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Relationship between extracellular matrix (ECM) components and mineralization in bone marrow stromal cells

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THESIS PURPOSE

The relations between cells and extracellular matrix seem to orchestrate tissue organization by regulating cell functions during fetal development and throughout normal adult life. Mesenchymal stem cells naturally reside within an extracellular matrix (ECM), which is a biological scaffolding material consisting of structural and functional molecules. Besides providing structural support to cells, the ECM is a dynamic microenvironment that also plays a role in modulating cell survival, migration, proliferation, and differentiation. Thus, focusing on the innate ability of the native ECM to better modulate cell behavior, the coating of synthetic biomaterials with cell-derived decellularized extracellular matrices is a promising approach to confer bioactivity to otherwise inert materials and direct the fate of host or transplanted cells in tissue engineering applications. Furthermore, mesenchymal stem cells (MSCs) are under investigation for possible uses in the production of decellularized matrix-coated substrates due to their high proliferative potential, ability to differentiate toward multiple lineages and extensive matrix production. My research activities regarded the adhesion and proliferation of human bone marrow stem cells grown on cell free extracellular matrix and the influence that these matrices have on the maintenance of cell stemness and biological functions, and their role during the induction of MSCs osteogenic differentiation.

1. INTRODUCTION

1.1 The extracellular matrix

Recently, it has become increasingly evident that the extracellular matrix (ECM) is an important component of the cellular niche within all animal tissues and organs, and provides not only essential physical scaffolding for the cellular constituents but also critical biochemical and biomechanical cues to initiate and sustain cellular functions such as tissue morphogenesis, differentiation and homeostasis (Kresse H and Schonherr E, 2001; Daley WP *et al.*, 2008). The importance of the ECM is vividly illustrated by the wide range of syndromes, from minor to severe, arising from genetic abnormalities in ECM proteins; indeed, any inherited or acquired structural defect, such as a single amino acid substitution, and/or metabolic disturbance in the ECM, may cause cellular and tissue alterations that may lead to the development or progression of a disease (Jarvelainen H *et al.*, 2009). The extracellular matrix consists of a variety of proteins and glycoproteins secreted locally and assembled in an organized network in close association with the surface of the cell responsible for their production. Although, fundamentally, the ECM is composed of water, proteins and polysaccharides, each tissue has an ECM with a unique composition and topology, which is generated during tissue development through a dynamic and mutual, biochemical and biophysical dialogue between the various cellular components (e.g. epithelial, fibroblast, adipocyte, endothelial elements) and the evolving cellular and protein microenvironment. Indeed, the physical, topological, and biochemical composition of the ECM is not only tissue-specific, but it is also markedly heterogeneous (Leitinger B and Hohenester E, 2007; Xian X *et al.*, 2010).

The connective extracellular matrix is often most abundant in cells which surround and determine the physical properties of the tissue. The connective tissues form the scaffolding in vertebrates, but the amount present in the various organs varies considerably, – from the cartilage and bone, containing the highest percentage, to the brain and spinal cord, where they are only minor constituents. The variations in the relative quantities of different types of matrix macromolecules and the way they are organized give rise to a surprising diversity of forms, each adapted to the functional requirements of the tissue in question. The matrix can become calcified to form the hard structures of bones and teeth, transparent to form the corneal stroma or its molecules can form a parallel structure to give tendons their enormous tensile strength (Adams JC and Watt FM, 1993; Geiger B *et al.*, 2001).

The extracellular matrix is composed of two main classes of macromolecules: glycosaminoglycans (GAGs) which are usually joined covalently to proteins in the form of proteoglycans (PGs), and fibrous proteins including collagens, fibronectins, elastins and laminins, which have both structural and adhesive functions (Schaefer L and Schaefer RM, 2010; Alberts B *et al.*, 2007).

Proteoglycans (PGs) consist of glycosaminoglycan (GAG) chains which, with the exception of hyaluronic acid, are covalently linked to a specific protein core. The GAG chains are unbranched polysaccharide chains composed of repeating disaccharide units, [sulfated N-aceltylglucosamine or N-acetylgalactosamine and D-glucuronic or L-iduronic acid] which can be divided further into sulfated (chondroitin sulfate, dermatan sulfate, heparan sulfate and keratan sulfate) and non-sulfated (hyaluronic acid) GAGs (Schaefer L and Schaefer RM, 2010). These molecules are extremely hydrophilic and, consequently, take highly extended conformations which are essential for hydrogel formation, and the matrices they form, are able to resist highly compressive forces.

There are several classification criteria of proteoglycans based on location, composition and function of the GAG chains. However, it is difficult to identify a single structure recurring within this class of macromolecules (Ruoslahti E, 1988); indeed there are different types of proteoglycans and they can also undergo modifications (substitution of sugar residues, phosphorylation, sulphation) during cell survival, adapted to different biological needs. A classification according to their core proteins, localization and GAG composition indentifies three main PGs families: small leucine-rich proteoglycans (SLRPs), modular proteoglycans and cell-surface proteoglycans (Iozzo RV and Murdoch AD, 1996; Iozzo RV, 1998).

Proteoglycan molecules have a wide variety of functions reflecting their unique buffering, hydration, binding and force-resistance properties; as a consequence, genetic diseases (congenital stromal dystrophy of the cornea, dyssegmental dysplasia, Schwartz-Jampel syndrome) have been linked to mutations in PG genes (Schaefer L and Schaefer RM, 2010; Kresse H and Schonherr E, 2001). PGs fill the majority of the extracellular interstitial space within the tissue forming a gel-like highly hydrated " basal substance" in which the fibrous proteins are embedded (Jarvelainen H *et al.*, 2009). The polysaccharidic gel resists compressive forces on the matrix while allowing rapid diffusion of nutrients, metabolites and hormones between blood vessels and tissue cells (Bishop JR *et al.*, 2007).

SLRPs, such as decorin, biglycan and lumican, have been involved in multiple signaling pathways including binding to and activation of epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGFIR) and low-density lipoprotein-receptor related protein 1 (LRP1), regulation of inflammatory response reaction, and binding to and activation of TGFβ (Goldoni S and Iozzo RV, 2008; Iozzo RV and Schaefer L, 2010 ; Schaefer L and Schaefer RM, 2010). Also, SLRPs seems to be engaged in binding collagens and in the modulation of fibrillogenesis, thus regulating the assembly of the interstitial matrix and its three-dimensional configuration according to the mechanical load that the tissue must sustain. For example, it was shown that decorin, in particular, when bound to collagen is capable of sequestering cytokines at the level of the ECM, exerting a role in the control of cell proliferation. Specifically, it inhibits the action of TGF (Yamaguchi Y *et al.*,

1990) and binds the EGF receptor, hindering its phosphorylation (Patel S *et al.*, 1998; Santra M *et al.*, 2002).

Modular PGs are a heterogeneous group characterized by the assembly of various protein modules in an elongated and often highly glycosylated structure. They can modulate cell adhesion, migration and proliferation (Schaefer L and Schaefer RM, 2010). Basement membrane modular PGs (perlecan, agrin and collagen type XVIII) have a dual function as pro- and anti-angiogenic factors (Iozzo RV *et al.*, 2009).

Cell-surface PGs (syndecans and glypicans), mainly with heparan sulfate chains, are also involved in the control of cell proliferation, acting as co-receptors since many growth factors bind with high affinity particular domains of the heparan sulfate chains on the one hand, and with their respective signaling receptors on the other (Kresse H and Schonherr E, 2001; Schaefer L and Schaefer RM, 2010).

Collagen is the most important fibrous protein within the extracellular matrix. As the main component of skin and bone, it is the most abundant protein in mammals, accounting for 25% of the total protein mass in these animals. To date, 28 different types of collagen have been identified in vertebrates (Gordon MK and Hahn RA, 2010).The structural unit of collagen is made of tropocollagen, a protein formed by three polypeptide chains wound spirally by hydrogen and covalent bonds to one another, and forming a triple-stranded helix. The tropocollagen molecules, synthesized within the cell as soluble precursors (procollagen), are associated longitudinally (head-to-tail) and in parallel, with an arrangement staggered, giving rise to the collagen fibrils. After exocytosis, the procollagen is cut and becomes collagen (Prockop DJ and Kivirikko KI, 1995); the majority of these molecules can assemble into supramolecular complexes, such as fibrils and networks, depending on the type of collagen. Fibrous collagens form the backbone of the fibril bundles within the interstitial tissue stroma, whereas network collagens are incorporated into the basal membrane (BM) (Frantz C *et al.*, 2010).

Collagen fibrils form structures which provide tensile strength, regulate cell adhesion, support chemotaxis and migration, and direct tissue development. The diameters and organization of these molecules vary according to the tissue considered; moreover, although within a given tissue, collagen fibers are generally a heterogeneous mix of different types, one type of collagen usually predominates (Rozario T and DeSimone DW, 2010). Cells can adjust the arrangement of the collagen molecules after secretion, guiding the formation of fibrils in close association with the plasma membrane. For example, synthesis of collagen type I involves a number of enzymatic posttranslational modifications (Gordon MK and Hahn RA, 2010; Myllyharju J and Kivirikko KI, 2004), mainly the hydroxylation of proline and lysine residues, the glycosylation of lysine and the cleavage of N- and C-terminal propeptides. Following their cleavage, collagen fibrils are strengthened by the covalent crosslinking between the lysine residues of the constituent collagen

molecules by lysyl oxidases (LOX) (Myllyharju J and Kivirikko KI, 2004; Robins SP, 2007). Furthermore, since the spatial organization of collagen fibrils partly reflects their interaction with other molecules of the ECM, cells can influence this organization by secreting, together with fibrillar collagens, different types and amounts of other matrix macromolecules (Prockop DJ and Kivirikko KI, 1995).

Elastin is another major ECM protein, its fibers provide recoil to tissues that undergo repeated stretches and, importantly, its elongation is crucially limited by tight association with collagen fibrils (Wise SG and Weiss AS, 2009). Secreted tropoelastin (elastin precursor) molecules assemble into fibers and become highly crosslinked to one another via their lysine residues by members of the lysyl oxidase (LOX) enzyme family, which include LOX and LOXL (Lucero HA and Kagan HM, 2006). Furthermore, elastin fibers are covered by glycoprotein microfibrils, mainly fibrillins, which are essential for their integrity (Wise SG and Weiss AS, 2009).

A third fibrous protein, fibronectin (FN) is intimately involved in directing the organization of the interstitial ECM and, additionally, has a crucial role in mediating cell adhesion and function (Vakonakis I and Campbell ID, 2007). It is also important for cell migration during development and has been implicated in cardiovascular diseases and tumor metastases (Rozario T and DeSimone DW, 2010; Tsang KY *et al.*, 2010). FN is a glycoprotein consisting of two polypeptide chains linked by disulphide bonds near the C-terminus. It has several binding sites to other FN dimers, to collagen, to glycosaminoglycans (GAGs), to heparin and also a sequence Arg-Gly-Asp (RGD) implicated in the interaction with specific receptors on the cell membrane; indeed, the molecules of fibronectin assemble into fibrils only on cell surface in a process guided by additional proteins, especially integrins (Schwarzbauer JE and Lichtman JW, 1999; Pankov R and Yamada KM, 2002).

The fibrils of fibronectin can be strongly stretched over its resting length and usually are aligned with adjacent stress fibers of intracellular actin. These, indeed, promote the assembly of secreted molecules of fibronectin into fibrils influencing their orientation (Smith ML *et al.*, 2007). The interactions between the extracellular fibronectin fibrils and intracellular actin filaments across the cellular plasma membrane are mediated mainly by transmembrane adhesion integrin. Cell contraction through the actomyosin cytoskeleton generates tension on fibronectin matrix resulting in FN fibril stretching. This mechanism causes the expure of cryptic binding sites on fibronectin molecules allowing them to bind one another, (Leiss M *et al.*, 2008; Mao Y and Schwarzbauer JE, 2005; Vakonakis I and Campbell ID, 2007) as well as the further exposure of integrin binding sites within the molecule which results in pleiotrophic changes in cellular behavior and implicate FN as an extracellular mechanoregulator (Smith ML *et al.*, 2007). Thus, actin cytoskeleton and the resulting integrin clustering promote FN fibril polymerization and matrix assembly.

Integrins are heterodimeric proteins composed of two trans-membrane subunits, α and β, not covalently linked. Each subunit consists of a large extracellular domain, a trans membrane domain

and a small cytoplasmic domain. To date 16 α and 8 β subunits have been identified and, depending on their combination, integrin binds specific proteins of the ECM. For example, α1β1 binds collagen, while α5β1 and αvβ3 are receptors for fibronectin and vitronectin respectively. On the one hand, integrins anchor the cell to the extracellular matrix proteins, on the other hand they bind proteins of the cytoskeleton (Anselme K, 2000).

The site of cell adhesion to the extracellular matrix via integrins is called "focal contact", and it regulates cellular behaviors by, for example, applying stronger traction forces to the substrate during cell migration. In cultured fibroblasts, there are remarkable differences between the 'classical' focal contacts – oval shaped, peripheral structures, regulated by the small G-protein Rho, enriched in activated αvβ3-integrin, paxillin, vinculin and tyrosine-phosphorylated proteins – and 'fibrillar adhesions', which are elongated or dot-like, central structures containing α5β1-integrin, tensin and parvin/actopaxin and attached to fibronectin fibrils. In addition, recent studies have shown, by using an antibody-chase technique, that whereas αvβ3 integrin remains in focal contacts, α5β1 integrin continuously translocates from peripheral focal contacts towards the cell center, forming fibrillar adhesions which indicates that this process plays a major role in fibronectin fibrillogenesis (Pankov *et al.,* 2000; Zamir E and Geiger B, 2001;Cukierman E *et al.*, 2001). Furthermore, a recent study by Roca-Cusachs P *et al.* (2009), on mouse embryonic fibroblasts has indicated the differential function of integrin species in adhesion processes. High matrix forces were found to be primarily resisted by clustered α5β1 integrins, while less stable αvβ3 integrin binding was shown to initiate mechanotransduction, resulting in a reinforcement of the integrin-cytoskeleton interactions. Indeed, these integrins have been identified as key regulators of osteoblast proliferation and differentiation (Biggs MJP and Dalby MJ,2010). Since the cytoskeleton can exert forces that are able to direct the ECM macromolecules, which, in turn, can organize the cytoskeleton of cells they came into contact with, the ECM can theoretically propagate order from cell to cell, creating structures oriented on a large scale. ECM components together with integrin receptors on the cell surface can be viewed as intricate nanodevices allowing cells to physically organize their 3D environment, as well as to sense and respond to various types of mechanical stress (Geiger B *et al.*, 2001).

1.2 Mesenchymal stem cells

The concept of mesenchymal cells has achieved wide popularity and the studies involving these cells are undergoing a rapid development. Despite the rapid growth of this field and the vast potential applications of mesenchymal stem cells from a scientific and medical point of view, uncertainties remain with respect to the defining characteristics of these cells, including their potency and self-renewal.

The history of mesenchymal stem cells originated at the end of the 19th century as a hypothetical assumption to explain the ability of certain tissues, such as blood, skin, etc., to regenerate for the lifetime of an organism even though they are made of short-lived cells. In these classical studies, the identification of stem cells as discrete cellular entities, resulting from the development of methods to isolate stem cells candidates, led the German pathologist Cohnheim JF in 1867 to suggest the presence of nonhematopoietic stem cells in bone marrow. His work showed that bone marrow could be the source of fibroblasts depositing collagen fibers as part of the normal process of wound repair (Prockop DJ, 1997). Around the same period, Goujon EJ (1869) demonstrated that autologous bone marrow (BM) transplanted into heterotopic anatomical sites formed de novo ectopic bone and marrow (Bianco P *et al.*, 2008).

