
Bone Engineering: A Matter of Cells, Growth Factors and Biomaterials

José A. Andrades, Lucía Narváez-Ledesma,
Luna Cerón-Torres, Anyith P. Cruz-Amaya,
Daniel López-Guillén, M. Laura Mesa-Almagro and
José A. Moreno-Moreno

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<http://dx.doi.org/10.5772/56389>

1. Introduction

1.1. A connective tissue

Bone is a highly specialized form of connective tissue that is nature's provision for an internal support system in all higher vertebrates. It is a complex living tissue in which the extracellular matrix (ECM) is mineralized, conferring marked rigidity and strength to the skeleton while still maintaining some degree of elasticity. In addition to its supportive and protective organic ions, it actively participates in maintaining calcium homeostasis in the body.

Bone is composed of an organic matrix that is strengthened by deposits of calcium salts. Type I collagen constitutes approximately 95% of the organic matrix; the remaining 5% is composed of proteoglycans and numerous noncollagenous proteins. The crystalline salts deposited in the organic matrix of bone under cellular control are primarily calcium and phosphate in the form of hydroxyapatite (HA).

Morphologically there are two forms of bone: cortical (compact bone) and cancellous (spongy bone). In cortical bone, densely packed collagen fibrils form concentric lamellae, and the fibrils in adjacent lamellae run in perpendicular planes as in plywood. Cancellous bone has a loosely organized, porous matrix. The differences between cortical and cancellous bone are both structural and functional. Differences in the structural arrangements of the two types are related to their primary functions: cortical bone provides the mechanical and protective functions and cancellous bone provides the metabolic functions.

1.2. Bone cell structure and function

Bone is composed of four different cell types (Fig. 1). Osteoblasts, osteoclasts, and bone lining cells are present on bone surfaces, whereas osteocytes permeate the mineralized interior. Osteoblasts, osteocytes, and bone lining cells originate from local osteoprogenitor cells, whereas osteoclasts arise from the fusion of mononuclear precursors, which originate in the various hemopoietic tissues.

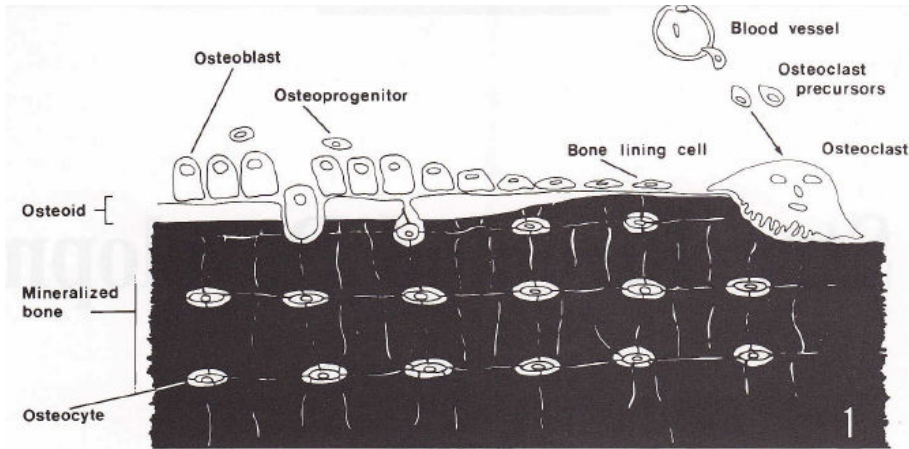


Figure 1. The origins and locations of bone cells. Taken from Academic Press Inc., with permission

Osteoblasts are the fully differentiated cells responsible for the production of the bone matrix. Portions of four osteoblasts are shown in Figure 2. An osteoblast is a typical protein-producing cell with a prominent Golgi apparatus and well-developed rough endoplasmic reticulum. It secretes the type I collagen and the noncollagenous proteins of the bone matrix.

The staggered overlap of the individual collagen molecules provides the characteristic periodicity of type I collagen in bone matrix. Numerous noncollagenous proteins have been isolated from bone matrix (Sandberg, 1991) but to date there is no consensus for a definitive function of any of them. Osteoblasts regulate the mineralization of bone matrix, although the mechanism(s) is not completely understood. In woven bone, mineralization is initiated away from the cell surface in matrix vesicles that bud from the plasma membrane of osteoblasts. This is similar to the well-documented role of matrix vesicles in cartilage mineralization (Hohling et al., 1978). In lamellar bone, the mechanism of mineralization appears to be different. Mineralization begins in the hole region between overlapped collagen molecules where there are few, in any, matrix vesicles (Landis et al., 1993) and appears to be initiated by components of the collagen molecule itself or noncollagenous proteins at this site. Whatever the mechanisms of mineralization, collagen is at least a template for its initiation and propagation and there is always a layer of unmineralized bone matrix (osteoid) of the surface under osteoblasts. Matrix deposition is usually

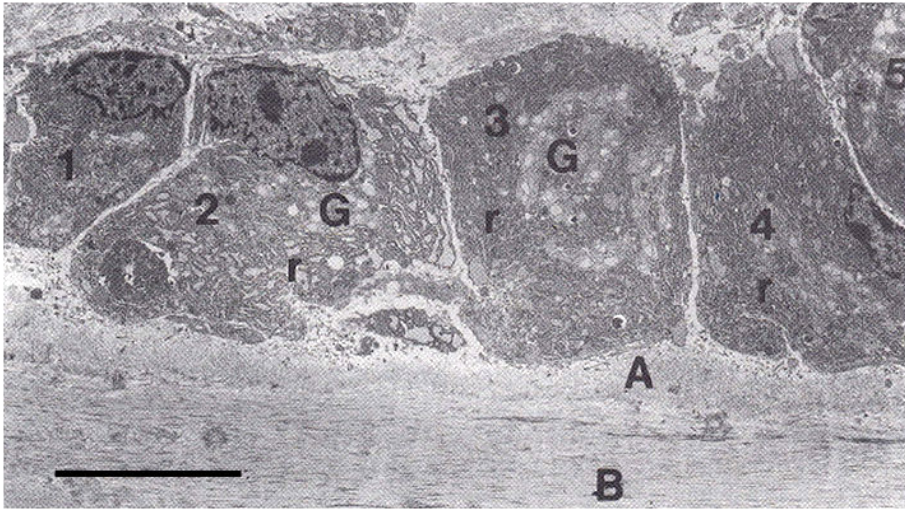


Figure 2. Transmission electron micrograph of osteoblasts (numbered) on a bone surface in which the collagenous matrix has been deposited in two layers (A and B) at right angles to each other. The Golgi apparatus (G) and rough endoplasmic reticulum (r) are prominent cytoplasmic organelles in osteoblasts. (Original magnification x2800, bar 0.1 μm). Taken from Academic Press Inc., with permission

polarized toward the bone surface, but periodically becomes generalized, surrounding the osteoblast and producing the next layer of osteocytes. Deposition of mineral makes the matrix impermeable and to ensure a metabolic lifeline, osteocytes establish numerous cytoplasmic connections with adjacent cells before mineralization.

The osteocyte is a mature osteoblast within the bone matrix and is responsible for its maintenance (Buckwalter et al., 1995). These cells have the capacity not only to synthesize, but also to resorb matrix to a limited extent. Each osteocyte occupies a space, or lacunae, within the matrix and extends filopodial processes through canaliculi in the matrix to contact processes of adjacent cells by means of gap junctions. Because diffusion of nutrients and metabolites through the mineralized matrix is limited, filopodial connections permit communication between neighbouring osteocytes, internal and external surfaces of bone and with the blood vessels traversing the matrix. The functional capacities of osteocytes can be easily ascertained from their structure.

