (Nanodrop). Two micrograms of RNA were retrotranscribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in accordance with the manufacturer's protocol. The gene expression of *Alpl*, *Osteocalcin* (*Bglap2*), *Runx2*, *Osterix*, *Bmp2, Bmp4*, *Fgf21* and *Axin2* was analysed using Taqman probes (Applied Biosystems) and normalised to *Gapdh* expression.

**Western blot assay**

BM-MSCs seeded in 3D gelatin scaffolds were cultured for 24 hours or 10 days. Cells were lysed with 75 µl of lysis buffer (PBS, 1% Triton X-100, 100 mM PMSF, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 mM of sodium orthovanadate, 10 mM NaF and 10 mM β-glycerophosphate) for one hour at 4ºC. Thirty micrograms of protein samples were subjected to SDS-PAGE and immunoblotting. Membranes were incubated with different antibodies: pGSK3α/β Ser9/21 (9331S), pSMAD1/5/8 Ser465/467 (9511S) and pS6 Ser235/236 (2211) and pp38 Thr180/Tyr182 (9211S) from Cell Signaling Technology, pErk1/2 (M5670) from Sigma, β-catenin (610154) from BD Transduction Laboratories and α-tubulin (T6199) from Sigma, all diluted to a ratio of 1:1000. Horseradish peroxidase-conjugated secondary antibodies were used, followed by

incubation with EZ-ECL reagent (Biological Industries). A chemiluminescent image of the immunoblots was captured with a Fujifilm LAS 3000 device.

**Calvarial critical-size bone defects and in vivo bone regeneration**

A surgical procedure was performed in 10-week old male BALB/c mice. Animals were housed individually and fed *ad libitum*. All procedures were performed in accordance with the protocols approved by the University of Barcelona Animal Research Ethics Committee and the Generalitat de Catalunya. Animals were anesthetised with isoflurane inhalation and an intraperitoneal injection of buprenorphine (0.05 mg/kg) was administered to provide intraoperative analgesia. To expose the parietal bones, a longitudinal midline incision was made and the tissues retracted. A circular critical-size bone defect with an outer diameter of 5 mm was carried out with a trephine bur on the left parietal. We cultured four $10^5$ BM-MSCs per scaffold in accordance with the protocol described above for 3D gelatin scaffold preparation. After cells had been exposed to the respective conditions for 48 hours, a 1% final concentration solution of low melting agarose at 36ºC was added as a bonding agent. Scaffolds were implanted to fill the bone defects, depending on the respective experimental group. The incised tissues were sutured and the animals monitored daily during the recovery phase. Five weeks after surgery, the animals were euthanised and the calvariae dissected.

**Histological analysis**

After skull fixation with 4% paraformaldehyde for 48 hours, the calvariae were decalcified with a Decalcifier II (Leica) for two days. They were then dehydrated, embedded in paraffin and cut into 6 µm sections to examine bone formation. Slides

were stained using hematoxylin/eosin and Masson's trichrome technique. Histological sections were evaluated using a light microscope (Nikon Eclipse E800).

**Statistical analysis**

Data were was obtained from at least three independent experiments and presented as mean ± SEM. The analysis was performed by Student's *t*-test using GraphPad software. Differences were considered significant at p values: #,* $p < 0.05$, ##,** $p < 0.01$, and ###,***$p < 0.001$.

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## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: RAM, JLR, FV. Performed the experiments: RAM, NA, BG, FV. Analyzed the data: RAM, NA, BG, JLR, FV. Contributed reagents/materials: PB. Wrote the paper: RAM, FV.

## DISCLOSURES

All authors state that they have no conflicts of interest.

## FIGURE LEGENDS

**Figure 1. Extracellular calcium induces expression of osteogenic markers on bone marrow mesenchymal stem cells**. Three different culture models were compared (cells in monolayer in plastic surface, cells in monolayer in gelatin-coated dishes and cells in 3D gelatin scaffolds). After 10 days, the mRNA expression of *Alpl*, *Osteocalcin* (*Bglap2*) and *Osterix* was analysed and normalised to *Gapdh* levels (n=3).

**Figure 2. Extracellular calcium increases the effects of BMP-2 on osteogenic marker expression**. Primary BM-MSCs were cultured on 3D gelatin scaffolds with 7.5 mM of $CaSO_4$ and/or 2 nM of BMP-2 for 10 days. The mRNA expression of *Alpl*, *Osteocalcin* (*Bglap2*), *Runx2* and *Osterix* was analysed and normalised to *Gapdh* levels (n=3).