Despite the importance of this first classical evidence, the ultimate proof of an innate osteogenic potential of the BM and, the idea of a "mesenchymal" stem cell came from the pioneering experiments of Tavassoli and Crosby in the 1960s. While investigating the significance of the specific localization of hematopoiesis in bone, they transplanted bone-less fragments of bone marrow into heterotopic sites, and observed the orderly formation of heterotopic bone at the graft site. This experiment revealed that bone marrow includes an entity, unknown at the time, endowed with the ability to generate histology-proven bone tissue (Tavassoli M and Crosby WH, 1968). However, because these tests were carried out with entire fragments of bone-free BM, the precise identity of any cell candidate to be the osteogenic progenitor could not be defined. Evidence that bone marrow contains cells able to differentiate into other mesenchymal cells, as well as fibroblasts, is now well-known, and was demonstrated in a series of studies by Friedenstein and colleagues in the 1960s and 1970s. They proved that the osteogenic potential repeatedly revealed by the previous classical experiments of heterotopic transplantation, was attributable to a subpopulation of BM cells, entirely distinguishable from the well-known hematopoietic stem cells (HSCs). Indeed, when whole bone marrow was placed in plastic culture dish, cells rapidly adhered to plastic, and medium changed 4 hours later, only removed most of the non adherent, hematopoietic stem cells. The remaining non-phagocytic, adherent cells seemed heterogeneous, but most were spindle-shaped and formed loci of two to four cells which, after an initial lag of 2-4 days, began to divide rapidly, with

population doubling time depending on the donor and the initial seeding density. Following several culture passaging, the adherent cells became more fibroblastic in appearance (Friedenstein AJ *et al.*, 1968). Friedenstein and his coworkers (1970) also highlighted another feature; specifically, they showed that cells, when grown in culture at low density, were able to form single-derived colonies designed as colony-formed unit fibroblastic CFU-F. When transplanted *in vivo,* strains derived from a single cell were able to generate a variety of fully differentiated connective tissues including bone, cartilage, adipose tissue, fibrous tissue and myelosupportive stroma. In other words, they realized that all these connective tissues had a single ancestral progenitor which Friedenstein and Owen (1987, 1988) called "osteogenic stem cell" or, later, "bone stromal stem cell".

Their observations were confirmed by other research groups throughout the 1980s (Ashton BA *et al.*, 1980; Castro-Malaspina H *et al.*, 1980), although the implications of these findings were initially appreciated merely in experimental hematology and only later for their relevance to bone biology and diseases. The innovative idea of the presence of nonhematopoietic stem cells in BM was accepted worldwide only after a study carried out and published by Pittenger *et al.*(1999). Actually, the repeatedly validated concept of Friedenstein and colleagues set a limit: the putative stem cell they isolated was a progenitor of all the skeletal tissues excluding all the mesodermal derivatives and, furthermore, it was located only in bone marrow. Subsequently, Caplan (1991) and Pittenger (1999), on the basis of Friedenstein's work, coined the widely used term "Mesenchymal stem cell" (MSC), and proclaimed that MSC was a common ancestor not only of skeletal tissues, but also of ''mesenchymal'' tissues, meaning substantially all nonhematopoietic derivatives of mesoderm: although found in bone marrow, it also resided in all tissues of postnatal organisms. As reported by Bianco *et al.* (2006 and 2008) the idea of a "mesenchymal stem cell" in postnatal tissues was easily accepted due to the acclaimed recent isolation of human embryonic pluripotent cells in culture, but remained essentially unverified because it was only demonstrable with a heterothopyc transplant of a single cell-derived colony. Hence, the term "skeletal stem cells" was suggested to refer to bone marrow-derived multipotent stromal cells with an *in vivo* demonstrable differentiative potential.

To date MSCs have been isolated not only from bone marrow but also from many other tissues and organs, including adipose tissue (Zuk PA *et al.*, 2002), umbilical cord blood, placental tissue, liver, spleen, testes, menstrual blood (Rossignoli F *et al.*, 2013), amniotic fluid, pancreas (Karaoz E *et al.*, 2010), synovial membrane, dermis, dental pulp (Shi S *et al.*, 2005) and periosteum. Furthermore, MSC-like cells have been isolated from pathological tissues (e.g. rheumatoid arthritis) and express bone morphogenetic protein receptors (Marinova-Mutafchieva L *et al.*, 2000).

Even though very few direct comparisons have made between MSCs isolated from different sources thus far (Kern S *et al.*, 2006; Rebelatto CK *et al.*, 2008), these studies agree that these cells show no significant differences in their morphology and immune phenotype, but they are heterogeneous in

their distinct success rates of isolation, proliferation and differentiation potential. Particularly, these reports demonstrate that bone marrow is thought to be the most available and abundant reservoir of MSCs as well as the major source for these precursor cells, which populate other adult tissues and organs (Prockop DJ, 1997). Adipose tissue-derived MSCs are considered an interesting alternative because they are abundantly distributed and easily accessible, but they have a lower osteogenic potential (Niemeyer P *et al.*, 2010). Overall, MSCs account for a small fraction in bone marrow and other tissues; the exact frequency is difficult to calculate due to the different methods of collection and separation. However, the frequency in human bone marrow has been estimated to be approximately 0.001-0.01% of the total nucleated cells, and therefore about 10-fold less abundant than haematopoietic stem cells (Pittenger MF *et al.*, 1999). Furthermore, the frequency of MSCs declines with age, from 1/10.000 nucleated marrow cells in a newborn to about 1/100.000 nucleated marrow cells in a 80 year old person (Caplan AI, 1994).

1.2.1 Characteristics of MSCs *in vitro*

The growing interest in the potential of MSCs has resulted in an exorbitant increase of scientific publications in a short time. However, the studies performed on MSCs used different isolation and expansion methods, as well as different approaches to cell characterization.

To allow for an easier comparison between the results of these studies and facilitate progress in the field, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT), as reported in Dominici M *et al.* (2006), has proposed three criteria to define MSCs. First, MSCs must be adherent to plastic when maintained in standard culture conditions. Second, MSC populations (≥ 95%) must be positive for several antigens such as CD105 ((known as endoglin), CD73 (known as ecto 5' nucleotidase) and CD90 (also known as Thy-1). Since new surface markers may be identified in future studies leading to changes in these criteria, the ISCT recommends that the expression of hematopoietic antigens should not be used as a further requirement to identify the MSC. In other words, these cells should not have the expression (≤ 2%) of hematopoietic antigens such as CD45 (a pan-leukocyte marker), CD34 (marks primitive hematopoietic progenitors and endothelial cells),CD14 or CD11b (expressed on monocytes and macrophages),CD79a or CD19(markers of B cells) and HLA class II. Third, the cells must be able to differentiate at least into osteoblasts, adipocytes and chodroblasts under standard *in vitro* differentiating conditions. The differentiation can then be demonstrated by well-accepted staining protocols (Dominici M *et al.*, 2006).

However, it should be said that the criteria introduced by the ISCT have several limitations. For example, although the ability of MSCs to adhere to plastic surfaces is accepted to define these cells,

also pre-B-cell progenitors and granulocytic/monocytic precursors show plastic adherence (Phinney DG *et al.*, 1999). Moreover, adherent cells capable of density-independent growth are found in a number of non hematopoietic tissues, such as periosteum and dental pulp, and probably in all connective tissues, and are also called CFU-Fs. In addition, not all cells within a given population are stem cells (Bianco P *et al.*, 2008).

Several similar works strongly suggest that MSCs and isolated clones are heterogeneous not only with respect to their self-renewal ability but also to their multi-potentiality (Bianco P *et al.*, 2001). Indeed, the concept of self- renew is often erroneously confused with the ability of a cell to give rise to a broad, long-term proliferation in culture, which is the number of the population doublings. Instead, self-renewal is to be understood as the ability of a stem cell to reconstruct *in vivo* a stem cell compartment with propriety and phenotype identical to the starting population, so that the cell maintains the stem cell pool while generation progenies undergo clonal expansion and differentiation. While self-renewal was widely confirmed for hematopoietic stem cells, recently it has been demonstrated also for bone marrow in the work of Sacchetti *et al.* (2007), which showed that BM stem cells can self-renew since they can be successfully explanted like cells expressing MCAM (marker that identifies all of the clonogenic stem cells), grown through several population doublings and then transplanted to recreate a compartment of identical cells *in vivo* while generating heterotopic "ossicles" (a shell of cortical bone with a cavity containing hematopoietic tissue). Sacchetti *et al.* also stated that BM MSCs can be directly identified with a specialist type of mural cells, also called pericytes, found in the sinusoid walls and long known as adventitial reticular cells, which act as organizers and regulators of the hematopoietic microenvironment/niche (Bianco P *et al.*, 2011).

The concept of multy-potentiality opens an additional controversy arising from the common place that BMSCs can give origin to all tissues of mesodermal origin. A differentiation assay, able to unequivocally demonstrate this feature, should be conducted through the use of clonal populations of cells; it must exclude the use of artificial factors stimulating the differentiation or factors which reprogram cell fate, such as bone morphogenetic proteins (BMPs), because spontaneous differentiation potential and responsiveness to reprogramming are equally important biological characteristics of a given cell, and yet they are radically distinct conceptually and experimentally (Bianco P *et al.*, 2006; Bianco P *et al.*, 2008). Finally, differentiation must be unequivocal, that is ideally coinciding with the generation *in vivo* of histological verified tissue and not only based on the expression of a number of tissue-specific proteins or mRNA (Sacchetti B *et al.*, 2007; Bianco P *et al.*, 2001).

In keeping with their anatomical origin, BMSCs have an osteogenic imprinting, but they are non-differentiated osteogenic progenitors (Satomura K *et al.*, 2000), which is suggested not only by in vivo experiments but also by the constitutive expression of the marker regulator of skeletogenesis

Runx2/Cbfa1; osteogenic commitment directed by Cbfa1 occurs upstream of the ontogeny of marrow stromal cells, which are the precursors of osteogenic cells. These cells retain expression of Runx2/Cbfa1, possibly as an inheritance of their osteogenic origins, but they remain capable of multy-differentiation so that osteogenic fate is not mandatory (Sacchetti B *et al.*, 2007 ; Bianco P and Pamela Gehron Robey, 2000).

With a view to the possible clinical applications of mesenchymal stem cells, it should be noted that their phenotype (indicated as MHC I +, MHC class II-, CD40-, CD80-, CD86-) is considered to be non-immunogenic; studies report that transplants in allogeneic host do not require to use of immunosuppressive drugs (Vater C *et al.*, 2011). Di Nicola *et al.* (2002) described MSCs as having immunosuppressive properties, and, specifically, that MSCs can modulate T-cell functions including cell activation; indeed when autologous or allogeneic BMSCs were added to T cells stimulated with dendritic cells (because these cells are considered professional antigen-presenting cells capable of modulating T-lymphocyte activation) and mitogens, a significant dose-dependent reduction of T-cell proliferation was evident. Also, by neutralizing monoclonal antibodies, these authors indicated transforming growth factor β1 and hepatocyte growth factor as the mediators of BMSC influence.

Many works have also shown that MSCs have immunomodulatory properties impairing maturation and function of dendritic cells (Jiang X *et al.*, 2005; Aggarwal S and Pittenger MF, 2005), and that human MSCs inhibit in vitro human B-cell proliferation, differentiation, and chemotaxis (Corcione A *et al.*, 2006). Even though, the mechanisms by which these cells exert their immunosuppressive function are still unclear, it is probable that they involve both cell-to-cell contact and soluble factors in antigen specific or non-specific manners (Yagi H *et al.*, 2010). Despite some disagreement, there is evidence that these *in vitro* observations may translate to the *in vivo* setting; in particular, autologous and allogeneic MSCs therapeutic potential has been investigated as a new therapeutic strategy for T cell-mediated diseases such as graft-versus-host disease (GVHD) (Toubai T *et al.*, 2009), Crohn's disease (Forbes GM *et al.*, 2013) and the prevention of organ transplantation rejection (Casiraghi F *et al.*, 2008).

Besides the immunomodulatory ability, another reason for us to believe in useful future applications of MSCs in cell therapy is the evidence that these cells are able to act as homing agents. Homing is the mechanism whereby exogenous MSCs migrate from circulation into damaged tissues, possibly in response to signals that are up-regulated in case of injury, and once arrived, they can exert local functional effects. Caplan AI (2007) was referring to the ability of MSCs to home to injured tissues or to participate in the injury response by providing a broad array of paracrine factors as their ‘‘trophic" activity. However, several concerns for an overall clinical approval remain at present; indeed while the homing of leukocytes to sites of inflammation has been studied in depth, the mechanisms of progenitor cell homing to sites of ischemia or injury are still poorly understood.

Moreover, starting from the niche hypothesis proposed by Schofield R in 1978, aimed to describe the physiologically limited microenvironment supporting stem cells, many works have been directed to support this idea by means of a variety of coculture experiments *in vitro* and by bone marrow transplantation, in which the niche is first "emptied" through irradiation or drug treatments (Dexter TM *et al.*, 1976; Dexter TM *et al.*, 1977; Moore KA , *et al.*, 1997).

Recent reports attempting to clarify the identity of the niche components and their localization have revealed an emerging role of bone marrow stromal cells and osteoblasts as stem cell niches, which would be able to act as organizers for the hematopoietic microenvironment within bone marrow (Sacchetti B *et al.*, 2007; de Barros APDN *et al.*, 2010). As consequence, attention has shifted from osteoblasts and endothelial cells (the former to be referred to as niche in bone marrow) to MSCs or osteoprogenitors, as providers of niche regulating hematopoietic stem cells while able to maintain their undifferentiated state (Omatsu Y *et al.*, 2010; Bianco P, 2011). In addition, these data demonstrate that specific perturbations in osteolineage cells can induce complex hematological disorders indicating the central role that individual cellular elements of 'stroma' can play in tissue homeostasis (Raaijmakers MH *et al.*, 2010). This is the first example of the interplay between two different systems of stem/progenitor cells that functionally interact in the regulation of hematopoiesis and bone physiology (Mendez-Ferrer S *et al.*, 2010).

This discovery, which is fascinating for its biological meaning, entails a new point of view on applicative translational approaches involving the use of bone marrow stem cells. Whereas it has been demonstrated that osteoprogenitors, which constitute a bone marrow microenvironment component, express all the genes implicated in a putative niche effect (Bianco P *et al.*, 2008), attempts have been made to manipulate the HSC niche using regulators of the physiology of osteogenic lineage such as parathyroid hormone (whose daily treatment is a clinically approved method for increasing osteoblast functions) in order to optimize physiological interactions leading to homing and engraftment of transplanted HSCs (Calvi LM *et al.*, 2003; Adams GB and Scadden DT, 2008). Genetic alteration make osteoprogenitors capable of directing an aberrant kinetics of HSC self-renewal, leading to myelodysplasia and leukemogenesis (Raaijmakers MH *et al.*, 2010). Thus, control of hematopoietic physiology by bone marrow stromal cells opens highly innovative prospects for understanding and targeting hematopoietic diseases (Lane SW *et al.*, 2009).

1.2.2 Differentiation of MSCs *In vitro*

A broader understanding of the molecular mechanisms driving the differentiation of these cells should significantly facilitate their use in clinical applications. The development of mesenchymal progenitors along with an osteogenic, chondrogenic and adipogenic linage occurs especially under the influence of chemical stimuli, for example dexamethasone, transforming growth factor β3 and

insulin, which is accompanied by profound changes in morphology, proliferation, gene expression, and molecular signaling events (Jaiswal N *et al.*, 1997; Mackay AM *et al.*, 1998; Jaiswal RK *et al.*, 2000). Cellular differentiation is induced by cues in the environment immediately surrounding cells; however, the underlying mechanisms governing mesenchymal stem cell phenotype *in vitro* and *in vivo* are not yet completely understood. Many of the soluble factors known to influence hMSC differentiation have been identified.