Bone lining cells are flat, elongated, inactive cells that cover bone surfaces that are undergoing neither bone formation nor resorption. Because these cells are inactive, they have few cytoplasmic organelles. Little is known regarding the function of these cells; however, it has been speculated that bone lining cells can be precursors for osteoblasts.

Osteoclasts are large, multinucleated cells which resorb bone. When active, osteoclasts rest directly on the bone surface and have two plasma membrane specializations: a ruffled border and a clear zone. The ruffled border is the central, highly infolded area of the plasma membrane where bone resorption takes place. The clear zone is a microfilament-rich, organelle-free area

of the plasma membrane that surrounds the ruffled border and serves as the point of attachment of the osteoclast to the underlying bone matrix. Active osteoclasts exhibit a characteristic polarity. Nuclei are typically located in the part of the cell most removed from the bone surface and are interconnected by cytoskeletal proteins (Watanabe et al., 1995). Osteoclasts contain multiple circumnuclear Golgi stacks, a high density of mitochondria, and abundant lysosomal vesicles that arise from the Golgi and cluster near the ruffled border.

When a fracture occurs, a set of signals is triggered. These are both local signals and systemic ones; some of these signals are mediated by neuronal impulses (Nordsletten et al., 1994), by the haematoma at the site of the fracture and by the trauma caused to the tissues surrounding the fracture (Einhorn, 1998). These signals can be divided into two interactive and interchangeable categories: inflammatory signals and bone building signals. These factors mitigate the migration of phagocytotic cells to the area of the fracture, removing the necrotic tissue and propagating the in-growth of new blood vessels to the site of the fracture, thus providing nutrients and cells to the fracture site and starting the healing cascade. If at the end of the healing process osteo-integration (of the new bone together with the native bone) is not achieved, even with the best type of scaffolds, the chances of long-term success are dismal (Avila et al., 2009).

2. Bone tissue engineering

2.1. The basic concepts

Today great hope is set on Regenerative Medicine in all medical fields and, of course, it has developed to be of interest in orthopedics, being bone defects one of the main focus. In the last two decades, Regenerative Medicine approaches have been extensively studied to improve bone healing, or even generate functional bone tissue to substitute lost bone in orthopedics, neurosurgery, and dentistry. These types of studies include two different strategies of cell-based therapy: in the first approach, called Cell Therapy, cells are applied to substitute damaged cells within a tissue to reconstitute its integrity and function. The second approach, called Tissue Engineering, is more complex and encompasses three approaches: bioactive molecules (growth factors (GFs), cytokines, ECM compounds and hormones) that encourage tissue induction; cells and cell substitutes that will respond to the signals; the seeding of cells into three dimensional matrices, with specific adhesion properties and degradation rates, to compose a tissue-like construct to substitute lost parts of the tissue, or even organs; and a good nutritious support (angiogenesis) (Fig. 3).

Stem cells (SCs) are of particular interest in Regenerative Medicine, since they inhere several unique characteristics that distinguish them from other cell types. SCs represent unspecialized cells, which have the ability to differentiate into cells of all three germ layers, different adult cell types, and represent the only cell type which has the ability to renew itself indefinitely. It is important to distinguish embryonic stem cells (ESCs), which are truly pluripotent from multipotent adult stem cells and only found in early developmental stages of the organism. The successful dedifferentiation of somatic cells into a pluripotent ESC-like status by trans-

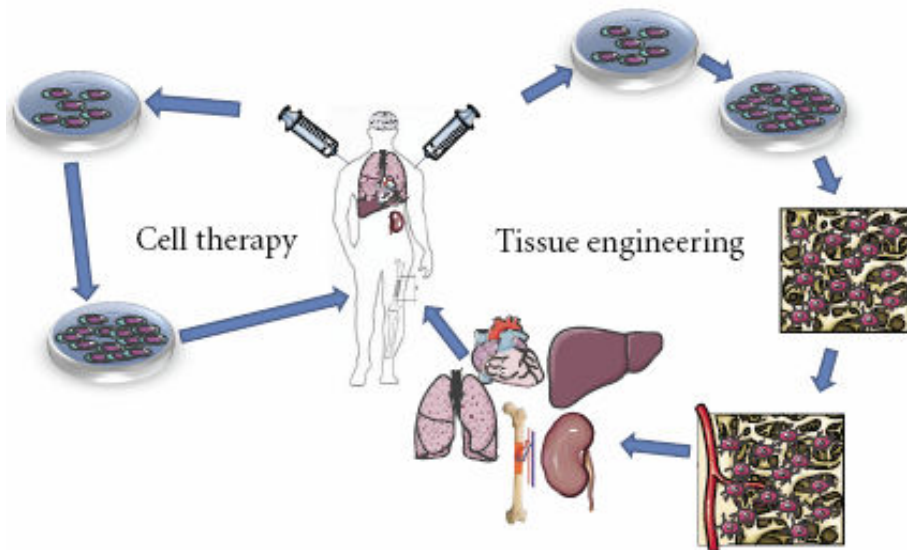


Figure 3. The two strategies of stem cell application in Regenerative Medicine. Stem cells are either isolated from the patient (autologous transplantation) or from other donors (allogeneous transplantation). The cells are expanded *in vitro* and either applied directly to the patient to substitute lost cells (Cell Therapy), or seeded into 3 dimensional scaffolds (Tissue Engineering) and differentiated into the demanded cell type. The composed artificial tissue construct is subsequently implanted into patients' tissue defect. Taken from A. Schmitt et al. 2012, with permission

fection with four embryonic transcription factors, the so-called induced pluripotent stem cells (iPS cells), provide the possibility of autologous therapy with pluripotent and easily accessible cells in the future. Beside the great potential this technique undoubtedly represents, it bears some essential safety problems which are currently far from being solved. In contrast, a variety of multipotent adult SCs exists in assumedly all tissues of the organism. They are responsible for maintaining the integrity of the tissue they reside in. Usually, these adult SCs show limited differentiation potential to tissues of one germ layer.

Bone regeneration is a physiological process which can be observed in healing fracture and continuous remodeling along by adult life. Bone holds the most regenerative ability of human tissues contained on the mayor source of osteogenic cells capable for forming bones, the bone marrow (BM). However, a loss considerable amount of bone due to any anomalies such as severe trauma, skeletal deformations, bone tumor resection or periprosthetic osteolysis can obstruct this capacity. Nevertheless, the capacity of proliferation and differentiation of BMSCs, as well as cells concentration, are reduced with the increase of the age of the patient. Many *in vitro* studies were performed to investigate applicability of different SC types for bone regeneration. Here, promising capacity for differentiating towards bone cells, enhancing bone healing and vascularization could be proven for ESCs and different adult mesenchymal stem cells (MSCs). However, due to the ethical and safety concerns, which currently forbid applications of ESCs or iPS cells in patients, we will focus on adult stem cells for therapeutic

applications. Therefore, MSCs presently seem to be the most promising candidates for bone regeneration, due to their excellent osteogenic differentiation capacity. They can be isolated from a number of adult mesenchymal tissues, among others, umbilical cord blood, peripheral blood, placenta, synovial fluid, adipose tissue, skeletal muscle or BM, as mentioned, where they contribute to normal tissue turnover and repair. Recently, the multitude of cell surface markers used in various studies has been limited to a marker panel representing, in addition to plastic adherence and differentiation capacity, the minimal criteria for the identification of MSCs. The molecular mechanisms of human MSCs regulation and the importance of specific GFs during the different stages of osteogenic differentiation, as well as the secreted signaling proteins known as *Wnts*, implicated in various differentiation programs including osteogenesis, are subjects of intensive research right now. Several studies have demonstrated improved results of MSCs therapy with genetically modified cells which produce osteogenic and angiogenic GFs in a local delivery of therapy strategy for bone healing. Also, there is recent information about the use of endothelial progenitor cells (EPCs) that improves the treatment of fracture and bone regeneration.