**Figure 3. Extracellular calcium increases the effects of BMP-2 on bone regeneration *in vivo***. Calvarial critical-size bone defects were generated in mice and implanted with cells cultured in 3D scaffolds pre-treated with 7.5 mM of $CaSO_4$ and/or 2 nM of BMP-2 for 48 hours. After five weeks, the implanted constructs were retrieved and processed for hematoxylin/eosin and Masson's trichrome staining. Scale bar, 200 μm.

**Figure 4. Early effects of extracellular calcium on cell signalling**. Cells were cultured in 3D gelatin scaffolds with $Ca^{2+}$ (7.5 mM) and/or BMP-2 (2nM) for 24 hours and extracts analysed by Western blot. Data was quantified relative to the levels of α-TUBULIN (n=3).

**Figure 5. Late effects of extracellular calcium on cell signalling**. Cells were cultured in 3D gelatin scaffolds with $Ca^{2+}$ (7.5 mM) and/or BMP-2 (2nM) for 10 days. Data was quantified relative to the levels of α-TUBULIN (n=3). Differences were considered significant at p values: * $p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$ when compared to

control and # $p < 0.05$, ## $p < 0.01$, and ###$p < 0.001$ when compared to cells treated with BMP-2 and $CaSO_4$.

**Figure 6. Extracellular calcium induces *Bmp2*, *Bmp4*, *Fgf21* and *Axin2* mRNA expression**. BM-MSCs were cultured on 3D gelatin scaffolds with different extracellular calcium concentrations for 10 days. The mRNA expression of *Bmp2*, *Bmp4*, *Fgf21* and *Axin2* was analysed and normalised to the levels of *Gapdh* (n=3).

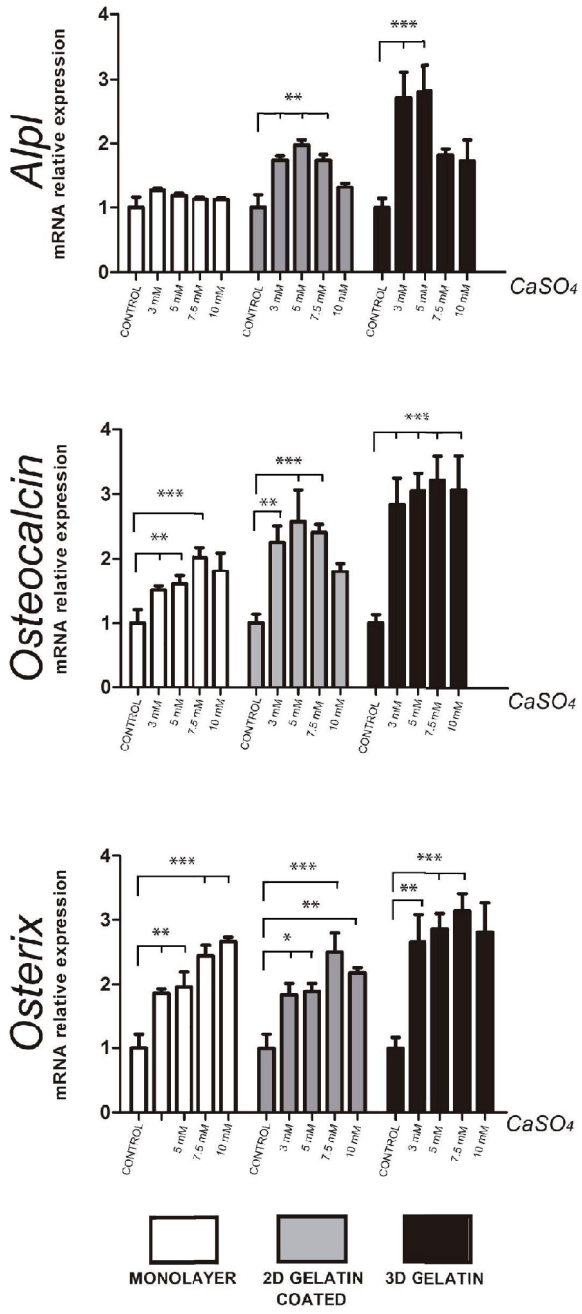

Alpl
mRNA relative expression
Osteocalcin
mRNA relative expression
Osterix
mRNA relative expression
CONTROL
3 mM
5 mM
7.5 mM
10 mM
CaSO4
MONOLAYER
2D GELATIN COATED
3D GELATIN