The classical method for osteogenic differentiation of MSCs *in vitro* involves incubating a confluent monolayer of MSCs with combinations of Dexamethasone (Dex), beta-glycerophosphate (β- GP) and ascorbic acid 2-phosphate (Asc- 2-P) for several weeks. When exposed to osteogenic medium hMSCs transform their shape from fibroblastic to cuboidal, produce extracellular matrix mainly composed of collagen type I, and, at a later stage, deposit calcium phosphate as hydroxyapatyte crystals which can be stained positively by alizarin red and von Kossa techniques (Bruder SP *et al.*, 1997). This medium also triggers a series of molecular events including the activation of signal transduction pathways and expression of osteogenic marker genes such as Runt-related transcription factor-2 (Runx-2) which, in turn, influences the expression of bone-specific genes, such as osterix (Osx), collagen type 1 alpha-1 (Col1a1), osteocalcin (OC) and bone sialoprotein (BSP), by binding to their promoters (Kern B *et al.*, 2001; Nakashima K *et al.*, 2002; Higuchi C *et al.*, 2002). Generally, Runx-2, ALP, Col1a1, transforming growth factor-beta 1 (TGF-β1), osteonectin (ON) and bone morphogenetic protein-2 (BMP-2), are known to be early markers of osteoblastic differentiation, whereas OC and osteopontin (OPN) are expressed later in the differentiation process (Spector JA *et al.*, 2001; Long MW, 2001). Dexamethasone is a synthetic glucocorticoid and has been reported to be an essential requirement for osteoprogenitor cell differentiation in MSCs (Leboy PS *et al.*, 1991; Herbertson A and Aubin JE, 1995). While MSCs cultured in basal medium without osteogenic supplements express increased levels of ALP, they fail to express mineralized ECM as well as other osteogenic markers such as Col1 (Hildebrandt C *et al.*, 2009).

Although the precise mechanisms of action of Dex on stem cell differentiation and skeletal function are unknown, it is thought to induce transcriptional effects. In rat osteoblast-like cells, for instance, Dex induces transcription of BSP by binding on the glucocorticoid response element (GRE) in the promoter region of the BSP gene (Ogata Y *et al.*, 1995). In addition, Dex improves the expression of the β-catenin-like molecule TAZ (transcriptional coactivator with PDZ-binding motif) and of integrin α5, both of which promote osteoblastic differentiation of MSCs by activating Runx-2-dependent gene transcription (Hong D *et al.*, 2009; Hamidouche Z *et al.*, 2009). While glucocorticoids clearly induce osteoblast differentiation under certain conditions, in supraphysiological amounts they have deleterious effects on bone, resulting in inhibition of the osteoblast function. In a study by Walsh *et al* (2001) MSCs were cultured in the presence and

absence of Dex at concentrations between 10 pM and 1 μM for up to 28 days. They demonstrated that at a physiological concentration (10 nM), Dex had no effect on the adhesion of hBMSCs or on their subsequent proliferation, but enhanced their osteogenic differentiation and further maturation. However, at a supraphyfisiological concentration, the effects of Dex on the osteogenic recruitment and maturation of cells and their progeny were maintained albeit with the disadvantage of a decrease in cell number. The authors suggested that a decrease in proliferation of the osteogenic precursors, but not in their differentiation, is likely to be a key factor in the genesis of glucocorticoid-induced osteoporosis.

Furthermore, glucocorticoids may suppress bone growth *in vivo* (Ng PC *et al.*, 2002), which may limit their usefulness for repairing bone in situ. Cheng and coworkers (2000) hypothesized that the detrimental effect of glucocorticoids on bone derived, at least in part, from decreased integrin matrix interactions. They demonstrated that Dex exhibited time-dependent regulation on the expression of αvβ3 and αvβ5 integrins in normal human osteoblastic cells. Short-term (two days) exposure to Dex increased the levels of αvβ3 and αvβ5 on the surface, cell adhesion to osteopontin and vitronectin, whereas long-term (8 days) exposure to Dex decreased the expression of integrins and inhibited the cell adhesion to matrix proteins. Response to this agent is biphasic and concentration-dependent, and varies according to the length of exposure (Aubin JE, 1998, 2001). In addition, at high concentration of Dex, proliferation seems to be negatively affected, mainly due to the inhibitory effect of glucocorticoids on collagen (type I and IV) synthesis through a direct effect on the collagen gene promoter and appears also to have a post-transcriptional effect on procollagen mRNA content (Weiner FR *et al.*, 1987). However, when MSCs are cultured in the presence of ascorbic acid, the effects of glucocorticoids on collagen production are markedly masked (Vater C *et al.*, 2011).

Recent studies have shown that in the presence of Asc-2-P MSCs upregulate genes related to cell cycle and mitosis, whereas absence of Asc-2-P leads to reduced ALP expression and inhibition of calcium accumulation (Fernandes H *et al.*, 2009). Usually, concentrations ranging from 50 to 500 μM are used to induce osteogenic phenotype of MSCs (Song I *et al.*, 2009; Pytlík R *et al.*, 2009). Furthermore, for matrix mineralization the presence of both calcium and phosphate ions is essential. β-GP, which is enzymatically hydrolyzed by alkaline phosphatase, serves as a crucial source of inorganic phosphate (Chang YL *et al.*, 2000). Chung *et al.* (1992) showed that osteoblast-like cells in culture medium containing β-GP undergo mineralization, lactate production, increased ALP activity, as well as protein and phospholipid synthesis, indicating enhanced osteogenic differentiation. Usually 5–10 mM beta-glycerophosphate is used for osteogenic differentiation of MSCs (Hildebrandt C *et al.*, 2009; Chen M *et al.*, 2009).

In addition, combinations of vitamin D3 (vit D3), transforming growth factor-beta (TGFβ) and bone morphogenetic proteins (BMPs), are also used for osteogenic differentiation. Through interaction

with a nuclear receptor, vitamin D in its active form [1α,25-dihydroxyvitamin D3(1,25-D3)] has been shown to stimulate the expression of bone-related transcription factors, i.e. Runx-2 and Osx, in addition to osteoblast differentiation markers, such as ALP, Col1a1, OC and OPN (Maehata *et al.*, 2006). Although vit 1,25-D3 synergized with both Dex and bone morphogenetic protein-2 promoting expression of osteoblastic markers, it was unable to induce matrix mineralization alone (Jørgensen NR *et al.*, 2004; Fromigué O *et al.*, 1997). In contrast to this, Jaiswal *et al.* (1997) reported that Dex may reduce vitamin D receptor expression in osteoblastic cells (Jaiswal N *et al.*, 1997), leading to a reduced uptake ability for 1,25-D3 and, therefore, decreased expression of differentiation markers such as OC. TGF-β1 influences cell growth and plays an essential role in the control of bone formation by modulating the synthesis and degradation of several bone matrix components, e.g. collagen type 1 and non-collagenous proteins (Centrella M *et al.*, 1987, 1991). Notably, although TGF-β1 stimulates the expression of Runx-2, it inhibits osteoblast differentiation in the late stages (Fromigué O *et al.*, 1997). Finally, BMPs are also members of the TGF-superfamily and can, in contrast to TGF-β1, induce ectopic bone formation in developed tissues (Hogan BL, 1996; Holleville N *et al.*, 2003). Recent reports investigating the role of BMPs in osteogenesis (Diefenderfer DL *et al.*, 2003; Knippenberg M *et al.*, 2006) have shown that it may have species-specific effect *in vitro*; in both mice and rats, BMPs promote osteoblast differentiation (Cheng H *et al.*, 2003; Osyczka AM *et al.*, 2004). Interestingly, there is body of evidence that BMP-2, BMP-4 and BMP-7 fail to induce osteogenic differentiation of human MSCs (Diefenderfer DL *et al.*, 2003; Osyczka AM *et al.*, 2004). In contrast, several studies have demonstrated that in cells of the osteoblast lineage, these BMPs are capable of inducing expression of ALP, Col1a1, OPN, BSP and other non-collagenous proteins found in bone (Hildebrandt C *et al.*, 2009; Cheng H *et al.*, 2003; Lecanda F *et al.*, 1997; Locklin RM *et al.*, 2001). There is a clear need to better understand the molecular mechanisms that control osteogenesis in MSCs. Moreover, insoluble cues affecting cellular differentiation arise largely from cellular binding to ECM proteins but the mechanisms linking ECM binding to osteogenic differentiation, especially in hMSC, are still largely unknown.

The adipogenic differentiation is enhanced by incubating MSC cultures with dexamethasone, insulin, isobutyl methyl xanthine, and indomethacin. Thus, an accumulation of lipid rich vacuoles occurs within cells, which express adipocyte-specific peroxisome proliferation- activated receptor γ2 (PPARγ2), lipoprotein lipase (LPL), and the fatty acid-binding protein-4 (FABP4/aP2). Eventually, the lipid vacuoles could combine and fill the cells. Accumulation of lipid in these vacuoles is assayed histologically by oil red O staining (Vater C *et al.*, 2011).

To promote chondrogenic differentiation, MSCs are centrifuged to form a pelleted micromass and cultured in the presence of transforming growth factor-β (Mackay AM *et al.*, 1998). The cell pellets develop a multilayered, matrix-rich morphology, and histological analysis shows strong staining

with toluidine blue, thus indicating an abundance of glycosaminoglycans within the extracellular matrix (Kopen GC *et al.*, 1999). The cells also produce type II collagen, which is typical of articular cartilage (Pittenger MF *et al.*, 1999). It has also been demonstrated that, when treated with 5-azacytidine and amphotericin B, MSCs differentiate into myoblasts that fuse into multinucleated myotubes (Wakitani S *et al.*, 1995).

In addition, differentiation into neuron-like cells expressing markers typical for mature neurons has been reported (Woodbury D *et al.*, 2000; Kohyama J *et al.*, 2001). However, Hofstetter and colleagues (2002) established that these neuron-like cells lack voltage-gated ion channels necessary for generation of action potentials; but, when delivered into the injured spinal cord of animals rendered paraplegic, MSCs survive well and form nerve fiber-permissive tissue bridges across areas of debris which are associated with a degree of long-term functional improvement. Therefore, these cells may not actually be classified as true neurons but a beneficial effect on the function of target organs has often been observed (Phinney DG and Prockop DJ, 2007; Picinich SC *et al.*, 2007). Further studies have also demonstrated that MSCs can also differentiate, under appropriate in vitro conditions, to form tenocytes and cells of visceral mesoderm (endothelial cells) (Pittenger MF *et al.*, 1999; Reyes M *et al.*, 2001).

1.2.3 Clinical applications of MSCs

Recognition of the broad growth, the phenotypic characteristics and differentiation potential of marrow stromal cells and the ease with which they can be obtained and increased in number has opened the door to at least three classes of clinical applications.

a) *Systemic delivery*

The first and perhaps most ambitious use for the mesenchymal stem cells would be to reconstitute some or all of the tissue to cure diseases by systemic delivery. A large number of studies were carried out on animal models (Pereira RF *et al.*, 1998; 95: Hou Z. *et al.*, 1999). Barbash IM and colleagues (2003) transfused labeled rodent BM-MSCs in rats subjected to myocardial infarction (MI) by direct left ventricular cavity infusion and intravenous infusion; they found that intravenous delivery of BM-MSCs is limited by the entrapment of the donor cells in the lungs, with a small amount of engrafting in the heart, but much smaller than after direct delivery into the ventricle.

However, previously Gao *et al.*(2001) had found that treatment with vasodilator sodium nitroprusside administered prior to cell infusion decreased the number of cells entrapped.

MSCs were also used to treat lung injury in mice: in their study Ortiz and colleagues (2003) demonstrated that murine MSCs home to lung in response to injury, adopt an epithelium-like phenotype, and reduce inflammation and collagen deposition in lung tissue of bleomycin treated mice, representing a model of pulmonary fibrosis. Despite evidence from animal models of the ability of stromal cells to colonize the target damaged organs once infused into the circulation is still missing, human bone marrow transplant (BMT) has already been attempted. Horwitz and colleagues (1999) administered systemically cultured MSCs after ablative chemotherapy to treat children with severe deforming osteogenesis imperfecta (OI), a disease in which osteoblasts produce defective type I collagen, which leads to osteopenia, multiple fractures, bone deformities, and shortened stature. Three months later they reported new dense bone formation, an increase in total body bone mineral content, growth velocity, and reduced frequency of bone fracture in all patients. Although there was an engraftment of 1–2% bone cells (estimated by ex vivo culture of recipient bone and bone marrow cells) and clinical improvements were evaluated over time, the clinical controls and the histological data lack in accuracy (Bianco P and Robey PG, 2000). In addition, increasing the time after infusion slowed down growth rate while bone mineral content continued to increase; as a consequence, it was hypothesized that additional therapy using isolated hMSCs, without marrow ablative chemotherapy, would enhance the responses after BM transplantation. Therefore, culture-expanded hMSCs were infused into children who had previously undergone conventional BMT. As a result, five out of six patients showed engraftment in one or more sites, including bone, skin, and marrow stroma, and had an acceleration of growth velocity during the first 6 months following infusion (Horwitz EM *et al.*, 2002). However, in both these works the authors failed to give sufficient evidence of the presence of donor cells and, since myeloablation apparently enhance osteogenic activity in several animal models, it remains to be determined whether clinical improvement was caused by the replacement of host osteoblasts with the administrated donor cells (Bianco P and Robey PG, 2000; Docheva D *et al.*, 2007). In other words, evidence for a biologically significant effect of the systemic infusion of bone marrow stromal cells is not available. The major limitation of this application depends on the commonplace that bone marrow stromal cells transplantation can take place using the same principles and procedures as transplantation of hematopoietic cells, which is most widely accepted. Although it was claimed that during BMT a small number of donor stromal cells could be found in the receiver, the majority of evidence indicates that marrow stromal cells are not transplanted during this procedure (Simmons PJ *et al.*, 1987; Agematsu K *et al.*, 1991).

The main point to consider is that this technique is based on the few accredited biological characteristics of hematopoietic stem cells which are completely different from those of stromal

cells. Whereas HSCs are known to circulate and pass the sinusoidal wall in the marrow via selective cell-cell interactions which allow them to locate in the extravascular compartment, circulating progenitors of the stromal system have not been identified conclusively (Luria EA *et al.*, 1971). Even assuming that such cells exist, there is little doubt that non circulating locally resident progenitors fabricate the majority of skeletal tissues during both development and postnatal growth. Another pertinent point is that while HSCs can replenish the whole hematopoietic system in a few weeks, renewal in an adult skeleton is markedly slower and much more complex. It requires 15 years and entails the creation of a complex physical structure whose precise spatial layout reflects an equally precise timing of events over a period of years. Consequently, we would expect replacement of skeletal tissue with infused BMSCs to occur over longer timescales compared to rapidly self-renewing tissues, even though issues related to efficient cell delivery and systemic engraftment were resolved (Bianco P *et al.*, 2001; Bianco P and Robey PG, 2001). Considering all the foregoing, systemic transplantation must follow precise guidelines and prove the homing ability of viable donor derived cells in the receiver as well as their presence in bone and bone marrow. Likewise, these cells must be shown to be competent for engraftment, generating a differentiated progeny in the recipient's marrow which must be sufficient to influence, in turn, tissue function. Finally, and to avoid the occurrence of any potential danger to humans, it must be proved that these cells produce the desired biological effect in appropriate animal preclinical models before clinical trials for these procedures are performed, although preliminary clinical studies are already underway (Bianco P and Robey PG, 2000).

b) *Gene therapy*

Due to their poor immunogenicity, MSCs may be also ideal carriers to deliver genes into the tissues of interest for gene therapy applications; this is probably the most difficult challenge. Several approaches have been examined and used to introduce exogenous DNA into MSCs to use them in tissue regeneration therapies. A popular option consists in viral transduction, particularly using adenovirus mediated gene transfer, which is able to generate stable cell clones with high efficiency and low cell mortality. For example, in the work of Chamberlain JR *et al.* (2004) an adenovirus vector was used successfully to disrupt dominant-negative mutant (*COL1A1*) collagen type I gene in MSCs from individuals with brittle bone disorder, osteogenesis imperfecta, where it acted as bone-repairer. However, the safety problems associated with viral transduction have led scientists to search for alternative non-viral gene delivery approaches. Traditional transfection methods, such as calcium phosphate coprecipitation, microinjection, lipofection, and electroporation, have had little success in delivering plasmid DNA into MSCs, usually resulting in less than 1% transfection efficiency and high cell mortality (Song L *et al.*, 2004). These methods have therefore proved to not be suitable for producing sufficient amount of engineered human stromal cells for gene delivery and

transplantation. Furthermore, usual regulation of expression of a desired gene in these cells appears to be difficult, and transgenes which are expressed successfully in standard, continuous, or immortalized cell lines cannot be used directly for *in vitro* models using human cells, let alone for clinical applications (Bianco P and Robey PG, 2000).

c) *in situ transplantation*

Finally, *in situ* transplantation is the most easily implemented use of marrow stromal cells and generally involves their osteogenic potential for the reconstruction of localized bone defects. The advantage provided over other existing methods (the use of uncultured marrow or biomaterials) is the hypothetical full biological compatibility provided by a device composed entirely of cells, which might overcome the limits in the size and shape of defects to be repaired (Bianco P and Robey PG, 2000). A number of preclinical studies in animal models have strongly supported the feasibility application of marrow cell grafts for orthopedics (Krebsbach PH *et al.*, 1997; Gazit D *et al.*, 1999; Kon E *et al.*, 2000) and led to preliminary studies in humans (Granchi D *et al.*, 2010). Besides osteoblastic cells, cardiomyocytes have been reported another possible target of stromal cell manipulation and transplantation. For instance, several researchers have used BMSCs to repair the infarcted myocardium (Orlic D et al. 2001 a;2001 b). Also, Hofstetter and colleagues (2001) injected MSCs into the spinal cords of rats rendered paraplegic one week after the injury. They found that MSCs formed robust bundles which bridged the epicenter of the injury guiding regeneration through the spinal cord lesion, thus promoting recovery. This phenotypic shift is explicable by the plasticity characterizing the bone marrow stromal system, which distinguishes it from the hematopoietic one, because its cells are able to differentiate into elements which are not phenotypically related to the cells in their tissue of origin (Bianco P *et al.*, 2001).