Besides their unique ability to differentiate into different cell types, MSCs secrete a variety of cytokines, showing anti-inflammatory activity and create an anabolic microenvironment. Furthermore, direct cell-cell contact immunomodulation has also been shown. On one hand, they indirectly influence tissue regeneration by secretion of soluble factors. On the other hand, they are able to modulate the inflammatory response. The differentiation potential of MSCs in bone engineering has been extensively studied *in vitro* and *in vivo*. By first time, Urist (1965), and Reddi and Huggins (1972) showed the capacity of a molecule, called bone morphogenetic protein (BMP), with potent osteoinductive properties in healing fractures and bone regeneration. Their experiments demonstrated the presence of osteoinductive cytokines in bone matrix that have abilities to induce MSCs differentiation into osteoblasts. The GFs, also including transforming growth factor-beta (TGF- β), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) or insulin-like growth factor (IGF), among others, are delivered of paracrine or autocrine manner, generating a chemotaxis process toward MSCs by recruitment, which induce their differentiation.

Extracellular matrix (ECM) is the native scaffold in most tissues. Besides the direct injection in the surrounding tissue, biomaterials are frequently used as carriers for cells, bioactive molecules and drugs. These materials have to be immune-compatible and nontoxic, whereas the bio-degradation process must neither release toxic substances nor tissue-toxic concentrations of degradation products. Scaffolds must be of three-dimensional structures, with great influence on cell growth and differentiation, and must be highly porous with interconnected pores of a diameter of at least 100 μm to allow ingrowth of cells and vessels. Despite the tissue engineering of bone, for which various inorganic materials, such as HA, calcium phosphate, calcium carbonate (due to their similarity to bone mineral, as well as their inherent biocompatibility and osteoconductivity), or glasses was tested, mainly organic biomaterials have been investigated for scaffold production. These are either naturally derived, for example, collagen, fibrin, agarose, alginate, gelatin, silk or hyaluronic acid; or produced synthetically, such as polyhydroxyacids. Since natural bone consists of an ECM with nanosized apatitic minerals

and collagen fibers that support bone cell functions, it is of interest to manufacture a synthetic biomimetic scaffold to i) contain nano-apatite crystals, together with fibers to form a matrix that supports cell attachment; ii) have mechanical properties similar to those of bone; and iii) encapsulate and support cells for osteogenic differentiation. Different methods have been employed in the fabrication of nanomaterials for bone engineering, such as the principle of electrospinning, that produce a variety of synthetic biomaterials, or the novel thermally induced phase separation (TIPS) technique to fabricate nanofibers to mimic natural collagen fibers. Rapid developments in this field of nanotechnology will be a key for many clinical benefits in the field of bone tissue engineering. The main advantage is that several novel biomaterials can be fabricated into nanostructures that closely mimic the bone in structure and composition. The optimization in the surface features of scaffolds has strongly improved cell behavior in terms of adhesion, proliferation, differentiation and tissue formation in three dimensions.

3. Stem cells as source

The popularity of SCs in the clinical arena has significantly increases, given the rapid improvement in our understanding of their biology. Classically, SCs are defined by their capacity to retain an undifferentiated state for a prolonged period while retaining the potential to differentiate along one lineage (unipotent), multiple lineages (multipotent), or into all three germ layers (pluripotent) (Young, 2003). These cells can be broadly categorized into two major classes: embryonic and adult SCs.

Embryonic stem cells (ESCs), isolated from the inner cell mass of the blastocyst, are pluripotent cells with the potential of differentiating into tissues from all three germ layers (Fig. 4) (Buehr et al., 2008).

While ESCs have significant regeneration capacity, their clinical application has been limited as a result of multiple factors including: 1) a propensity to form teratomas, 2) ethical concerns with isolation, 3) rejection by the host immune system after transplantation, and 4) the use of a feeder layer to retain an undifferentiated state *in vitro* (Cho et al., 2010). Recently discovered, another source of pluripotent SCs are induced pluripotent stem (iPS) cells, derived from somatic cells treated with few defined factors (Hamilton et al., 2009). While iPS cell-based therapy has the potential to revolutionize the field of Regenerative Medicine, many obstacles must be overcome before their clinical application can be realized (Lengner et al., 2010).

3.1. Mesenchymal stem cells as candidates

Furthermore, naturally occurring adult SCs have also been identified and categorized into their hematopoietic stem cells (HSCs), a source of various hematopoietic cell lineages, and nonhematopoietic SCs, which can give rise to cells of mesenchymal origin (de Barros et al., 2010). Many reports have suggested that these nonhematopoietic SCs, also known as mesenchymal stem cells (MSCs), can be isolated from a wide variety of adult tissues such as blood, adipose, skin, mandible, trabecular bone, fetal blood, liver, lung and even the umbilical cord and placenta

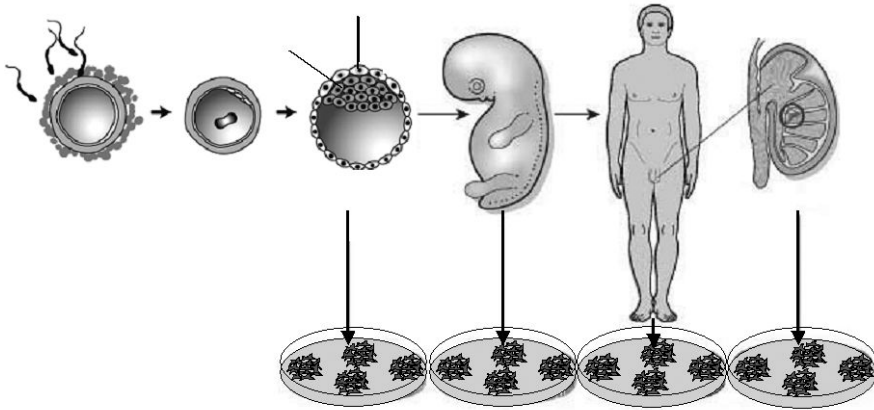


Figure 4. Origin of the different types of stem cells available. Derivation of embryonic stem cells (ESCs), embryonic germ cells (EGCs) and adult stem cells (SCs). Taken from M.E. Arias and R. Felmer 2009, with permission

(Steinhardt et al., 2008). The wide range of sources, methods, and acronyms are standardized by the International Society for Cellular Therapy in 2005.

Upon harvest, these cells can be expanded *in vitro* with high efficiency without sacrificing differentiation capacity (Kassem, et al., 2004). While these multipotent progenitor cells share many similar characteristics, they can be differentiated based on their expression profile and differentiation propensity along various lineages (Wagner et al., 2005). Amongst the various sources, MSCs isolated from the BM are considered to have the greatest potential for multilineage differentiation and have been the most characterized (Kuznetsov et al., 2009).