FIGURE 1

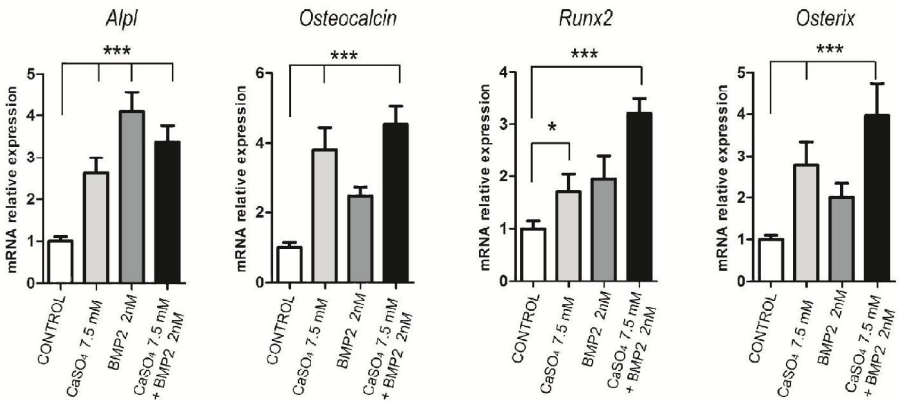

Alpl
Osteocalcin
Runx2
Osterix
mRNA relative expression
***
*
CONTROL
CaSO4 7.5 mM
BMP2 2nM
CaSO4 7.5 mM + BMP2 2nM