Generally, the cells of connective tissues are characterized by a slow turn-over and most are exposed to an abundant extracellular matrix (ECM) that helps maintain their differentiated phenotype, but the marrow stroma is perhaps the only connective tissue with a remarkable paucity of ECM, which may in part explain the facility with which these cells can pass from one phenotype to another (Bianco P and Robey PG, 2000). Nonetheless, the ideal *ex vivo* expansion conditions, the number of cells required for the regeneration of a volume of bone and the composition and structure of the ideal carrier are still under investigation.

1.3 Biomaterials for tissue engineering

The recent advances in stem cell biology and recognition of their unique properties have opened important prospects about their applications in tissue and organ disorders repairs.

For over 50 years patients suffering from diseased and injured organs have often been treated with organ transplants. This practice has been in use since 1954 when Murray successfully transplanted a kidney from one identical twin to another. It was the first entire organ to be replaced in a human. Several years later, Murray performed an allogeneic kidney transplant from a non-genetically identical patient to another. This transplant, which overcame the immunologic barrier, marked a new era in medicine and opened the door for the use of transplantation as therapy for different organ systems (Murray JE *et al.*, 1976). Since current medicine has increased human life expectancy, the aging population has grown, as has the need for donor organs, because aging organs are generally more prone to failure. As organ transplants became increasingly widespread, the most significant problem related to them was the shortage of available organs. Furthermore, patients fortunate enough to receive a donor organ are at risk of pathogen transfer and acute or chronic rejections, and even if they does not occur, immunosuppressive therapy is still needed throughout the patients' lives, which also entails associated morbidity and many unknown variables in the process of new organ maturation and development (Badylak SF *et al.*, 2012). To overcome these difficulties, physicians and scientists are searching for new techniques as alternatives to organ transplantation.

In the 1960s, a natural evolution occurred whereby researchers began to combine new devices and materials with cell biology, thus creating a new field which is now termed "tissue engineering". The most common concept in tissue engineering is the creation of a living device, combining a scaffold or a matrix, living cells and/or bioactive factors (such as growth factors or other biological molecules) in order to restore, maintain or improve injured tissue or organ functions (Langer R and Vacanti JP, 1993). The cell based nature of tissue engineering, not necessarily stem cell based, serves to specify, and distinguish it from 'guided tissue regeneration' in which a scaffold is designed to support regeneration solely by cells residing at the site of its transplantation (Stock UA and Vacanti JP, 2001). Since the fields of stem cells, cell transplantation, cloning and tissue engineering all have the common aim of living tissues and organs regeneration, in 1999, William Haseltine, the then scientific founder and chief executive officer of Human Genome Sciences, coined the expression "regenerative medicine", to group all these fields together under one term.

In recent years, a variety of different biomaterials have been investigated for their scaffolding ability in tissue engineering. The scaffold supports cell colonization, migration, growth and differentiation and often guides the development of the required tissue or acts as a drug delivery vehicle (Hutmacher DW and Garcia AJ, 2005). For this use, materials have to fulfill some

fundamental requirements. They have to be biodegradable to allow replacement by regenerated tissue; they must be immune-compatible, and they must neither be toxic nor release toxic substances when they are degraded. Besides these features, matrices formed from biomaterials must have distinct properties with regard to the desired kind of tissue (Ehnert S *et al.*, 2009).

In the past, synthetic biomaterials such as ceramics, bioglass and metals were introduced to replace or rebuild diseased tissues or parts in the human body thus opening a new field of research that led to the development of a wide array of devices for human use (Bose S *et al.*, 2012). Although these devices were capable of providing structural support, they typically lacked the innate capacity to actively modulate cell phenotype, making it difficult to effectively control cell behavior *in vitro,* and not allowing the complete restoration of the original tissue (Olson JL *et al.*, 2011). Subsequently, the use of synthetic polymers as scaffold has greatly impacted the advancement of modern medicine; in particular, polymeric biomaterials, such as polylactide (PLA), polyglycolide (PGA), and poly (lactide-co-glycolide) (PLGA), –which are biodegradable–, are especially advantageous because they can be broken down and removed after they have served their function, thus providing a variety of clinical applications such as surgical sutures and implants (Ulery BD 2011).

However, while a number of synthetic polymer-based scaffolds possess desirable physical properties, because they are biodegradable and capable of providing structural support (Chan G and Mooney DJ, 2008), many of them lack biocompatibility and cannot be used for the delivery and subsequent cellular growth, particularly given the intrinsic challenges in maintaining the viability and biological functions of the transplanted cells at the disease-compromised tissue site (Noth U, 2010). In addition to their biological inertness which makes them unable to actively modulate cell phenotype *in vitro,* the presence of acidic moieties, residual catalysts, and microscale particulates accompaning degradation limited their clinical application (Williams DF, 2008).

The need to provide signals to cell populations *in vitro* in order to direct their responses, as well as, the improved understanding of the interactions between the cells and their micro environments have led scientists to focus on the role of the extracellular matrix. The ECM, an important component of the cellular niche in a tissue, plays a central role in regulating the maintenance and behavior of progenitor cells via physical interactions with cell surface proteins and modulation of soluble growth factor (Chen XD, 2010; Guilak F *et al.*, 2009). Traditionally, ECM proteins, such as collagens and fibronectin, were perceived as the ECM scaffold with a mainly structural role, but now they are known to control many different functions such as cell proliferation, growth, cell survival, migration and differentiation (Fernandes H *et al.*, 2009). Therefore, the possibility of taking advantage of the ECM potential has become of great interest for tissue engineering scientists who have begun to focus on developing novel biomaterial surfaces which are better able to direct cell phenotype by mimicking the *in vivo* cellular environment.

Until now, this purpose has been primarily pursued by the deposition of individual purified ECM proteins or peptides on a substrate surface. ECM biopolymers have been broadly investigated as potential adhesive scaffolds for bone defect healing and implant integration; for example, there are many studies that use collagen to assess the mechanisms of cell motility and contraction thanks to its abundance in bone matrix (Grinnell F, 2003; Wolf K *et al.*, 2003). Collagen can be readily purified from animal tissues, such as skin and tendon, as well as from human tissues such as placenta, and reconstituted into gels by changing the pH and temperature of suspension of the precursor components. For use in situ tissue repair and regeneration the elasticity of collagen gels can be easily adjusted by means of chemical glycation methods to obtain matrices possessing high mechanical strength. Also heat and chemical treatments have been developed to produce cross-linked collagen sponges (d'Aquino R *et al.*, 2009). Glycosaminoglycans (GAGs) such as chondroitin sulfate and hyaluronic acid are often applied in cartilage tissue engineering as natural components of hydrogel like scaffolds, because they promote chondrocyte redifferentiation (Wang D-A *et al.*, 2007 ; Hwang S *et al.*, 2007). Hyaluronic acid can be isolated from animal tissue, such as rooster comb, and from microbial cultures; by absorbing enormous amounts of water and it causes an osmotic swelling which, in turn, provides compressive strength. Attempts have been made to create chemical derivatives with the aim of rendering the polymer more hydrophobic and, thus, less soluble, for example by functionalizing with hydrophobic esters (Vindigni V *et al.*, 2009) or by cross-linking the material into an elastic gel (Bulpitt P and Aeschlimann D, 1999). These materials have been used as barriers to prevent postoperative adhesion formation in internal healing (Johns DB *et al.*, 1997) and as delivery vehicles to transplant cells for *in situ* tissue formation; for instance Solchaga *et al.* (2001) transplanted both chondrocytes and mesenchymal stem cells within matrices constructed from hyaluronic acid derivatives to repair articular cartilage. Chondroitin sulfate has also been purified from animal sources and utilized in matrices with collagen as a structural component in skin (Butler CE *et al.*, 1999) and peripheral nerves repair (Chamberlain LJ *et al.*, 2000).

Fibrin is a fibrillar protein which shows a great potential in wound healing and tissue engineering. It is formed by polymerization of fibrinogen in the presence of thrombin; subsequently it undergoes crosslinking mediated by transglutaminases contributing to clot formation during wound healing (Lord ST, 2007; Lorand L and Graham RM, 2003). Fibrin is available from autologous sources and cryoprecipitated blood plasma, and alone or in combination with other materials it has been successfully used as a biological scaffold for skin repair, e.g. in the fixation of skin grafts (Currie LJ *et al.*, 2001) and as an effective cell transplantation matrix in dermal burns repair with autologous keratinocytes isolated from healthy skin (Horch RE *et al.*, 2001; Currie LJ *et al.*, 2003). Also fibrin matrices are currently used in clinics as drug delivery systems for proteins such as bone morphogenetic proteins (BMPs), and for grow factors in vascular graft engineering (Schmoekel HG

et al., 2005). In general, these proteins have obtained some degree of success because of their inherent properties of biological recognition, including presentation of receptor-binding ligands and susceptibility to cell-triggered proteolytic degradation and remodeling. However, despite their natural derivation from the ECM, the widespread use of natural macromolecules in clinical applications has been prevented by several factors. ECM proteins have low solubility, the processes of extraction and purification in large quantities are expensive, they undergo batch-to-batch variation, they allow for the possibility of pathogen transmission and potentially suffer from immunogenicity (Shekaran A and AJ García, 2011; Lutolf MP and Hubbell JA, 2005). Nevertheless, greater control over materials properties and tissue responses could be achieved with available synthetic analogs (Lutolf MP and Hubbell JA, 2005). To avoid ECM molecules limitations, methods for synthesis of the recombinant expression of proteins, such as collagen or for ECM-derived peptides have been recently described; these synthetic analogs have the minimal functional sequence of their original protein in order to render functionalized materials bioactive (Shakesheff K *et al.*, 1998; Yang, C. *et al.,* 2004; Sano A *et al.*, 2003). Indeed, while natural proteins, such as fibronectin or collagen, are enormous molecules consisting of thousand of aminoacids, only a few short peptide sequences within them work as integrin recognition and binding sequences able to trigger downstream processes such as adhesion, signaling and spreading. Moreover, compared to ECM polymers, these peptides and protein fragments, can be synthesized in larger quantities via chemical synthesis or recombinant protein expression; they can be immobilized on appropriate surfaces at high densities, and tailored in composition for specific applications (Shekaran A and Garcia AJ. 2011b).

Since first evidences (Pierschbacher MD and Ruoslahti E, 1984; 1987) have identified the domains on ECM adhesion proteins that mediate receptor–ligand bond, perhaps best represented by the RGD tripeptide (Arginine-glycine-aspartic acid) of fibronectin (FN), knowledge of the molecular interactions between cell surface receptors and ECM adhesion molecules has quickly evolved (Guilak F *et al.*, 2009). Thus, several approaches have been developed to incorporate these domains into materials (Hubbell JA, 1999; Lee JY *et al.*, 2011), many of which aim to reconstitute the ECM cell adhesion character in matrices for tissue engineering applications. Although there are many ECM-derived cell-binding motifs, most bioadhesive tissue engineering studies have been restricted to fibronectin and collagen sequences. Many biomaterial strategies have incorporated RGD as an adhesive ligand. In 1984, Pierschbacher and Ruoslahti used enzyme techniques to reduce the cell-binding domain of fibronectin to the RGD segment, and showed that most of the cell adhesion activity of fibronectin can be attributed to this tripeptide. Since then, cell adhesive RGD sites were identified in many other ECM proteins, including fibronectin, vitronectin, bone sialoprotein and osteopontin (Pytela R *et al.*, 1987). Also RGD can bind to multiple integrins such as αvβ3, αvβ1, α8β1, αvβ8, αvβ6, αvβ5 and αIIbβ3. However, for certain integrins, binding to RGD is strongly

modulated by another sequence, such as the PHSRN synergy site for α5β1 (Redick SD *et al.*, 2000; Petrie TA *et al.*, 2006).

The RGD motif has shown controversial results in bone regeneration experiments. Elmengaard B *et al.* (2005) reported enhancements in osseointegration for implants with cyclic RGD peptides, but other studies using cyclic RGD have also failed to show improvements in implant fixation in rat tibiae (Rammelt S *et al.*, 2006) and canine mandibles (Schliephake H *et al.*, 2002). Also, Hennessy KM and coworkers (2008) implanted hydroxyapatyte (HA) disks functionalized with RGD peptides into rat tibiae and demonstrated that after 5 days these implants significantly inhibited total bone formation and reduced the amount of new bone. Thus, RGD peptide alone, which is widely believed to promote cell/biomaterial interactions, seems to have a negative effect in bone formation and osseointegration responses on HA implant performance. Although fibronectin and its RGD peptide are widely used for cell adhesion, the promiscuity with which they engage integrins leads to difficulty in controlling receptor-specific interactions. Hence, many attempts have been made to design specific recognition sequences for integrins as a promising approach to control cellular processes. In their work Petrie *et al.* (2006; 2008) have engineered a recombinant fragment of fibronectin, FNIII7-10, which includes the 7–10th repeats of native fibronectin and binds specifically to the α5β1 integrin. This fragment enhances implant osseointegration in a rat cortical model when compared to titanium implants modified with RGD at an equivalent molar surface density, as well as both osteoblast adhesion strength and differentiation *in vitro*. Also Martino and colleagues, using surfaces and hydrogels functionalized with fibronectin (FN), fibronectin fragments (FNIII9–10 and FNIII10) and a more α5β1-specific mutated fibronectin fragment (FNIII9*-10) demonstrated that the level of osteoblastic differentiation for each fragment was correlated with its degree of binding specificity for the α5β1 integrin which supports other studies suggesting that α5β1 engagement may enhance osteogenesis (Hamidouche Z *et al.*, 2009; Petrie TA *et al.*, 2008). Thus, the engineered peptide FN III9*-10 provides more α5β1-integrin-specific instructions to MSCs capable of supporting proliferation and enhancing differentiation, while maintaining similar attachment and spreading capacities compared to FN (Martino MM *et al.*, 2009).

Other engineered peptides which have been extensively studied derived from collagen. The hexapeptide sequence Gly-Phe-Hyp-Gly-Glu-Arg (GFOGER) is found on residues 502-507 of the α1(I) chain of type I collagen and serves as the major recognition site for α2β1 integrin binding (Knight CG *et al.*, 1998; 2000). Reyes and Garcìa (2003) produced a Col I-mimetic GFOGER containing peptide which summarizes the triple helical tertiary structure of native collagen as an adhesive ligand for biomaterials; surfaces including adsorbed or covalently immobilized GFOGER peptide supported α2β1 integrin mediated cell adhesion and focal adhesion assembly as native collagen I. This engineered peptide also promotes osteoblastic differentiation of

MC3T3-E1 and primary bone marrow stromal cells *in vitro* (Reyes CD *et al.*, 2004; 2007). Furthermore, Wojtowicz and colleagues (2010) demonstrated that GFOGER enhances bone repair *in vivo* within rigorous critical-sized rat femur defect models without the delivery of cells or growth factors.