MSCs were initially described by Friedenstein and colleagues more than 40 years ago as adherent cells, with a fibroblast-like appearance capable of differentiating *in vitro* into osteoblasts, chondroblasts, adipocytes, and tenocytes (Friedenstein et al., 1968; Alonso et al., 2008; Prockop et al., 2009; Andrades et al., in press (a)). Unlike ESCs, MSCs provide the flexibility of autologous transplantation, circumventing ethical concerns or immunological rejection (Igura et al., 2004). These cells also play a sentinel role in proliferation and differentiation of hematopoietic cells (Briquet et al., 2010). Mankani et al. illustrated that the formation of both hematopoiesis and mature bone organ is correlated with the high local density of MSCs (Mankani et al., 2007). Additionally, MSCs are considered to be immune privileged and have the capacity for allogeneic transplantation a property has been used in the clinical setting for the treatment of various autoimmune diseases (Le Blanc et al., 2008). While many studies have suggested that MSCs are immunoprivileged and do not undergo rejection, others have cast doubt on this notion, showing that in certain scenarios, MSCs induce immune rejection (Nauta

et al., 2006) (Fig. 5). More investigations should be conducted to provide further insight into the specific interaction between these progenitor cells and the host immune system.

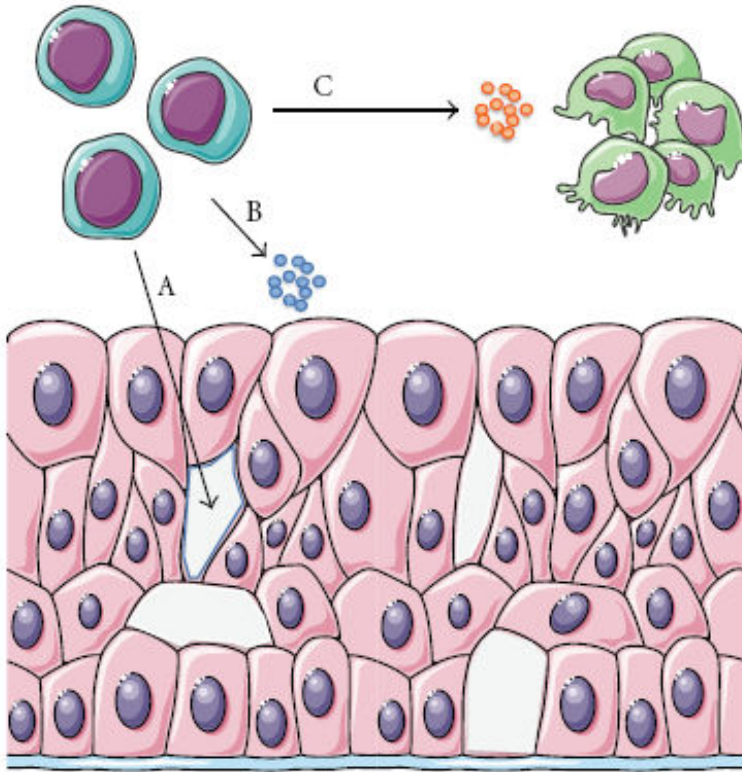


Figure 5. Stem cells participate in tissue regeneration in different ways. They directly differentiate into tissue-specific cells and thus substitute damaged or lost cells (A). They indirectly influence tissue regeneration by secretion of soluble factors. Here they promote vascularization, cell proliferation, differentiation within the tissue (B) and modulate inflammatory processes (C). Taken from A. Schmitt et al. 2012, with permission

Considerable effort has been put forth to identify specific surface markers that characterize MSCs, yet disagreement within the literature has prevented the creation of definitive standards. The minimal criteria identified by the International Society for Cellular Therapy for identifying MSCs requires the cells: 1) to be plastic adherent while maintained in cell culture; 2) to express CD73, CD90, and CD105, and lack expression of CD11b, CD14, CD19, CD34, CD45, CD79-alpha, and HLA-DR; and 3) to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro* (Dominici et al., 2006; Claros et al., 2008; 2012). Additional studies have also suggested that CD146 is considered an important marker of BM progenitor cells (Sorrentino et al., 2008). These guidelines were set in place to enable a unified approach for comparison amongst different studies.

BM is generally considered milieu plentiful for various cell types, collectively referred to as stromal cells. Amongst these, the multipotent subset of MSCs comprises a small fraction (<0.01) (Dazzi et al., 2006), yet despite their small numbers, the relative ease with which MSCs can be harvested has propelled their experimental use. Researchers have pioneered the creation of stable animal models aimed at mimicking human conditions to study the therapeutic capacity of these BM-derived cells (Kadiyala et al., 1997). Because of their ubiquity, tolerance of expansion, paracrine capabilities, and multipotency, the potential for clinical applications of MSCs in the orthopaedic realm is countless (Becerra et al., 2011).

The first problem that arises when Cell Therapy methods are used to rebuild bone tissue is how to obtain a sufficiently large number of osteocompetent cells for the intervention to be successful. Hence, the idea of using SCs, which are self-renewing and differentiate into various tissues, surfaced.

4. Direction by growth factors

Growth factors (GFs) serve a critical role in Regenerative Medicine, facilitating tissue growth *in vitro* and repair *in vivo*. In the case of skeletal tissues, they are being used to regulate chemotaxis, proliferation, and differentiation of MSCs. Also, selected hormones, cytokines, and nutrients are potentially useful in controlling MSCs growth.

A GF is a signaling biomolecule, commonly polypeptide, that is not a nutrient. Typically they act as signaling molecules via binding to specific receptors on the same cells that secrete the factors (autocrine signaling) or on neighboring cells (paracrine signaling). The binding of the receptor initiates a cascade of cellular reactions, often involving the activation of specific gene transcription. These cellular activities lead to alterations in cell proliferation, differentiation, maturation, and production of other GFs and ECM, all of which result in the formation of specific tissues. Unlike hormones, which act on cells distant from the source (endocrine signaling), GFs have a local (nonsystemic) effect and are often secreted at low concentrations (Fig. 6).

4.1. Transforming growth factor-beta (TGF- β) superfamily

Members of this superfamily, as bone BMPs, growth differentiation factors (GDFs) and TGF- β s, are involved in the different stages of repair bone (intramembranous and endochondral bone ossification) during bone repair (Gerstenfeld et al., 2003).