FIGURE 2

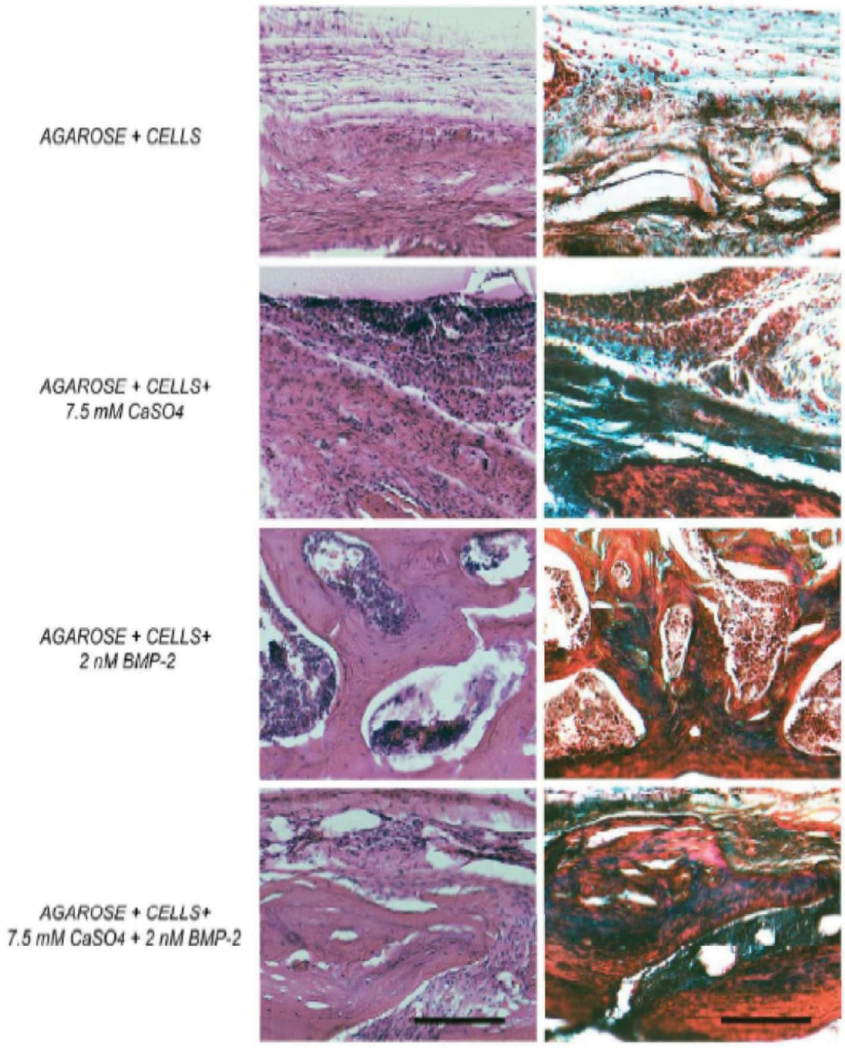

AGAROSE + CELLS
AGAROSE + CELLS+
7.5 mM CaSO4
AGAROSE + CELLS+
2 nM BMP-2
AGAROSE + CELLS+
7.5 mM CaSO4 + 2 nM BMP-2

FIGURE 3

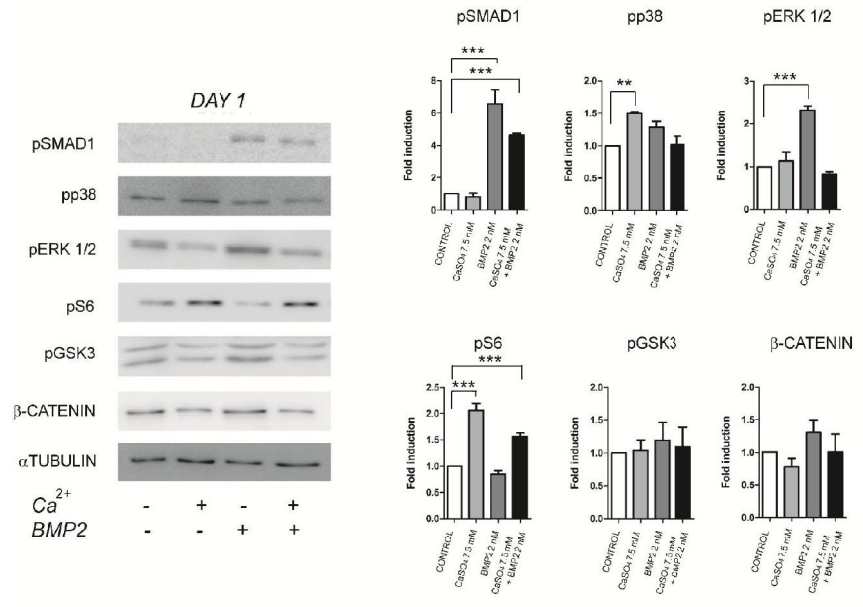

DAY 1
pSMAD1
pp38
pERK 1/2
pS6
pGSK3
β-CATENIN
αTUBULIN
Ca2+ - + - +
BMP2 - - + +
pSMAD1
pp38
pERK 1/2
pS6
pGSK3
β-CATENIN
Fold induction
CONTROL
CaSO4 7.5 mM
BMP2 2 nM
CaSO4 7.5 mM + BMP2 2 nM

FIGURE 4

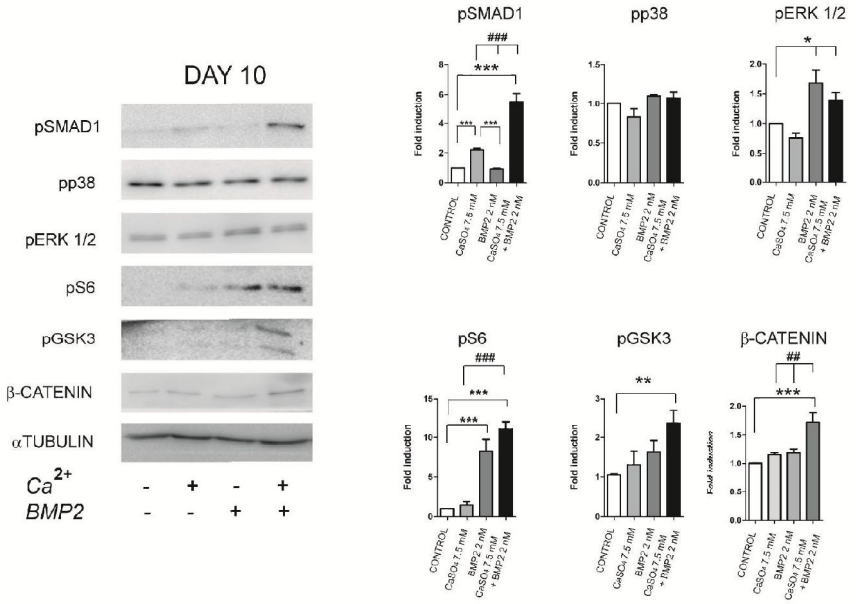

DAY 10
pSMAD1
pp38
pERK 1/2
pS6
pGSK3
β-CATENIN
αTUBULIN
Ca2+ - + - +
BMP2 - - + +
pSMAD1
pp38
pERK 1/2
pS6
pGSK3
β-CATENIN
Fold induction
CONTROL
CaSO4 7.5 mM
BMP2 2 nM
CaSO4 7.5 mM + BMP2 2 nM