Besides the large number of ECM-derivative peptides, in recent years, an increasing trend has been observed toward the creation of bioactive scaffolds via incorporation of growth factor-derived peptides. Growth factors are soluble signals which not only provide physical support but also express biological signals to modulate tissue regeneration. Soluble signals include growth factors such as fibroblast growth factor (FGF), epidermal growth factor (EGF) insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), transforming growth factor- β (TFG-β) and vascular endothelial growth factor (VEGF), as well as cytokines and chemokines (Whitaker MJ *et al.*, 2001; Boehler RM *et al.*, 2011; Sikavitsas VI *et al.*, 2001). Growth factors are naturally occurring polypeptides that may act through autocrine or paracrine mechanisms with the primary result of activating cellular growth, proliferation and/or differentiation. They are often stored and sequestered in the extracellular matrix and interact with cells through receptor tyrosine kinases (RTKs). Growth factor signaling pathways overlap to a large extent with that of integrins, thus cell responses to many growth factors are dependent on integrin-mediated adhesion (Discher DE *et al.*, 2009).

Many growth factors are quite versatile, stimulating division in numerous cell types; while others are specific to a particular cell-type bind to receptors on their plasma membrane. Therefore, a crucial component in planning a controlled delivery system is the selection of the appropriate single or combination of growth factors to optimize tissue repair (Lee K *et al.*, 2011). For example, bone morphogenetic proteins (BMPs), multi-functional growth factors belonging to the TGFβ superfamily, are the proteins most investigated for bone regenerative therapies as they regulate key steps in the process of bone morphogenesis, such as mitosis, chemotaxis, cartilage induction, osteoblastic differentiation and bone formation (Cunningham NS *et al.*, 1992; Gautschi OP *et al.*, 2007). It was demonstrated that a peptide derived from amino acids 73-92 of BMP-2, designed as P24, enhances *in vivo* ectopic bone formation within poly-lactic-co-glycolic (PLGA) implants, hydroxyapatite/recombinant collagen/poly-lactic acid scaffolds and PLGA/polyethylene oxide-aspartic acid scaffolds (Duan Z *et al.*, 2007; Wu B *et al.*, 2008; Lin ZY *et al.*, 2010) and that MSCs cultured i*n vitro* in osteogenic media including P24 peptide show higher alkaline phosphatase activity than cells in osteogenic media alone. Furthermore, Lee *et al.* (2009) identified a peptide sequence derived from BMP-2 (30-34), DWIVA, osteopromotive domain (OPD), strongly supports human BMSCs adhesion *in vitro* and enhances their alkaline phosphatase activity. Although this approach has led to improvements in cell adhesion and differentiation, single proteins or peptides are unable to accurately mimic the complex native ECM composition, a milieu that can be

efficiently generated only by a certain number of cellular populations. Therefore, cells cultured within a functionalized biomaterial have to synthesize a much more complex ECM within their microenvironment before reaching their ultimate functional state, a process that can prolong the maturation time of engineered tissues (Decaris ML *et al.*, 2012).

Decellularized tissues offer an alternative by providing a complex ECM-based scaffold similar to that of the native tissue. It involves the use of natural extracellular matrices obtained by the decellularization of whole allogeneic or xenogeneic tissues and organs. Once obtained, the 3D scaffold should be ideally repopulate with autologous cells thus representing a potential solution to the shortage of allogeneic donor organ; this construct is allowed to mature *in situ* or *in vitro* in a bioreactor for a short time before implantation *in vivo*, even though very few bioreactor systems comply at present with good manufacturing process regulations (Crapo PM *et al.*, 2011). If autologous cells are unhealthy or cannot be satisfactorily expanded *ex vivo*, endogenous organ specific progenitor cells or multipotent stem cells, derived from bone marrow or other tissues, might be usable to recellularize the ECM-scaffold with consequent guided differentiation along organ-specific or tissue-specific lineages. Another possibility would be using of differentiated autologous induced pluripotent stem cells (IPS). Whichever cell sources is used, none of them requires immunosuppression therapy after implantation in the host (Olson JL *et al.*, 2011).

The method of cell reintroduction into a three-dimensional scaffold derived from tissue or organ, will take advantage of the retained vascular structures; for example, perfusion via the native vascular system provides the necessary way to deliver cells to all the scaffold regions. Other organ-specific methods (e.g, airway delivery in the lung) might also be used but they have not been supported by systematic studies evaluating cell concentrations in the perfusate, perfusion pressures, flow rates, or other variables that may affect cell survival (Badylak SF *et al.*, 2012).

The main advantage of decellularised scaffolds is probably to preserve or enhance site-pertinent cell phenotypes during the process of cell repopulation through presentation of the ligands and bioactive molecules that are necessary for resident or migrant cell populations to create a functioning organ able to respond effectively to the demands of a recipient after *in vivo* implantation (Badylak SF *et al.*, 2009). Although notable scientific and ethical challenges remain as this approach advances to clinical use, successful proof of principle for organs such as lung (Petersen TH *et al.*, 2010; Daly AB *et al.*, 2012), liver (Uygun BE *et al.*, 2010; Soto-Gutierrez A *et al.*, 2011) and heart (Ott HC *et al.*, 2008), and complex tissues such as trachea (Macchiarini P *et al.*, 2008), esophagus (Badylak SF *et al.*, 2011), and skeletal muscles (Mase VJ Jr *et al.*, 2010) have been reported.

In spite of the great potential of this strategy, to obtain the removal of cells from their integrin-bound anchors and intercellular adhesion complexes while maintaining extracellular matrix surface topography and resident ligands, is a challenge. Moreover, the methods used to achieve scaffold decellularisation (physical, ionic, chemical, and enzymatic) are often harsh and also long procedure

times (ranging from 5 h to 7 weeks) can damage the ability of the residual ECM to modulate cell behavior. Finally, failure to effectively remove cellular remains can cause a proinflammatory response in the recipient that interferes with the structure and function of the recellularised organ (Badylak SF *et al.*, 2012).

To avoid these problems scaffold coated with cell-derived decellularized matrix (DM) should be a safe and reliable biomaterial candidate. In particular the ECM secreted by autologous cells would be a potential option to acellular autologous tissues and organs because autologous cells can be expanded *in vitro*, deposit extracellular matrix and maintaining under a pathogen-free condition. Once secreted, these ECM have to be decellularized to obtain a free cells matrix and then utilized as substrate for cell growth and differentiation (Decaris ML and Leach JK 2011).

Chen X-D *et al.* (2007) prepared, from murine BMSCs, a cell-free extracellular matrix that was revealed to have a composition reflecting that of native ECM; indeed it was made up of collagen types I, III, and V, syndecan-1, perlecan, fibronectin, laminin, biglycan, and decorin. Interestingly, semiquantitative immunostaining procedure demonstrated that, for the majority, the cell extraction procedure did not seem to affect the composition of the cell-free ECM. Moreover, when murine MSCs expanded on free cell ECM were transplantated *in vivo* in immunocompromised mice generated more bone compared with cells grew on plastic. Cell free extracellular matrix obtained from human BMSCs was also able to strongly promote cell proliferation retaining cells in a more multipotent state during culture as demonstrate by telomerase activity that remained highly stable in cells maintained on the ECM, but rapidly decreased in cells on plastic (Lai Y *et al.*, 2010).

Lu H and colleagues (2011) underlining the importance of the development of autologous scaffolds and restricted availability to obtain it from a patient tissue decellularization, prepared an autologous extracellular matrix (ECM) scaffolds. It was made by culturing human autologous cells (BMSCs, chondrocytes, and fibroblasts) and mouse fibroblast-derived ECM scaffolds in a three-dimensional PLGA template, decellularization, and template removal. Authors reported that ECM scaffolds derived from mouse fibroblast showed excellent biocompatibility when implanted into mice.

Furthermore these ECM derived scaffolds have also been used with the purpose of inducing differentiation of cells cultured on them. For instance Datta N *et al.* (2005) created bone-like cell free ECM seeding rat BMSCs, previously cultured in osteogenic medium, on titanium fibers scaffold and found that they were able to enhance the osteoblastic differentiation of rat bone marrow stem cells. Hoshiba T *et al.* (2009; 2010) obtained cell free ECM that mimic the stepwise tissue development of extracellular matrix during osteogenesis and adipogenesis and demonstrated that these coatings had different effects respectively on the osteogenesis and adipogenesis of MSCs, and that the early stage matrices provided in both cases a favorable microenvironment for the differentiative process. On the contrary, Pei M and coworkers (2011) evaluated differentiative potential of hBMSCs isolating cells from either ECM or plastic and re-plating they on plastic. They

founded that hBMSCs expanded on cell free ECM had an increased osteogenic potential but a decreased adipogenic ability compared to cells grown on Plastic.

Attempts were also made to creating transferable decellularized matrix, by culturing human mesenchymal stem cells (hMSCs) on tissue culture plastic (TCP) followed by collection, mechanical homogenization and transfer to a secondary culture surface. They established that transferred decellularized matrix had the ability to accelerate hMSC osteogenic differentiation thus demonstrating that ECM instructive potential was maintained also after its transfer to another surface (Decaris ML *et al.* 2012).

2. MATERIALS AND METHODS

The experimental protocol consists in two different steps:

1) The construction of a cell free extracellular matrix using a monolayer of bone marrow mesenchymal stem cells on glass or TCP.

2) ECM coated scaffolds were seeded with an appropriate number of BMSCs, in comparison to uncoated tissue culture plates (TCP) and investigations have been performed both in normal medium and under osteogenic treatment.

2.1 Mesenchymal Stem Cell Culture

Cells were obtained from human bone marrow aspirates of healthy donor, seeded in 75 cm^3 flasks and expanded in growth medium (GM) that is minimum essential alpha medium (αMEM) supplemented with L-Glutamine, Nucleosides, Earle's salts, antibiotic-antimycotic (10,000 U/mL Penicillin, 10,000 µg/ml Streptomycin, 25 µg/ml Fungizon), 10% fetal bovine serum (FBS), 100 µM ascorbic acid and maintained at 37ºC in a humidified atmosphere of 5% CO2 and 95% air. Medium changes were performed after 3-4 days to remove non adherent cells and replaced with fresh media. Upon confluence cells were detached by enzymatic digestion in Trypsin/EDTA.4Na (0.05% / 1x), fluo-cytometrically characterized for surface marker pattern expression typical for human MSCs (positive for: CD105, CD90W, CD73 and negative for CD34, CD14, Gly A) and used for experiments. Vital cell count was performed in Bürker chamber using Trypan blue. All products used were purchased from GIBCO (Life technologies).

2.2 Cell-free extracellular matrix preparation

The adherent stromal cell layer was dispersed with Trypsin/EDTA·4Na (0.05% / 1x) then 1,0-1,5x10^4 cells/cm^2 were seeded on glass coverslips previously sterilized or plastic culture plates and cultured for an additional 15 days. The medium, α-MEM with 15% FBS, was changed every 3–4 days; ascorbic acid (100 µM; Sigma) was added during the final 8 days of culture. After extensive washing with PBS, cells were permeabilized by incubation with 0.4% Triton X-100 containing 20 mM NH_4OH in PBS for 7 min at 37°C. Plates were then rinsed with PBS and treated with bovine Deoxyribonuclease I (Invitrogen, 100 units/mL PBS) for 1 h at 37 °C to obtain the digestion of the DNA. After 3 washes in PBS, plates were allowed to dry within a sterile biosafety cabinet for up to 12 h. Matrix-coated substrates were stored at room temperature in the dark (according to Chen *et al.*, 2007 modified by Decaris and Leach, 2011).

2.3 Osteogenic Differentiation Medium

Osteogenic medium (OM) used to induce differentiation consists in growth medium (minimum essential alpha medium (αMEM) supplemented with L- Glutamine, Nucleosides, Earle's salts, antibiotic-antimycotic (10,000 U/mL Penicillin, 10,000 μg/ml Streptomycin, 25 μg/ml Fungizon), 10% fetal bovine serum (FBS), 100 μM ascorbic acid) supplemented with 100μM Dexamethasone (Sigma) and 10mM β-glycerophosphate (Sigma).

The osteogenic induction was accomplished maintaining MSCs in culture under discontinuous treatment with osteogenic medium, that consisted in 3 days of osteogenic medium followed by a change with normal medium for 4 days.

- ✓ ***Experimental protocol for MSCs***

a) cell survival and proliferation was assessed via MTT assay. In this case $3x10^4$ cells/well were seeded 6 well plates and examined at 3,6 and 15 days.

b) ultrastructure of the cell free extracellular matrix and of seeded cells using scanning electron microscopy (SEM); Calcium content was determined by X- Ray microanalysis. For this purpose $2.5x10^3$ cells were seeded and observed after 3 hours, 6 and 15 days.

c) microfilaments and adhesion cell surface integrin α5 were evaluated by immunofluorescence. For this aim we seeded $1x10^4$ cells on the slides that were analyzed after 3 hours and 6 days.

d) activity of Alkaline Phosphatase, early osteogenic marker was performed by the Enzymatic Assay of ALP (Sigma). To do this $1x\ 10^4$ cells were cultured on plates and analyzed for 6, 15 and 21 days.

e) matrix mineralization was evaluated using Alizarin red staining. Thus, $1x\ 10^4$ cells were seeded and examined at 6, 15 and 21 days.

f) osteogenic gene expression by Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with $3x10^5$ cells at 1 and 6 days of culture under treatment with osteogenic medium. Osteogenic gene expression was also analyzed in $3x10^5$ cells of cell suspension (time 0) used for this experiment.

2.4 MTT assay

MTT Assay (SIGMA) is a cell viability test that takes advantage of a colorimetric indicator of growth to assess the metabolic activity of cells. The thiazolic dye MTT (3*,(4,5-dimethylthiazol-2)2,5 difeniltetrazolium bromide)* is a yellow salt, soluble, non-toxic towards the cells that, in contact with the mitochondrial dehydrogenases, is reduced to formazan salts which accumulates in the form of blue crystals. To accomplish this protocol cells were washed in PBS and 0.5 mg/ml MTT in serum free medium (1:10) was added to each well. After incubation at 37 °C for 2h and 30 min, formazan salts were solubilized with 0.1 N isopropanol/HCl.

Successively the formazan salts were quantified spectrophotometrically at 570nm and 650 nm. The values obtained were expressed as corrected optical density (Δ OD: λ570-λ650).

2.5 Scanning Electron Microscopy (SEM)

Samples were fixed in 2% glutaraldehyde in 0.1M sodium-cacodylate buffer (EMS), pH 7.2, for 1 h at 4°C and then post-fixed in 1% osmium tetroxide (EMS) for 1 h at 4°C. After dehydration in graded ethanol and critical point drying using CO_2 (Emscope CPD 750), samples were mounted on stubs and sputter coated (Sputter Coater, Polaron SC7640) with gold to achieve a 100 nm coating. Scanning electron microscopy (SEM) imaging was accomplished using a Field Emission Scanning Electron Microscope (FESEM) - Hitachi S4000.

2.5.1 X-Ray Microanalysis

Elemental analyzes were carried out using a SEM-EDX, that is a scanning electron microscope (Cambridge) equipped with an X-Ray detector (Inca X-Sight, Oxford instrument). Samples, investigated especially for their content in Calcium, were the same previously prepared and observed by SEM. All samples were analyzed on two different area of the diameter of about 470 µm and also each fields was further examined in four different points.

2.6 α5 integrin and microfilaments Immunofluorescence

For this purpose $1x10^4$ cells were seeded on extracellular matrix coatings and TCP. Samples were rinsed gently with PBS, fixed with 4% paraformaldehyde/PBS for 15 minutes and permeabilized with 0.5 % Triton X-100/PBS for 5-10 minutes. After blocking with 5% BSA in PBS (30 minutes), primary antibody polyclonal anti Integrin α5 subunit, cytoplasmatic, (1:1000) was added to each sample for 1 hour at room temperature followed by fluorescent secondary antibody Goat anti-rabbit IgG -Alexa Fluor 594 (2.5 µg/ml) for 1 hour at room temperature. Finally, samples were washed and incubated with Fitc-phalloidin (20 µl / ml

PBS) (Sigma) for 30 minutes at 37ºC. After two wash, samples were allowed to dry and mounted with mounting medium Fluoro Gel with DAPI (EMS). All antibodies were purchased from Immunological Science. The immunofluorescence was observed with a fluorescence microscope Olympus BX50 equipped with a DC500 camera (Leica).