4.1.1. Transforming growth factor-beta (TGF- β)

The term transforming growth factor beta is applied to the superfamily of length well-known growth factors involved generally with connective tissue repair and bone regeneration present in many types of tissue (Lieberman et al., 2002). TGF- β exists as five isoforms, three of them have received the most attention regarding fracture repair and proliferation of MSCs (TGF- β 1, TGF- β 2, TGF- β 3), although TGF- β 3 has the most pronounced effect on increases proliferation

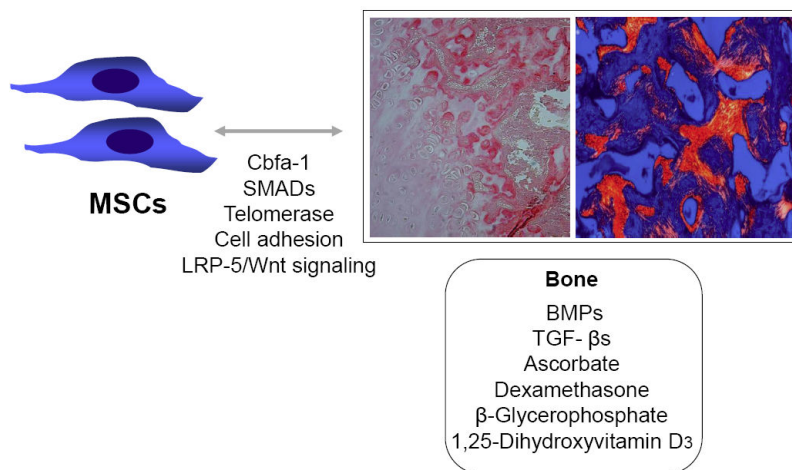


Figure 6. Growth factor regulation of BM-derived MSCs differentiation. Among the classes of bioactive factors, such as matrix ligand, mechanical stimulation, and cell shape, GFs exert strong effects on the regulation of the lineage differentiation of MSCs. Boxed GFs and hormones are used to control bone differentiation *in vitro*. Factors under the arrow have been implicated in promoting regulation differentiation. Pictures represent two histological sections, stained with Sirius red and observed under light (left, femur segmental resection) and polarized (right, profile of a hydroxyapatite implant) microscopes. New bone tissue appears in red. *Results obtained in LABRET-UMA*

of MSCs and chondrogenesis (Weiss et al., 2010). All TGF- β members superfamily are synthesized as large precursors which are proteolytically cleaved to yield mature protein dimers (Massague et al., 1994). TGF- β signaling that involves two receptor types, TGF- β receptor type I and type II, occurs when factors from the family bind a type II serine/threonine kinase receptor, recruiting another similar transmembrane protein (receptor I). Receptor I phosphorylates the primary intracellular superfamily signal effector molecules, SMADs, causing their translocation into the nucleus and specific gene transcription (Valcourt et al., 2002). TGF- β and members of this growth factor family can also signal via the mitogen activated protein tyrosine kinase (MAPK), Rho GTPase and phosphoinositide 3kinase (PI3K) pathways (Zhang, 2009).

Like PDGF, they are synthesized and found in platelets and macrophages, as well as MSCs and some other cell types (Barnes et al., 1999), acting as paracrine and autocrine fashion (Fig. 7). TGF- β s inhibit osteoclast formation and bone resorption, thus favoring bone formation over resorption by two different mechanisms (Mohan & Baylink, 1991). The TGF- β activates fibroblasts and preosteoblasts to increase their numbers, as well as promoting their differentiation toward mature functioning osteoblasts. It influences the osteoblasts to lay down bone matrix and the fibroblast to lay down collagen matrix to support capillary growth (Marx et al., 1996). They also play a role in osteogenesis, its actions are diverse and it is thought to influence the activity of BMPs (Salgado et al., 2004). TGF- β 1 plays a pivotal role in the process and site of fracture healing where appears elevated levels in humans, as well as in other mammals, as it enhances the proliferation and differentiation of MSCs and is chemotaxis on bone cells (Sarahrudi et al., 2011).

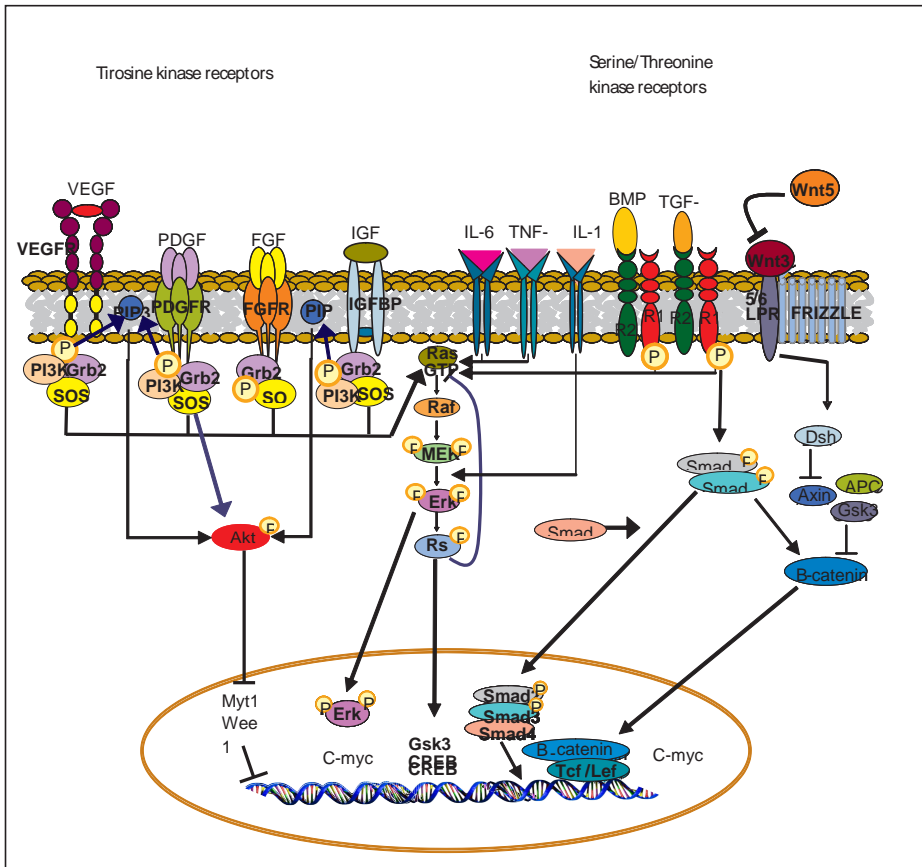


Figure 7. The signal cascade inside the cell after the receptor binding of GFs involves in bone repair. Taken and modified from L. Barnes et al. 1999, and Rodrigues et al. 2010, with permission

Also, our group have demonstrated that osteogenic precursor cells can be selected from a mixed population of BM MSCs by virtue of their distinctive survival responses in the presence of a recombinant human TGF- β 1 fusion protein (Andrades et al., 1999a; 1999b; Andrades and Becerra, 2002a; Andrades et al., 2003; Becerra et al., 2006; Claros et al., in press), engineered to contain an auxiliary collagen binding domain (rhTGF- β 1-F2) (Fig. 8), and further, that these selected cells exhibit unique properties in the chondroosteogenic lineage that can ultimately be utilized to therapeutic advantage

4.1.2. Bone morphogenetic proteins (BMPs)

The first BMP was identified by Urist (1965). He observed the ability from demineralized bone matrix (DBM), to induce ectopic bone formation when implanted under the skin of rodents, and showed that there was a recapitulation of all the events that taking place during skeletal

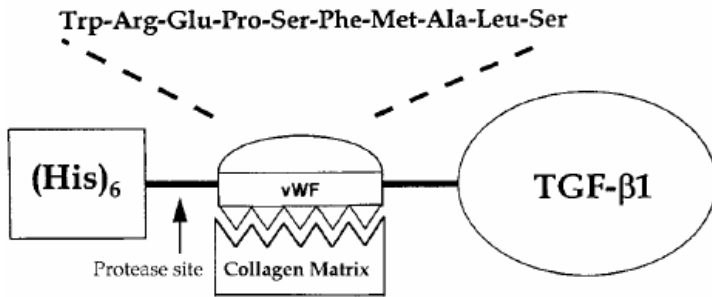


Figure 8. Schematic representation of the genetically engineered fusion construct, containing a histidine purification tag, a protease site, an auxiliary von Willebrand Factor collagen binding domain. Recombinant hTGF- β 1-F2 applied to a bovine collagen matrix as vehicle and delivery system could be of advantage in promoting the survival, proliferation, differentiation, and colony mineralization of the osteogenic precursor cell population. It plays a crucial role in early stages of osteogenic commitment and differentiation. *Results obtained in LABRET-UMA*

development. In 1971, it was named as the responsible factors BMPs. More lately, others searchers, as Reddi and Huggins (1972) demonstrated that these molecules are important during development. Even present, at least many than 30 BMPs have been identified and BMP's functions have been studied by means of analysis of mutant genes and knockout experiments in mice. Different BMPs, among others member of the TGF- β s superfamily, trigger a serine/threonine kinasa cascade of events that induce the formation of cartilage and bone (Fig.7).