FIGURE 5

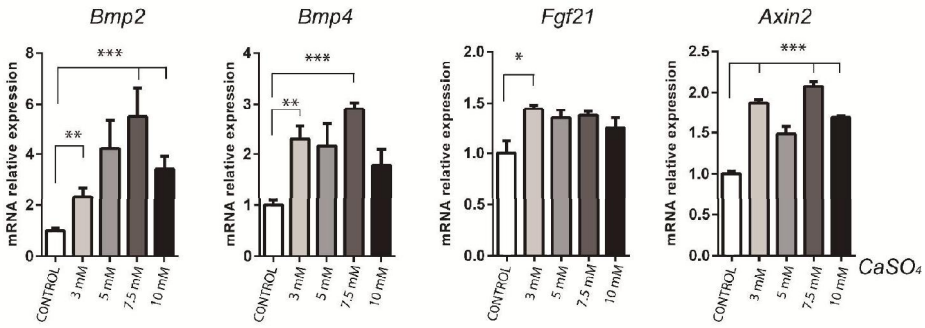
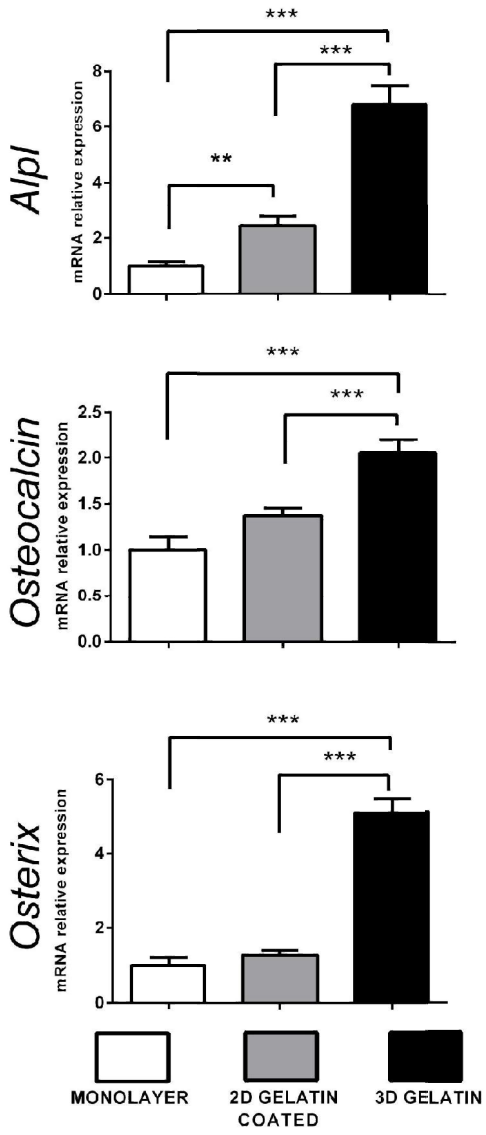
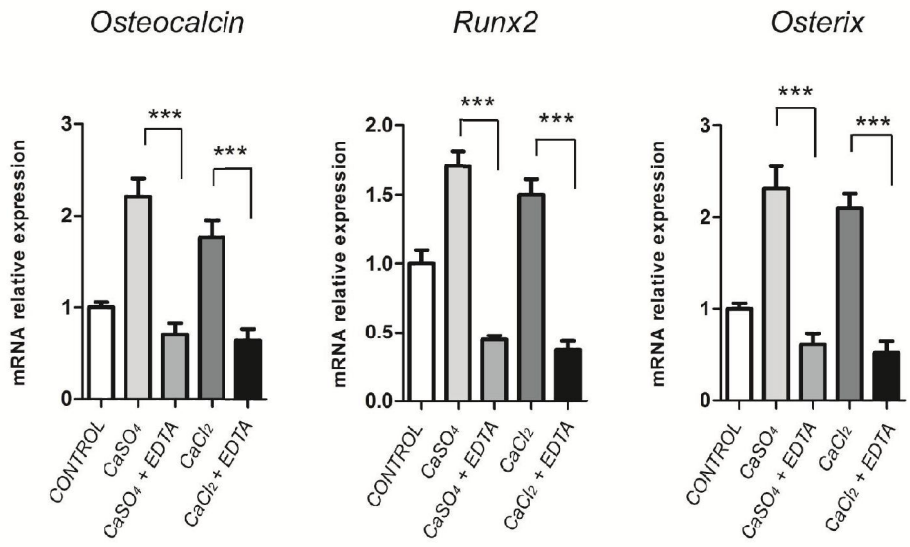

Bmp2
Bmp4
Fgf21
Axin2
mRNA relative expression
CONTROL
3 mM
5 mM
7.5 mM
10 mM
CaSO4

FIGURE 6




SUPPL. FIGURE 1




SUPPL. FIGURE 2