2.7 Histochemistry

2.7.1 Alkaline Phosphatase Staining and Measurement

The analysis was carried out following the Alkaline Phosphatase Kit (Sigma-Aldrich) specifications. Sites of alkaline phosphatase activity were revealed through the formation of stable diazonium salts which appear as red granulation when observed under light microscopy.
The staining was successively quantified incubating cells with NaOH/EtOH solution (50 mM/100°), until complete dissolution of stain, then the solution was measured spectrophotometrically at a wavelength of 550 nm (Conn PM 2010).

2.7.2 Alizarin Red staining and measurement

For visualization of Calcium deposits, cells were fixed in 4% formalin in distilled H_2O for 10 minutes at 4°C, washed with cold H_2O, and stained with 2% Alizarin Red S (Sigma), pH 4.2 for 5 minutes. After extensive washes to remove the unbounded dye, cells were air-dried and evaluated by light microscopy Olympus BX50 equipped with a DC500 camera (Leica). Quantification of staining was performed incubating cells with a solution of 10% acetic acid and 20% methanol to extract the calcium-chelated Alizarin Red stain for 15 min. Samples were analyzed spectrophotometrically at 450 nm as described in Pei *et al.*, 2011.

2.8. Reverse transcriptase-polymerase chain reaction (RT-PCR)

2.8.1 RNA Isolation

Total RNA was isolated using PureLink® RNA Mini Kit (Ambion®) following manufacturer instructions. RNA concentration was assessed at 260 nm by NanoDrop 1000 Spectrophotometer. Furthermore, each sample was also read at a wavelength of 280 nm to determine the ratio 260/280 nm, a parameter evaluating the purity of isolated RNA.

2.8.2 cDNA Synthesis

The reverse transcription reaction was performed using the M-MLV Reverse Transcriptase Kit (Invitrogen). The mixture used for each sample was reported in Table I:

M-MLV reverse transcriptase buffer 5x	5 µl
Random primers 50 ng/ µl	3 µl
dNTPs mix 2,5 mM	3 µl
DTT 0,1 M	2,5 µl
M-MLV reverse transcriptase 100U/µl	1 µl
RNA 250-300 ng	5 µl
H2O-DEPC	5.5 µl

Table I - List of reagent used for cDNA Synthesis

cDNA synthesis was carried out according thermal profile reported in Table II:

denaturation	75 ° C	3 min
reverse transcription	37 ° C	60 min
enzyme inactivation	90 ° C	2 min

Table II - thermal profile used for reverse transcription step

2.8.3 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

10 µl cDNA, previously obtained were amplified in the following reaction: 5 µl Buffer 10X, 3 µl dNTPs mix (2,5 mM), 1 µl of each primer (20 µM), 0,5 µl Taq Polymerase (5 U/ µl), and ultrapure H_2O to a final volume of 50 µl. A C1000 Biorad Thermal Cycler was utilized.

The thermal profile used was described in the Table III:

Initial denaturation	94°C	3’	1cycle
Denaturation	92°C	1’	35 cycles
Annealing	65°C	1’	
Extention	72°C	1’	
Final extention	72°C	10’	1cycle

Table III - thermal profile used for RT PCR

For each target gene, primers were selected using the software "Primer" and blasted on the National Center for Biotechnology Information (NCBI) database to rule out any non-specific amplification. All the primers pairs used are shown in Table IV.

The PCR products were separated by electrophoresis on 1% agarose gel in 1x TBE buffer and a 100 bp ladder (Invitrogen) was used as marker. Bands were visualized in presence of an intercalating dye (Syber safe) by exposure to UV rays. The levels of RNA expression were evaluated by densitometry and normalized according to the housekeeping gene.

Name	nucleotide sequence	Expected product (bp)
PGK_fwd	AGGTGCTCAACAACATGG	198
PGK_rev	CCAGTCTTGGCATTCTCA	
ALP_fwd	GGACATGCAGTACGAGCTGA	562
ALP_ rev	GACGTAGTTCTGCTCGTGGA	
RUNX2_fwd	CCTCGGAGAGGTACCAGATG	526
RUNX2_rev	GGTGGTAGAGTGGATGGACG	
α5_fwd	GCTTCAACTTAGACGCGGAG	526
α5_rev	GTCTTGGTGAACTCGGCACT	

Table IV - List of synthetic oligonucleotides pairs assayed for PCR amplification.

3. RESULTS

Premise

All results presented in this PhD thesis were aimed to achieve a better understanding of the interactions between the components of the extracellular matrix produced by *in vitro* culture of human mesenchymal stromal cells derived from bone marrow and mineralization process which characterizes the late stage of osteogenic differentiation.

Since a broad literature on the ability of cell free extracellular matrix to influence cell biology still lack, I have firstly investigated the role of the ECM in the adhesion and proliferation of cell expanded in normal culture conditions and mechanisms governing hBMSCs behavior when compared to a uncoated substrate.

After examining the mesenchymal stem cells response on cell free ECM, their morphology, microfilaments and the main adhesion molecules to the extracellular matrix, I began to study the ability of ECM coatings to direct cell fate underlying in particular modifications which occur when these coated surfaces were used in combination with the osteogenic medium.

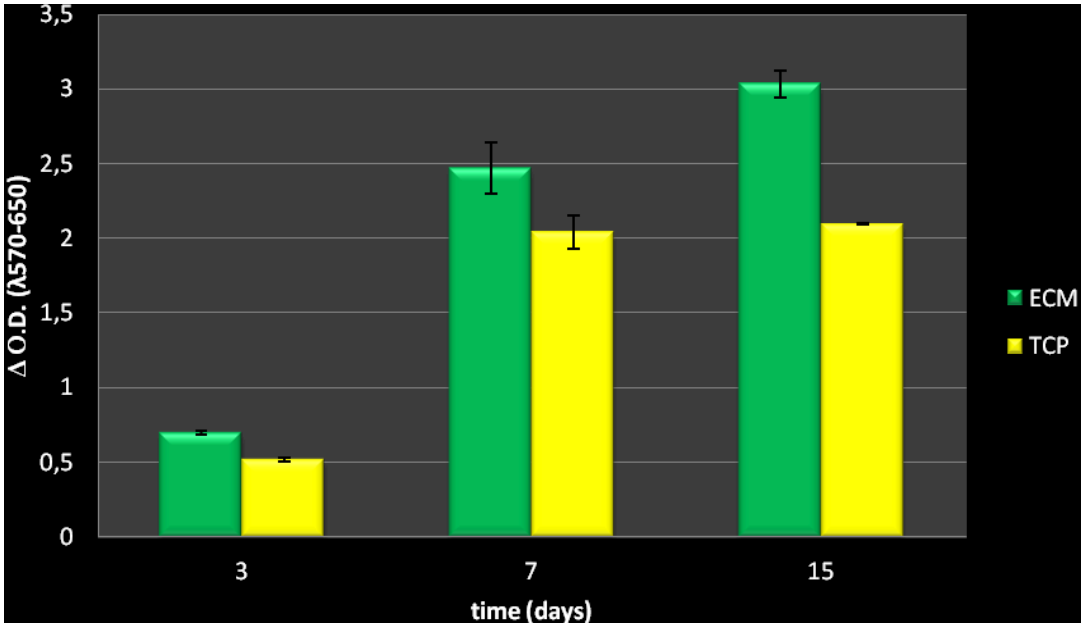
3.1 Analysis of hBMSCs on ECM coatings and TCP in growth medium

3.1.1 Cell Survival and Proliferation

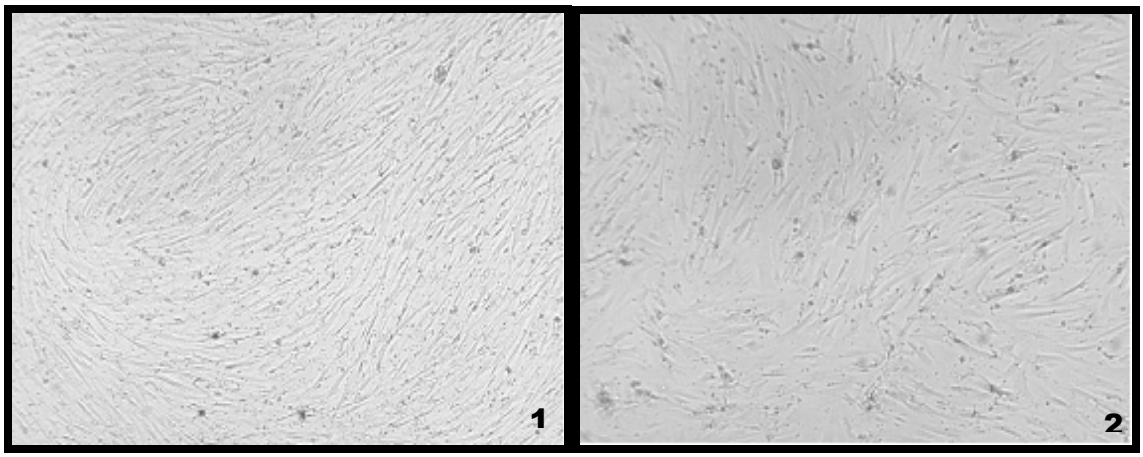
The viability of human bone marrow derived mesenchymal stromal cells grown on extracellular matrix coating surfaces or on uncoated tissue culture plates was evaluated via MTT assay at three different times in growth medium and results obtained were showed in Graphic I.

ECM coated substrate enhanced the proliferation of MSCs above all between day 3 and day 7 and a further increase was visible at the end of the experimental time. Whereas cell proliferation on TCP underwent an increase between day 3 and day 7 (Δ D.O. 0.52 to 2.04 respectively) and maintained the same value of correct optical density (Δ D.O. 2.09) until day 15. These values were in any case lesser than that observed for the ECM.

These results were also confirmed by optical microscopy (Figure 1 and Figure 2) where hBMSCs appeared forming a confluent oriented monolayer on ECM coatings and a semi confluent layer on tissue culture plate with cells arranged in a disorderly manner after 15 days of culture.



Graphic I – hBMSCs proliferation on ECM coating and tissue culture plates in growth medium at different time points. MTT assay.



Figures 1 and 2 – Appearance of cells cultured on ECM coatings and on TCP observed by light microscope after 15 days of culture. **1.** hBMSCs on ECM were numerous and oriented (8 x). **2.** Cells on TCP appeared in lower number and disorderly arranged (8 x).

3.1.2 Morphological Investigations

As revealed by scanning electron microscopy, extracellular matrix elaborated by human bone marrow mesenchymal cells and decellularized with previously described procedures, appeared composed of a fibrillar stroma where thick bundles followed the orientation of cellular bodies and also were irregularly intersected by thin filaments (Figure 3). Consequently, the network resulted constituted by fibers and filaments of various sizes, among which collagen fibers were particularly identifiable due to their characteristic banded structure and their size (arrows in figure). Permeabilization and DNase method used for the free cell ECM preparation did not allow a total removal of cellular material; indeed body cellular residues appeared interspersed in the stroma. Very thin slightly banded filaments were present inside these cytoplasmatic residues.

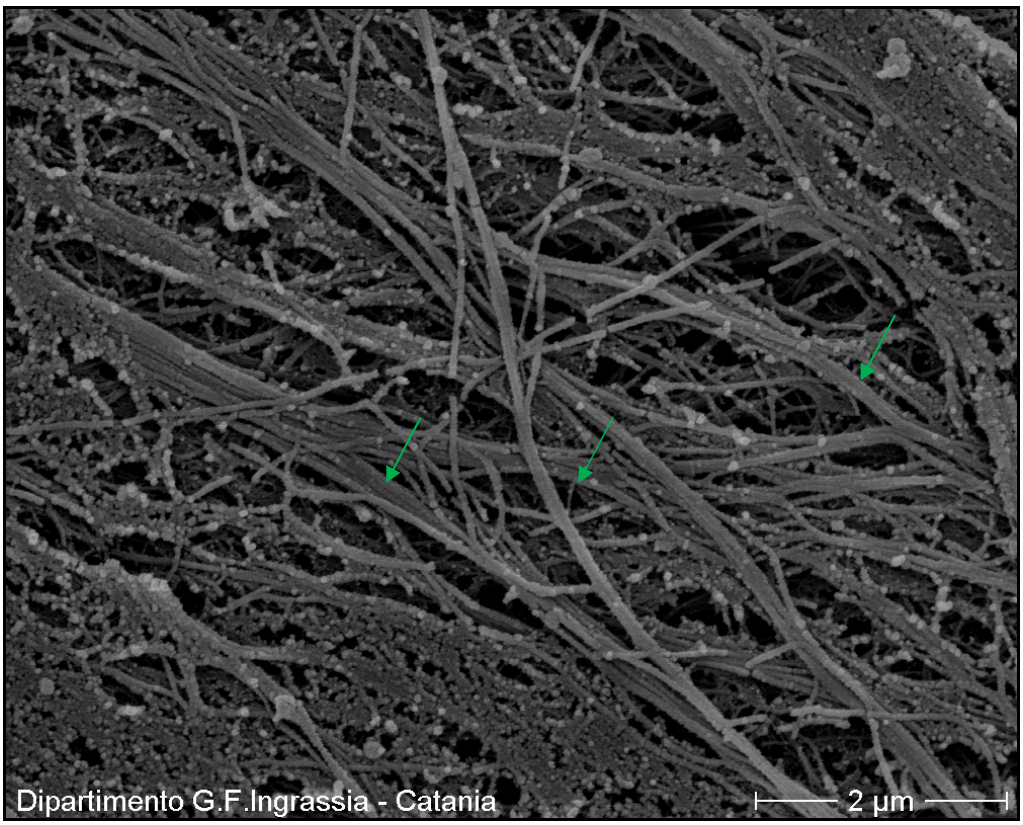


Figure 3– Cell free extracellular matrix coating obtained after cell layer decellularization (20000 x).

hBMSCs behavior during their adhesion and proliferation on extracellular matrix coatings was examined by electron microscopy until day 15 of culture.

In the early hours following the seed, cells on ECM coating represented the various degrees of cell adhesion to the substrate and although some of them appeared more attached to the matrix, all had a very evident cytoplasmic body (Figure 4). hBMSCs showed a wide variety of shapes and sizes;

some were polygonal and strictly spread on the extracellular matrix presenting cytoplasmic prolongations that connect cells together, others retained rounded shapes with irregularly ragged cytoplasmic borders.

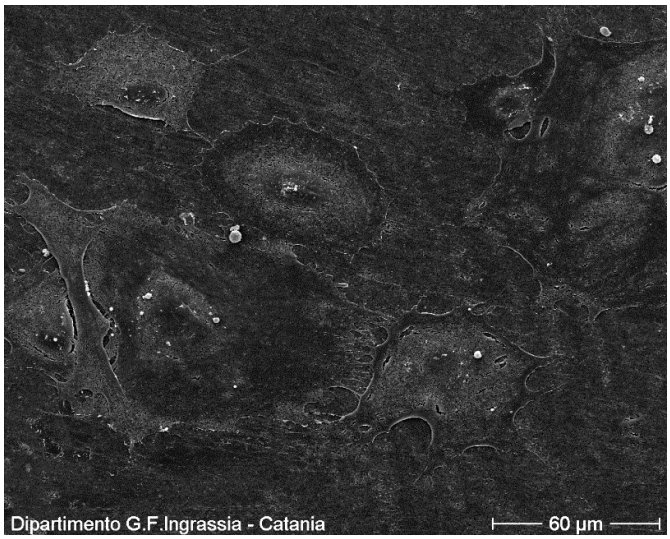


Figure 4. Cells after 3 hours showed different shapes and sizes (500x).