During fracture repair, BMPs are produced by MSCs, osteoblasts, and chondrocytes, and bind to cells by direct interaction or are accumulated and subsequently delivered of ECM to promote the bone generation. These proteins induce a cascade of cellular pathways that promote cell growth, migration and differentiation of MSCs to repair the injury, stimulates angiogenesis, as well as synthesis of ECM and play a regulatory role in tissue homeostasis (Reddi, 2001). The different BMPs act in different temporal scale during bone repair. In studies of fracture healing, BMP-2 mRNA expression showed maximal levels within 24 hrs of injury, suggesting that this BMP plays a role in initiating the repair cascade. Other *in vitro* studies examining marrow MSCs differentiation have shown that BMP-2 controls the expression of several other BMPs, and when its activity is blocked, marrow MSCs fail to differentiate into osteoblasts (Edgar et al., 2007).

BMP-3, BMP-4, BMP-7, and BMP-8 are expressed during bone repair, from days 14 to 21, when the resorption of calcified cartilage and osteoblastic recruitment are most active, and bone formation takes place. Our group has demonstrated that BMP-7 is capable of selecting a cell population from BM which, in a three dimensional collagen type I gel, achieves skeletogenic potential under *in vitro* and *in vivo* environments (Andrades et al., 2001; Andrades and Becerra, 2002b; Andrades et al., 2003). BMP-5 and BMP-6 and other members of the TGF- β s superfamily are constitutively expressed from days 3-21 during fracture in mice, suggesting that they have a regulatory effect on both intramembranous and endochondral ossification. BMP-2 to BMP-8 show high osteogenic potential, however BMP-2, BMP-6, and BMP-9 may be the most potent

inducers of MSCs differentiation to osteoblasts, while the others, stimulate the maturation of osteoblasts (Cheng et al., 2003).

The first BMP extracted in a highly purified recombinant form was BMP-2. In preclinical models, BMP-2 has the ability to induce bone formation and heal bone defects and promote the maturation and consolidation of regenerated bone. Recombinant human BMP-7 and BMP-2 are among the first growth factor based products available for clinical use to treat patients afflicted with bone diseases. A large number of studies have been performed to determine appropriate carriers for BMPs (Cheng et al., 2003).

In vitro cultures, MSCs and osteoblasts exhibit a high number of BMP receptors and synthesize the BMP antagonist's noggin, which are capable of blocking osteogenesis as MSCs differentiate into osteoblasts. BMP antagonists are important in normal bone turnover and regulation. The expression of the BMP antagonists, as noggin, which blocks BMP-2, BMP-4, and BMP-7 interaction with its receptor, also is modulated during bone repair (Balemans et al. 2002).

4.1.3. Wnt proteins

The Wnt pathway was initially identified as a proto-oncogene in mammary tumors that was activated by integration of the mouse mammary virus (Nusse & Varmus, 1982). Since then, it has been the subject of many studies. It knows Wnt proteins are secreted cysteine-rich glycosylated family proteins to share a highly conserved pattern of 23–24 cysteine residues and several asparagines-linked glycosylation sites (Li et al., 2006). In mature tissues, Wnt pathway play a regulator role of osteogenesis and stem/progenitor cells self-renewal, it is involved in bone formation, and also cellular adhesion and migration through their indirect interactions with the cadherin pathway (Arnsdorf et al., 2009).

Wnt proteins are divided towards to activate one of two main signaling pathways that consist of the Wnt1 class, also called Wnt/ β -catenin or canonical Wnt pathway and Wnt5a class, Wnt/ Ca^{2+} or non-canonical pathway. Several lines of evidence have demonstrated the importance of canonical Wnt signaling in promoting osteogenesis *in vitro* and *in vivo* (Chung et al., 2009). Wnt signaling is a prime target for bone active drugs and the approaches include inhibition of Wnt antagonist like Dkk1, sclerostin, and Sfrp1 with neutralizing antibodies and inhibition of glycogen synthase kinase 3 β (GSK-3 β), which promotes phosphorylation and degradation of β -catenin. Enhancement of Wnt signaling either by Wnt overexpression or deficiency of Wnt antagonists (ten Dijke et al., 2008) is associated with increased bone formation in mice and humans. Gain of function mutations of *LRP5/6* that lead to impaired binding of Dkk-1 (Dickkopf-1 is a secreted Wnt antagonist that binds LRP5/6) to this Wnt coreceptor are associated with increased bone mass (Boyden et al., 2002).

In spite of osteogenic inhibitory function of canonical Wnts, this pathway plays a positive role in bone homeostasis *in vivo* (Liu et al., 2009). Canonical Wnt signaling in osteoblast differentiation is modulated by Runx2 and osterix transcription factors (Hill et al., 2005). Quarto et al. (2010) have shown canonical Wnt signaling can either inhibit or promote osteogenic differentiation depending on the status of cell (cellular differentiation degree undifferentiated vs. differentiated), the threshold levels of its activation (existence of a differential activation of

canonical Wnt signaling between an undifferentiated MSC and osteoblast), and Wnt ligands concentration showing *in vitro* and *in vivo* data correlated results for Wnt3a treatment of calvarial defects created in juvenile mice where rise activation of canonical Wnt signaling inhibited osteogenic differentiation of undifferentiated MSCs, whereas increased the mineralization of differentiated osteoblasts.

4.2. Growth hormone and insulin-like growth factors (GH and IGF)

In clinic, the patients that present short stature are treated with the Growth Hormone (GH); for this reason, many researcher study the effects of GH in the treatment for osteoporosis and repair bone fracture. It is released by pituitary gland and travels through the circulation to the liver, where target cells are stimulated to release IGF. There are two IGFs identified: IGF-I and IGF-II. Various studies have shown that both IGF-I and IGF-II (Swolin et al., 1996;) are delivered by osteoblasts, chondrocytes, endothelial cells, and bone matrix, and they are detected by recruitment MSCs and bone cells in a paracrine/autocrine manner thanks to the presence of six insulin growth factor-binding proteins (IGFBPs), which modulate their action by intracellular tione kinase cascade.