With the experimental time progression, mesenchymal stromal cells showed a proliferative activity with consequently changes in their morphology; indeed they assumed a more elongated shape and exhibited cytoplasmic bodies stretched in many directions; each cell, through these prolongations, was strictly in contact with the underlying extracellular matrix and with the neighboring cells (Figure 5).

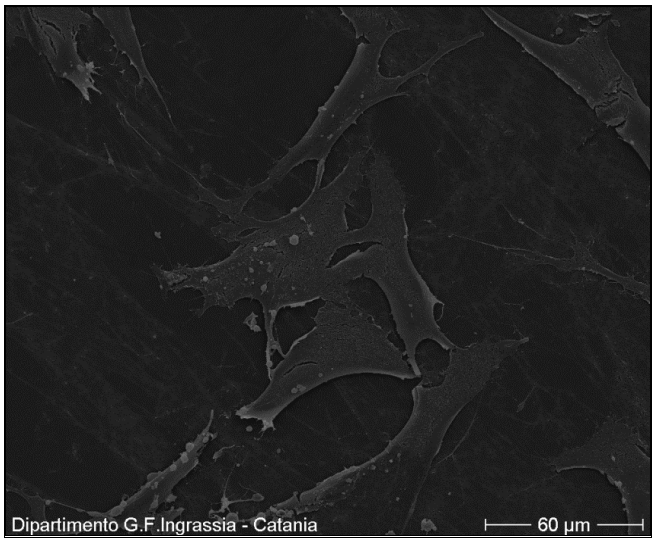
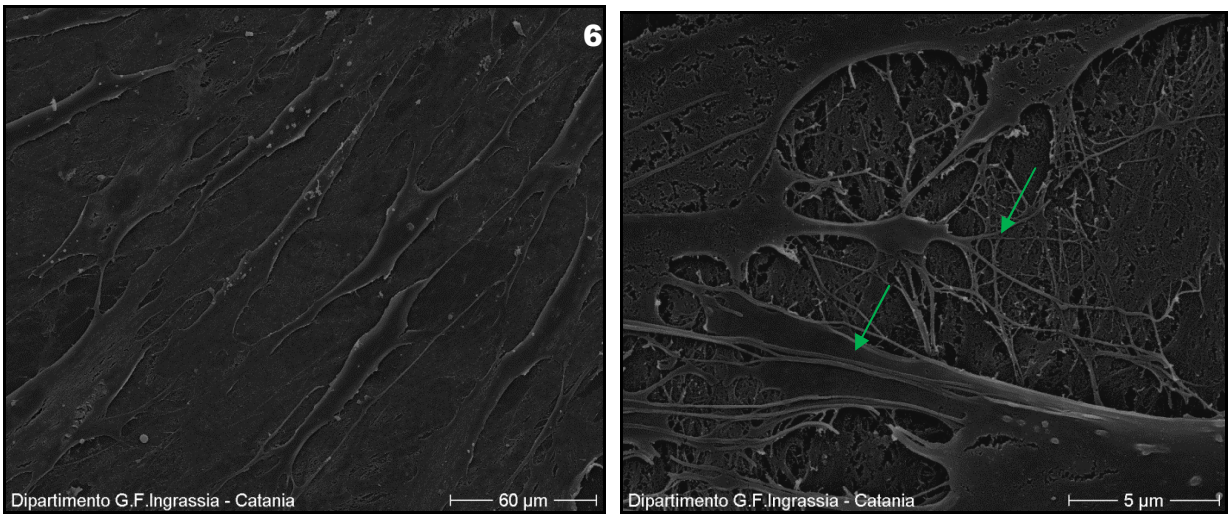


Figure 5. Cell spreading at day 6 (500 x).

hBMSCs, at the end of experiment, covered the ECM surface with a nearly continuous cellular monolayer constituted by very spread elements and above this layer other cells were visible. These

cells appeared widely elongated and, although some had a more stretched body on the extracellular matrix coating, most of them were characterized by a fibroblastic-like shape. Particularly, hBMSCs were arranged in an ordered orientation following that of decellularized ECM fibrous components (Fig 6).

Interestingly, an extracellular matrix of new production was detected (Fig 7); it was constituted by numerous fibers which branched off from the periphery of the cells, among which collagen fibers were particularly evident, connected to each other and also with the preexisting ECM components.



Figures 6-7 – hBMSCs cultured on extracellular matrix coatings after 15 days of culture. **6.** Cells were arranged in an ordered orientation following that of decellularized ECM components (500 x). **7.** Collagen fibers (green arrows) which branch off from the periphery of the cells (6000 x).

The overall behavior of mesenchymal stromal cells maintained on tissue culture plates revealed that their morphology was clearly distinguishable from that of cells on ECM coatings; indeed cells since the early hours and for all the experimental time appeared extensively flat without any membrane specialization. Human BMSCs showed a proliferative activity during culture progression and were connected each other through cytoplasmic prolongations (Figure 8), they did not present distinguishable cellular boundaries and there was no great evidence of fibers originating from the cell edges.

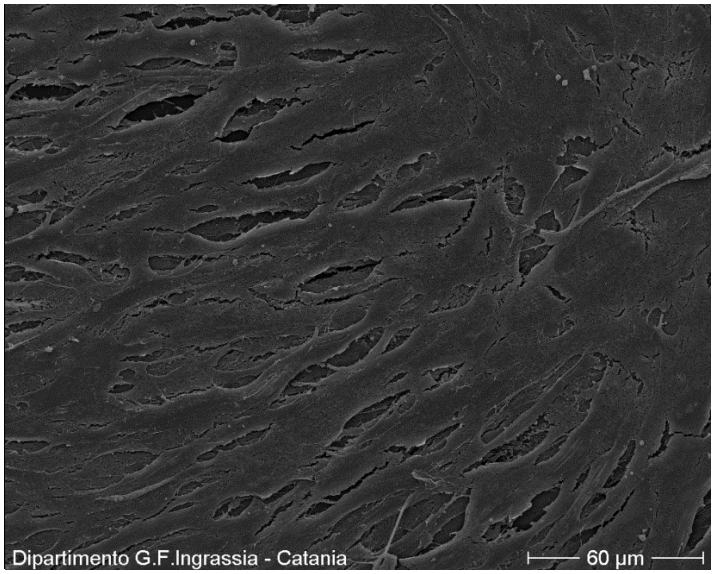


Figure 8 – hBMSCs cultured on tissue culture plates at day 15 appeared extensively flat (500x).

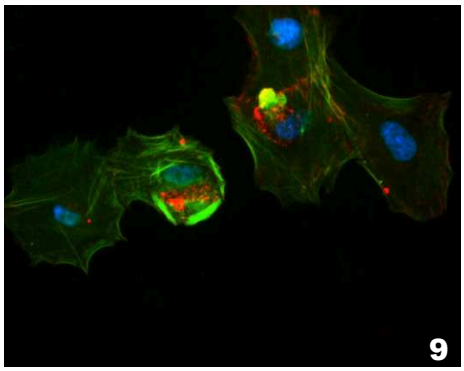
3.1.3 Integrin α5 and Microfilaments: cell adhesion to substrate

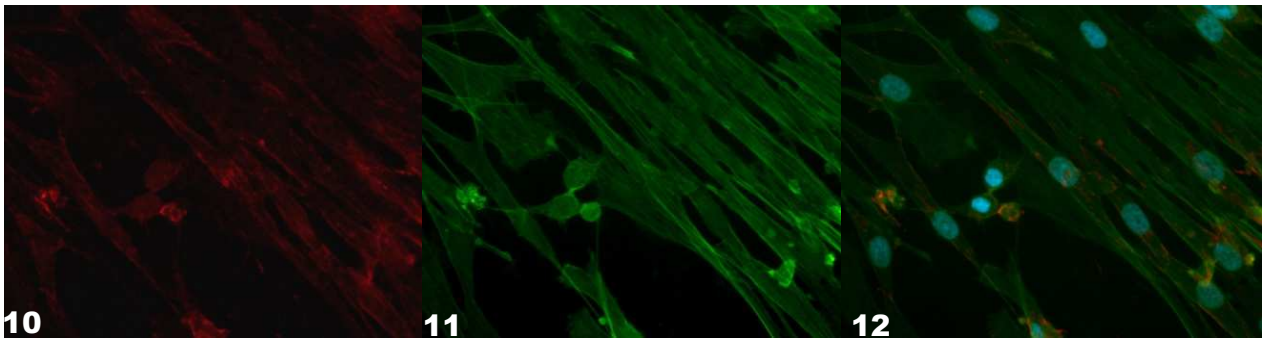
Integrin α5 has the binding sites on fibronectin molecules and is closely correlated with the microfilaments in correspondence of focal contacts.

During their adhesion to extracellular matrix coatings, bone marrow stem cells showed a different distribution of Integrin α5 linked to the time of seeding and consequently to the anchoring process.

Initially, Integrin molecules corresponded to intense fluorescent spots located preferentially in the central area of cells and, in those more spread, they were localized in submembranous termini of the actin filaments, which are attached to the focal cell substrate contacts. Cytoskeleton actin components was differently organized consequently to the cellular adhesion degree; some cells had particularly fluorescent spots corresponding to the contact area between the cell plasma membrane and the extracellular matrix, others although presenting some distension on the substrate, showed an initial organization of microfilaments in stress fibers, which appeared slightly fluorescent (Figure 9).

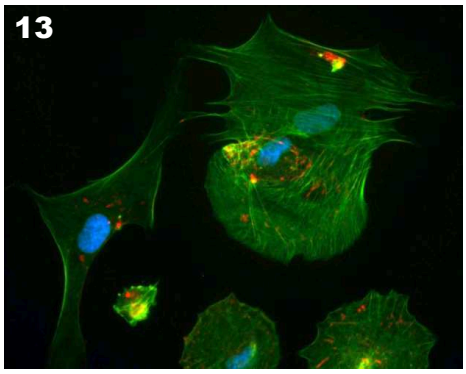
After 6 days, Integrin α5 formed fluorescent rod-like clusters all oriented in the same direction corresponding to the microfilament s arrangement now observable as evident stress fibers. Also α5 receptor was localized on the whole plasma membrane that adhere on the extracellular matrix coatings and also its fluorescence was particularly present on the filipodia, namely the long bold processes, observable in electron micrographies, which contain a fluorescent actin core (Figg. 10-12).

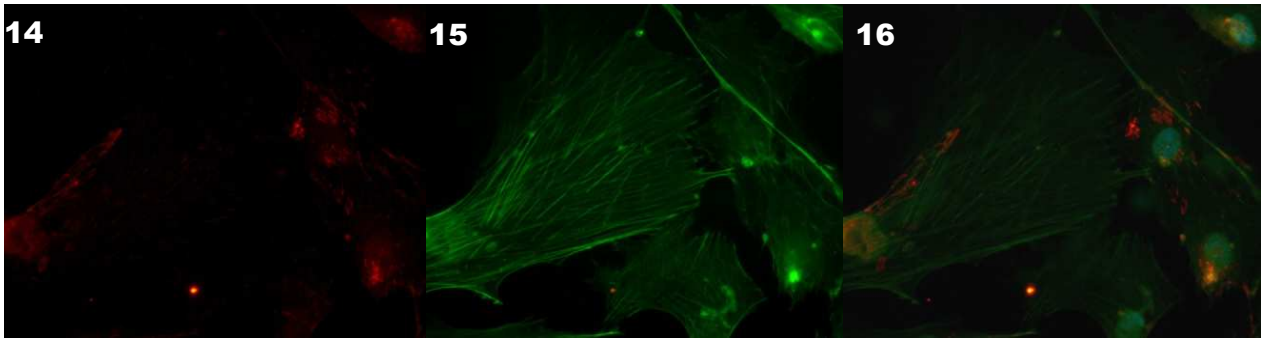




Figures 9-12– Integrin α5 and Microfilaments analysis in hBMSCs on ECM coatings, normal medium. Double immunofluorescence technique (40x). **9.** Cells after 3 hours, Integrin α5 (red) central location (merge). **10 -** Cells after 6 day. Integrin α5 formed rod-like clusters in the whole plasma membrane. **11.** Microfilaments (green) observable as evident stress fibers and oriented following the ECM compounds. **12.** Merge.

Mesenchymal stromal cells, cultured directly on glass coverslips and analyzed by immunofluorescence, presented Integrin α5 and microfilaments organization different from those of cells on ECM. After 3 hours, Integrin α5 was only poorly detectable like fluorescent spots nearly the nuclear area and microfilaments were often organized in stress fibers or bundles (Figure 13). With the progression of experimental time, Integrin α5 localization pattern was similar to those observed on ECM coatings, even if it was less homogenously diffused (Figures 14-16) .



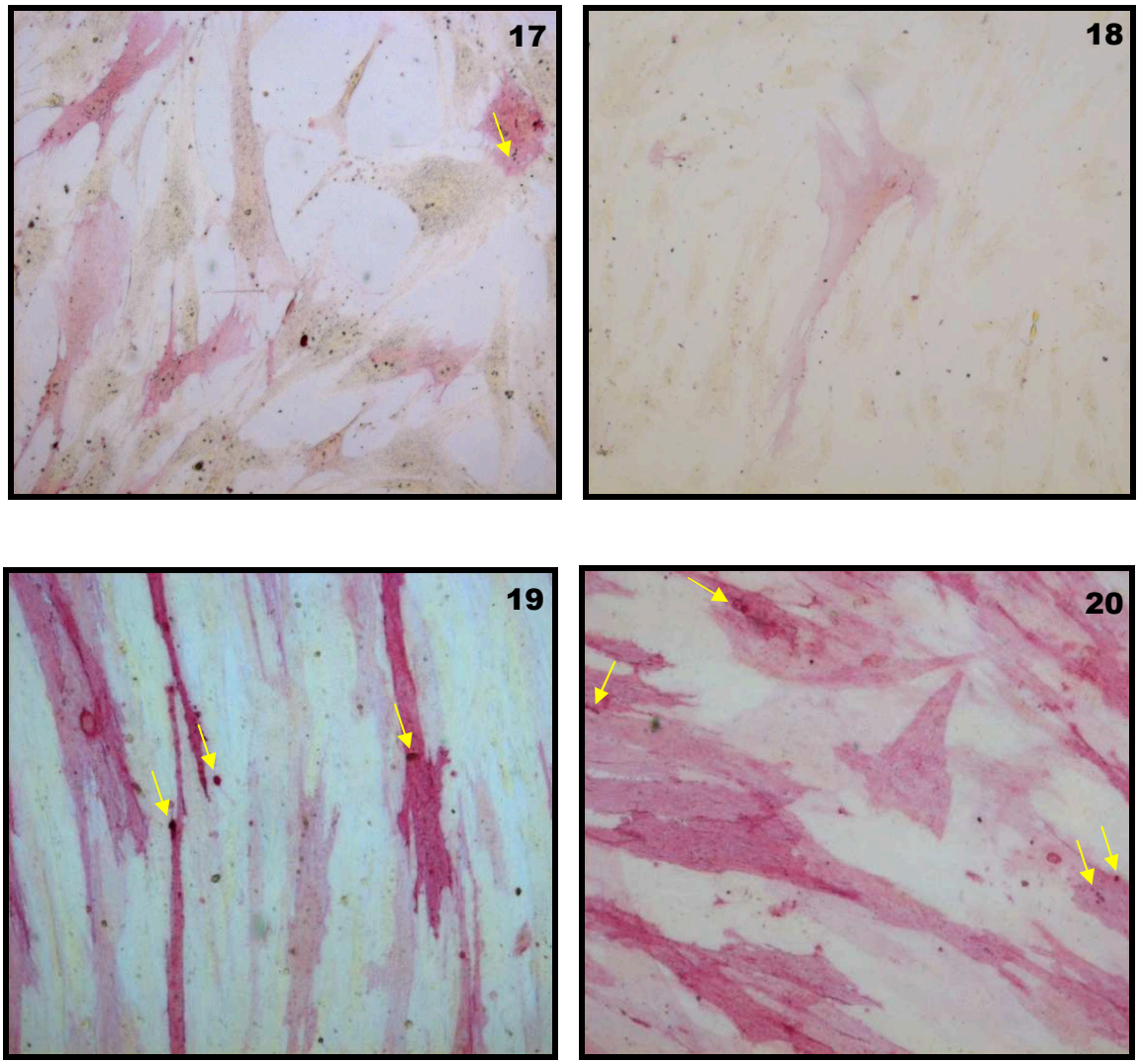


Figures 13-16– Integrin α5 and Microfilaments analysis in hBMSCs on TCP, normal medium. Double immunofluorescence technique (40x). **13.** hBMSCs after 3 hours, Integrin α5 (red) poorly detectable (merge). 1**4.** hBMSCs at day 6. Integrin α5 was not homogenously diffused. **15 -** Microfilaments (green) organized in stress fibers or thick bundles. **16.** Merge.