IGF-II is the most abundant GF in bone matrix. However, IGF-I is 4 to 7 times more potent in synthesis of bone matrix (type I collagen and non-collagen matrix proteins) (Lind, 1996). IGF-II acts on phase of endochondral bone formation and induces type I collagen production, stimulates cartilage matrix synthesis, and cellular proliferation. Both factors have been localized in bone studies of animals and humans with GH-deficient levels. The expression and secretion of IGFBPs, IGF-I and IGF-II (Birnbaum et al., 1995) changes during *in vitro* MSCs cultures. Prisell et al. (1993) showed that IGF-I mRNA was expressed during the MSCs recruitment and proliferation; however IGF-II mRNA expression happened later, during endochondral bone formation by osteoblasts and chondrocytes. IGF production is not only under the control of GH, is also regulated by estrogen, PTH, cortisol (inhibits IGF-I synthesis), local GFs and cytokines (Ohlsson et al., 1998). This abundant supply of IGFs is necessary to promote bone formation, bone repair, and MSCs cell proliferation and differentiation.

4.3. Fibroblast growth factor (FGF)

FGF is a secreted glycoproteins family whose signals are implicated in wound healing and angiogenesis, which influence in cellular proliferation, differentiation, migration, survival and polarity transduced through their receptors (FGFR1, FGFR2, FGFR3 and FGFR4). These receptors are constituted of extracellular immunoglobulin-like (Ig-like) domains and cytoplasmic tyrosine kinase activity domain. FGF proliferation signals occur through the tyrosine kinase cascade in various target cell types (Ng et al., 2008).

The various FGF receptors display varying affinities for each of the members of the FGF family and are expressed in a wide variety of tissues including indeed, bone. As with many of the tyrosine kinase receptors, activation of the intrinsic tyrosine kinase activity occurs through receptor dimerization in response to ligand binding. An additional complexity may be added to the receptor-ligand association through the binding of FGF li-

gand by either secreted or membrane-bound proteoglycans, heparin-like proteoglycans in particular because their high affinity (Givol & Yayon, 1992). Nine members of the FGF family have been identified of which, the most abundant in human tissue are FGF-1 (acid character) and FGF-2 (basic character) (Lieberman et al., 2002). FGFs are important regulators of fracture repair expressed by MSCs, maturing chondrocytes and osteoblasts and have been demonstrated to enhance TGF- β expression in osteoblastic cells (Bolander, 1998). They play a role in maintaining the balance between bone-forming cells and bone-resorbing cells and promote angiogenesis. Specifically, FGF-2 not only maintains MSCs proliferation potential, it also retains a slight osteogenic, adipogenic and chondrogenic differentiation potentials through the early mitogenic cycles; eventually, however, all of the MSCs differentiate into the chondrogenic line (Yanada et al., 2006).

4.4. Platelet derived growth factor (PDGF)

PDGFs are potent mitogens of MSCs (Ng et al., 2008) which express all forms of the GF: PDGF-A and PDGF-C at higher levels, and PDGF-B and PDGF-D at lower levels, such as both receptors type PDGFR α and PDGFR β through which PDGF signaling is transduced (Tokunaga et al., 2008). PDGF is a dimeric molecule can exist either as a homodimeric (PDGF-AA, PDGF-BB, etc) or a heterodimeric form (PDGF-AB) according to the relative levels of each subunit generating a level of ligand complexity in cells in which both polypeptides are expressed. The different PDGF isoforms exert their effect on target cells by binding with different specificity to two structurally related protein tyrosine kinase receptors, denoted as the α -receptors and β -receptors, which are autophosphorylate ligand bound (Tokunaga et al., 2008). Several groups have found PDGF-BB to induce both proliferation and migration in MSCs (Fierro et al., 2007). While PDGFR β inhibits osteogenesis, however, PDGFR α has been observed to induce osteogenesis. Akt signaling has been proposed to mediate both the suppression and induction of osteogenesis by PDGFR signaling (Tokunaga et al., 2008).

These molecules acts as paracrine manner stimulating mitogenesis of the marrow SCs and endosteal osteoblasts transferred in grafts to increase their numbers by several orders of magnitude. It also begins an angiogenesis of capillary budding into the graft by inducing endothelial cell mitosis and macrophage activator effect. It is known to emerge from degranulating platelets at the time of injury. PDGF also increased hMSC proliferation like Wnt (Liu et al., 2009). PDGF recruits MSCs and promotes chemotaxis and angiogenesis (Salgado et al., 2004).

5. Biomaterials as support

Natural bone consists of an ECM with nanosized apatitic minerals and collagen fibers that support bone cell functions. It is advantageous for a synthetic biomimetic scaffold to: (1) contain nano-apatite crystals similar to those in bone, together with fibers to form a matrix that supports cell attachment; (2) have mechanical properties similar to those of bone; and (3) encapsulate and support cells for osteogenic differentiation and bone regeneration. The success

in regenerating a damaged tissue using the tissue engineering approach depends on the various types of interactions between the cells, scaffolds, and GFs. Besides, an understanding of the phenomena of cell adhesion and, beyond, the function of the proteins involved in cell adhesion on contact with the materials and the purpose depends of supramolecular assembly (scaffolding) of biomimetic biomaterials such as collagens, proteoglycans, and cell adhesion glycoproteins such as fibronectins and laminin.

Osteogenesis is highly dependent on the substrate carrier used, which has to provide a favorable environment into which bone cells can migrate before proliferating, differentiating, and depositing bone matrix (i.e., osteoconduction) (Ono et al., 1999). At the cell level, substrates of this kind must have specific biochemical (molecular) properties, physicochemical characteristics (surface free energy, charge, hydrophobicity, and so on), and a specific geometric conformation (they must be three dimensional and show interconnected porosity) (Jin, 2000). From the biomaterial point of view, the scaffolds used for bone engineering purposes have to meet a number of criteria, including (1) biocompatibility (nonimmunogenicity and nontoxicity); (2) resorbability (showing resorption rates commensurate with the bone formation rates); (3) preferably radiolucency (to allow the new bone to be distinguished radiographically from the implant); (4) osteoconductivity; (5) mechanical properties to match those of the tissues at the site of implantation; (6) easy to manufacture and sterilize; and they must be (7) easy to handle in the operating theater, preferably without requiring any preparatory procedures (in order to limit the risk of infection).

The bone substitute materials intended to replace the need for autologous or allogeneic bone, consist of bioactive ceramics, bioactive glasses, biological or synthetic polymers, and composites of these. Biological polymers, such as collagen and hyaluronic acid provide guidance to cells that favors cell attachment and promotes chemotactic responses, but, a disadvantage is immunogenicity for the potential risk of disease transmission. On the other hand, other alternative is synthetic polymers such as polyfumarates, polylactic acid (PLA), polyglycolic acid (PGA), copolymers of PLA and PGA (PLGA), and polycaprolactone. Nevertheless, there are a wide range of bioactive inorganic materials similar in composition to the mineral phase of bone, for example, tricalcium phosphate, HA, bioactive glasses, and their combinations; and all of these can be tailored to deliver ions such as Si at levels capable of activating complex gene transduction pathways, leading to enhanced cell differentiation and osteogenesis. Hydrogels, such as polyethylene glycol or alginate-based, are to provide a three-dimensional cellular microenvironment with high water content, this is suitable for cartilage regeneration. Polyethylene glycol (PEG) hydrogels were investigated as encapsulation matrices for osteoblasts to assess their applicability in promoting bone tissue engineering. Non-adhesive hydrogels were modified with adhesive Arg-Gly-Asp (RGD) peptide sequences to facilitate the adhesion, spreading, and, consequently, cytoskeletal organization of osteoblasts. Finally, mineral deposits were seen in all hydrogels after 4 weeks of *in vitro* culture, but a significant increase in mineralization was observed upon introduction of adhesive peptides throughout the network. Potentially, the cell suspension could be injected into the body through a needle and photopolymerized through the skin to provide a non-invasive technique to enhance bone regeneration.

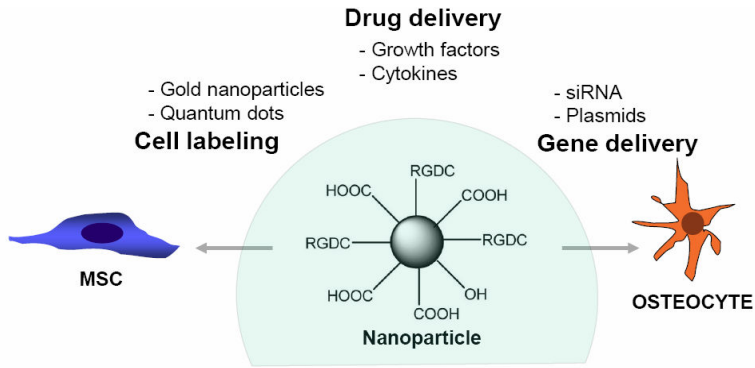


Figure 9. Overview of nanoparticle applications in bone regeneration

Biomaterials such as polymers, ceramics, and metals are widely used in bone for regenerative therapies, including in bone grafts and in Tissue Engineering as well as for temporary or permanent implants to stabilize fractures (Navarro et al., 2008). In recent years, biomaterials in general and bone-related implant materials in particular have been considerably refined, with the objective of developing functionalized materials, so-called smart materials, containing bioactive molecules to directly influence cell behaviour (Mieszawska and Kaplan, 2010). Rapid developments in nanotechnology have yielded many clinical benefits, in particular in the field of bone tissue engineering. The main advantage is that several novel biomaterials can be fabricated into nanostructures that closely mimic the bone in structure and composition. The optimization in the surface features of biomaterials has strongly improved cell behaviour in terms of adhesion, proliferation, differentiation and tissue formation in three dimensions. In this context, nanoparticles that are in the same size range as integral parts of natural bone, such as HA crystals or cellular compartments, are promising candidates for local applications. In bone, locally applied nanoparticles may be suitable for numerous potential uses with respect to the improvement of tissue regeneration, the enhanced osseointegration of implants, and the prevention of infections.

Increasingly refined nanoparticles are being developed for a wide range of applications (Fig. 9). These include cell labelling to broaden research possibilities as well as to improve and noninvasively monitor cell therapy approaches (Bhirde et al., 2011; Andrades et al., in press (b)). Moreover, drug delivery systems with improved pharmacologic characteristics are being developed. They promote enhanced therapeutic outcome by providing controlled release of bioactive molecules, such as growth factors or anticancer drugs (Allen and Cullis, 2004). In addition, gene therapy concepts with good prospects are required for future treatment options based on intracellular manipulation (Evans, 2011).

The heterogeneous picture of research on the interactions of nanoparticles with MSCs makes it difficult to draw general conclusions. However, it becomes clear that parameters such as chemistry, size, and shape in some cases greatly affect the particle uptake behaviour of MSCs as well as their natural differentiation potential. Different strategies for nanoparticle applica-

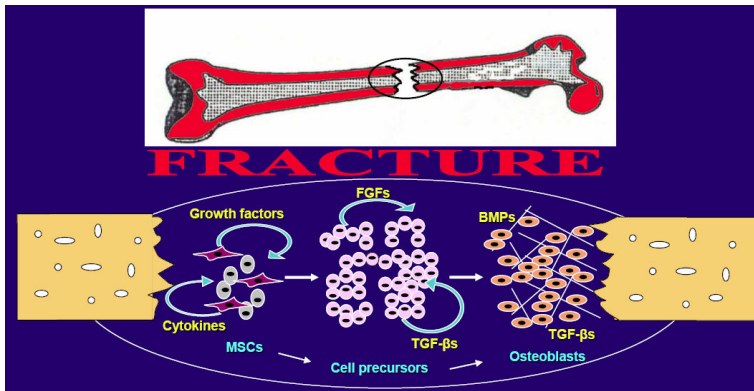


Figure 10. Bone fracture repair and regeneration is a question of balance among cells, growth factors and biomaterials.

tion in bone (i.e., as cell-labeling agents and for drug or gene delivery) have great potential for monitoring and supporting tissue regeneration.

6. Conclusion

Over the last decades we have advanced in many aspects of bone defects treatment. We have good understanding of the components involved in the healing of bone. Osteoprogenitor cells are necessary to replace the inserted scaffold and to create new bone tissue. These cells, MSCs, can come from the periosteum, the BM, or from chemotaxis and blood vessels entering the haematoma at the fracture site. Specific mechanical and biological stimulants cause the cells to differentiate into osteoblasts, which are the bone forming cells (Fig. 10). However, in critical size bone defects the natural migration of osteoprogenitor cells does not suffice for fracture healing. In normal conditions MSCs are rare (one in 10 million cells) (Pittenger et al., 1999). However, when a bone is broken, these cells, using special probing signals, roam in the blood and settle in the fracture site, differentiate into bone cells and start to construct the callus. The number of stem cells differs from person to person and is affected by age, sex and environmental factors.

Also, we have strived forward in defining different components of bone regeneration and have achieved a good combination of biology and technology leading to solid and reproducible answers to the *in vitro* and animal *in vivo* problem of bone defects. However, there is still one more step to take (the human *in vivo* step). There are scant data with respect to this part of the question, and in the next few years this field must undergo a transition, giving clinicians tools to deal with these critical everyday problems. The solution will come from a collaborative work of biologist, surgeons, engineers and chemists who possess the social understanding that there has to be a limit to the cost that the patient (and the society) can bear for healing a fracture.

Consequently, the search for the new bone regeneration strategies is therefore a key international priority fuelled by the debilitating pain associated with bone damage, and the increasing medical and socioeconomic challenge of our aging population.

Acknowledgements

Laboratory of Bioengineering and Tissue Regeneration-University of Málaga (LABRET-UMA) is supported by grants from the Spanish (PI10/02529, and Red de Terapia Celular, RD06/00100014), and the Andalusian Governments (PI-0729-2010, and PAID BIO217). CIBER-BBN is an initiative funded by the VI National R&D&I Plan 2008-2011, *Iniciativa Ingenio 2010*, *Consolider Program*, *CIBER Actions* and financed by the Instituto de Salud Carlos III (Ministry of Economy and Competitiveness) with assistance from the *European Regional Development Fund*.

This chapter has been designed, coordinated and written by Professor José A. Andrades and some of his students, in a novel educational initiative at the University of Málaga (Spain), academic course 2011/12.

Author details

José A. Andrades^{1,2}, Lucía Narváez-Ledesma³, Luna Cerón-Torres³, Anyith P. Cruz-Amaya³, Daniel López-Guillén³, M. Laura Mesa-Almagro³ and José A. Moreno-Moreno³

1 Laboratory of Bioengineering and Tissue Regeneration (LABRET-UMA), Department of Cell Biology, Genetics and Physiology. Faculty of Sciences, University of Málaga, Spain

2 Networking Biomedical Research Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain

3 Faculty of Sciences, University of Málaga, Spain

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