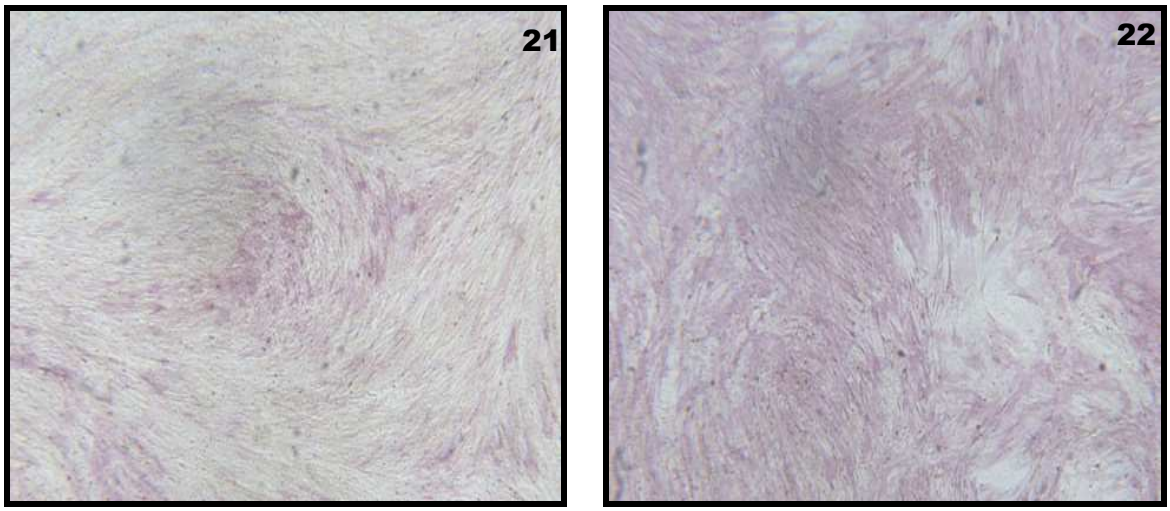
3.1.4 Alkaline Phosphatase Activity and Staining Measurement

ALP is an early marker of osteogenic differentiation and sites of phosphatase activity are detectable as pink-red vesicles on a pale pink cytoplasm both on the cell body and on cell prolongations.

Bone marrow mesenchymal cells grown on ECM coating at day 6 (Fig 17) appeared slightly more stained than those on tissue culture plate (Fig 18), but the situation inverted at day 15 where, compared to ECM samples (Fig 19); hBMSCs on TCP revealed a greater number of positive elements which were characterized by a more strong pink cytoplasm (Fig 20). Additional analysis accomplished at day 21 continued to display a similar trend with less intense ALP staining on ECM coatings (Fig 21) and greater positivity on TCP (Fig 22).

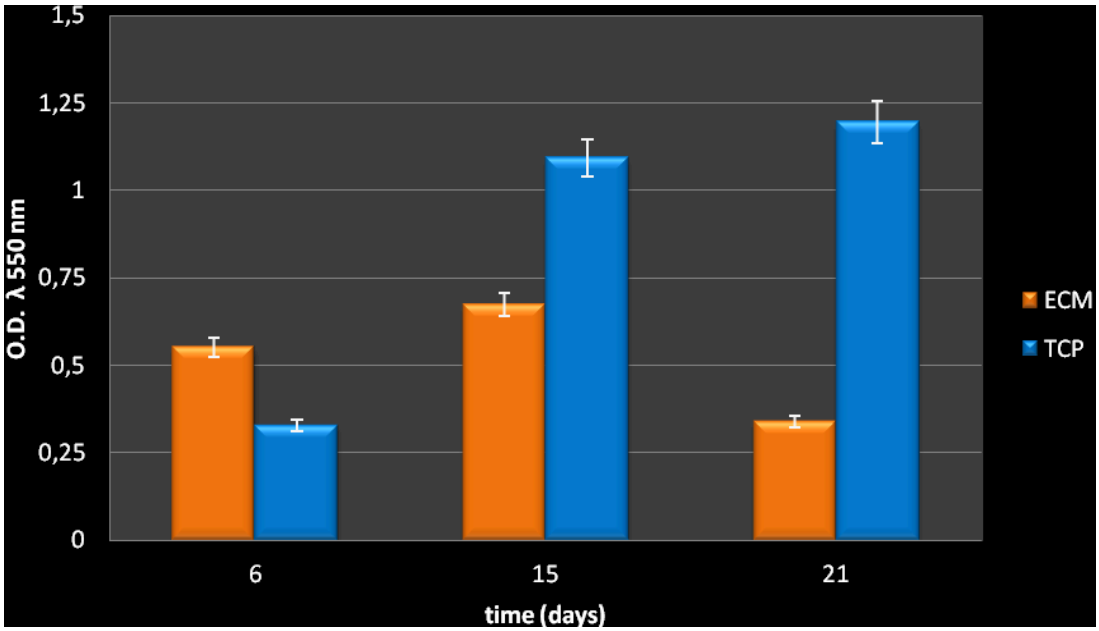


Figg. 17-20 – ALP staining of hBMSCs cultured on ECM coatings and TCP in growth medium. ALP granulations (yellow arrows). Light microscopy (20 x). **17.** Cells on ECM substrates after 6 day of expansion, few ALP red granulation. **18.** hBMSCs cultured on TCP at day 6. **19.** Cells on ECM coatings at day 15 showed a typical oriented disposition **20.** hBMSCs stained on TCP after 15 days displayed a greater ALP positivity as revealed by strong pink colored cytoplasm.



Figg. 21-22 –ALP staining of hBMSCs on ECM coatings and TCP at day 21. Light microscopy images (8 x) **21.** Cells confluent layer on ECM of culture. **22.** hBMSCs on TCP showed intense staining.

The spectrophotometric quantification of the staining, which aimed to further clarify the previous results obtained by light microscopy, supported the observation that alkaline phosphatase activity followed different trends in the two substrates (Graphic II). Optical density measured in cells on ECM coatings showed an increase at day 15 and a decrease at day 21, whereas O.D. related to cells on TCP underwent a continuous increase.



Graphic II – Alkaline phosphatase staining measurement in hBMSCs on extracellular matrix coating and plastic culture plates in growth medium at different time points. Spectrophotometric analysis.

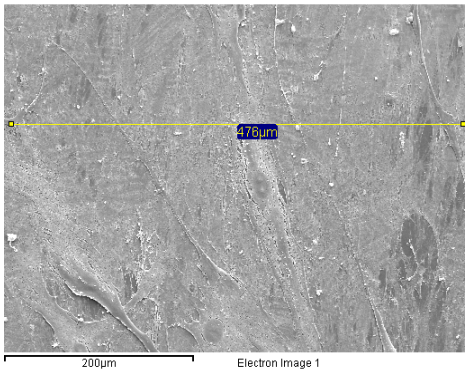
3.1.5 Calcium content revealed by Alizarin Red Staining and Measurement and X-Ray Microanalysis

Alizarin red staining was performed on the two substrates without osteogenic induction. The results obtained both by staining and following spectrophotometric analysis showed that, even though bone marrow mesenchymal stem cells have a slight osteoblastic commitment as revealed by ALP results both on ECM and TCP, no relevant Calcium deposits were detected by the Alizarin Red after 6, 15 and 21 days of culture in non osteogenic medium (data not shown).

X-Ray analysis was carried out on cells growing on ECM coating after 3 hours, 6 and 15 days in growth medium. The analysis were performed taking into consideration cell surfaces, the granules above cellular membranes and those present on the extracellular matrix coatings. All the samples examined did not showed Calcium at detectable levels. The spectrum of an examined area referring to the longest experimental time and the relative revealed elements (weight%) were reported as demonstration (Table I).

Spectrum	C	Al	Tc	Hf	Hg	Pb	O	Total
Sum Spectrum	27.01	0.04	0.13	0.00	0.42	0.27	72.12	100.00
Mean	27.01	0.04	0.13	0.00	0.42	0.27	72.12	100.00
Std. deviation	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
Max.	27.01	0.04	0.13	0.00	0.42	0.27	72.12	
Min.	27.01	0.04	0.13	0.00	0.42	0.27	72.12	

All results in weight%



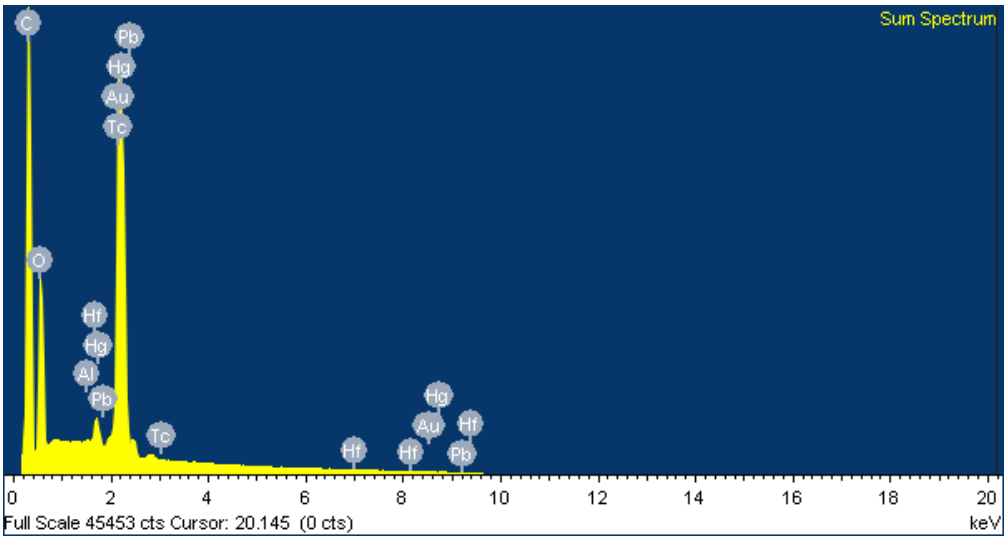


Table I– X-ray microanalysis. hBMSC on ECM after 15 days in growth medium. Map, table of the elements and spectrum corresponding to area 1 (476 µm diameter).

3.2 Analysis of hBMSCs on ECM coatings and TCP in osteogenic medium

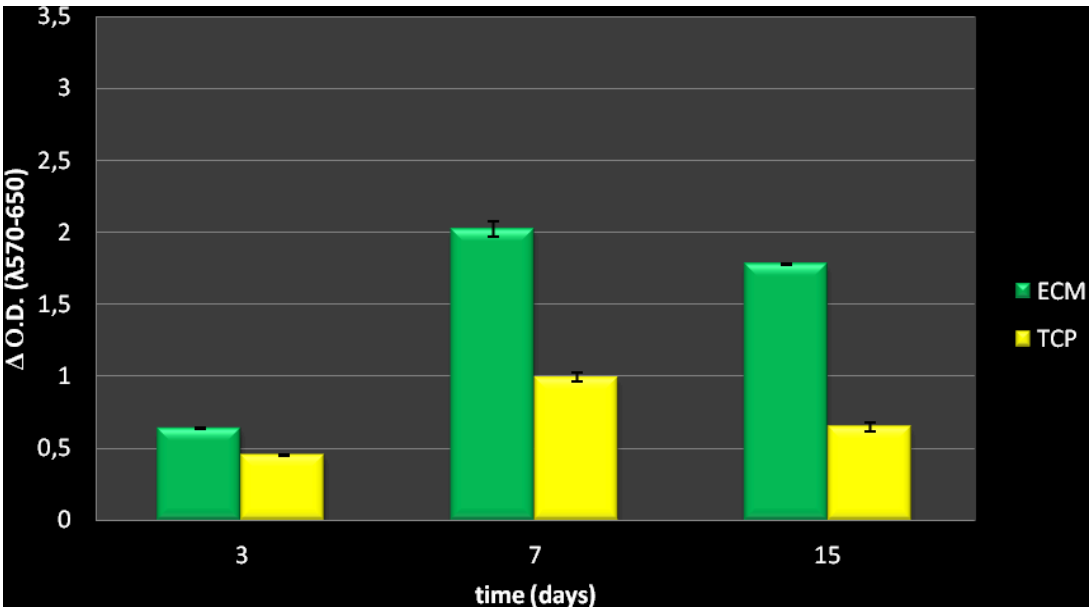
3.2.1 Cell Survival and Proliferation

Mesenchymal stem cells viability on both ECM coatings and TCP was evaluated after exposure to discontinuous treatment with dexamethasone by MTT assay (Graphic III) at day 3, 7 and 15 of culture.

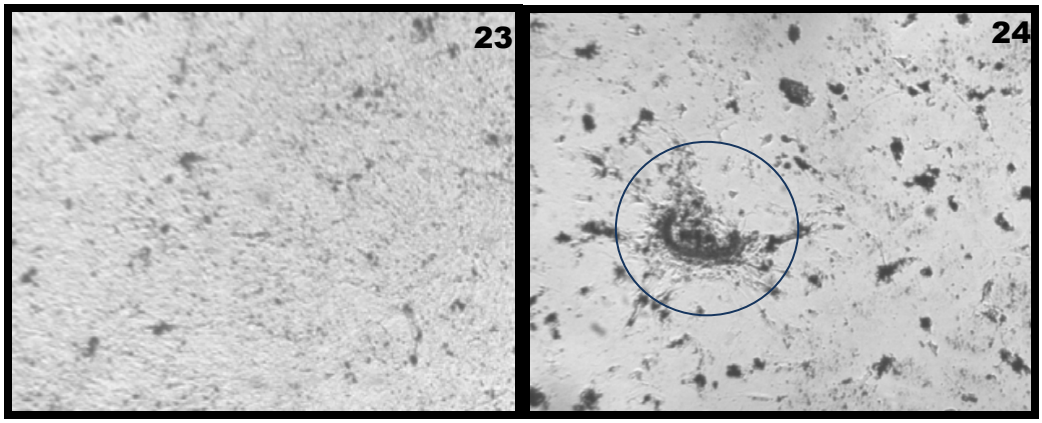
Results revealed that the survival of hBMSC cultured on extracellular matrix coatings was slightly reduced in the third (about 8 %) and seventh days (about 18%) compared to values obtained when cells were maintained in growth medium (Graphic I), whereas a more pronounced viability decrement of about 42% was observed in the fifteenth day of treatment.

Instead, cell survival on tissue culture plates showed grave variations compared to values obtained for hBMSCs maintained in growth medium. Indeed viability, with the exception of day 3 (about 12%) in which was only lightly reduced, underwent a marked decrease of about 52% already at day 7 that became more pronounced at the end of the experimental time with a difference of about 69 % compared to values in normal medium at the same time point (Graphic I).

The proliferative inhibition effect of osteogenic treatment, probably do to the used concentration of Dexamethasone, on bone marrow mesenchymal cells seeded on tissue culture plates was also visible at light microscopy at the end of the experimental time. Cells grew on ECM coatings formed a confluent monolayer entirely covered by numerous dark granulations (Fig 23); whereas hBMSCs on TCP constituted small cellular islets whose surfaces were also covered by black granular material (Fig 24).



Graphic III – hBMSCs proliferation on ECM coating and plastic culture plates under osteogenic treatment at different time points. MTT assay.



Figg. 23-24 – Cells cultured on ECM coatings and plastic observed by light microscope after 15 days of culture under osteogenic treatment (8x). **23.** hBMSCs on ECM were numerous and covered by granulations . **24.** Cells on TCP appeared lower in number and arranged to form small islets (circle).

3.2.2 Morphological Investigations

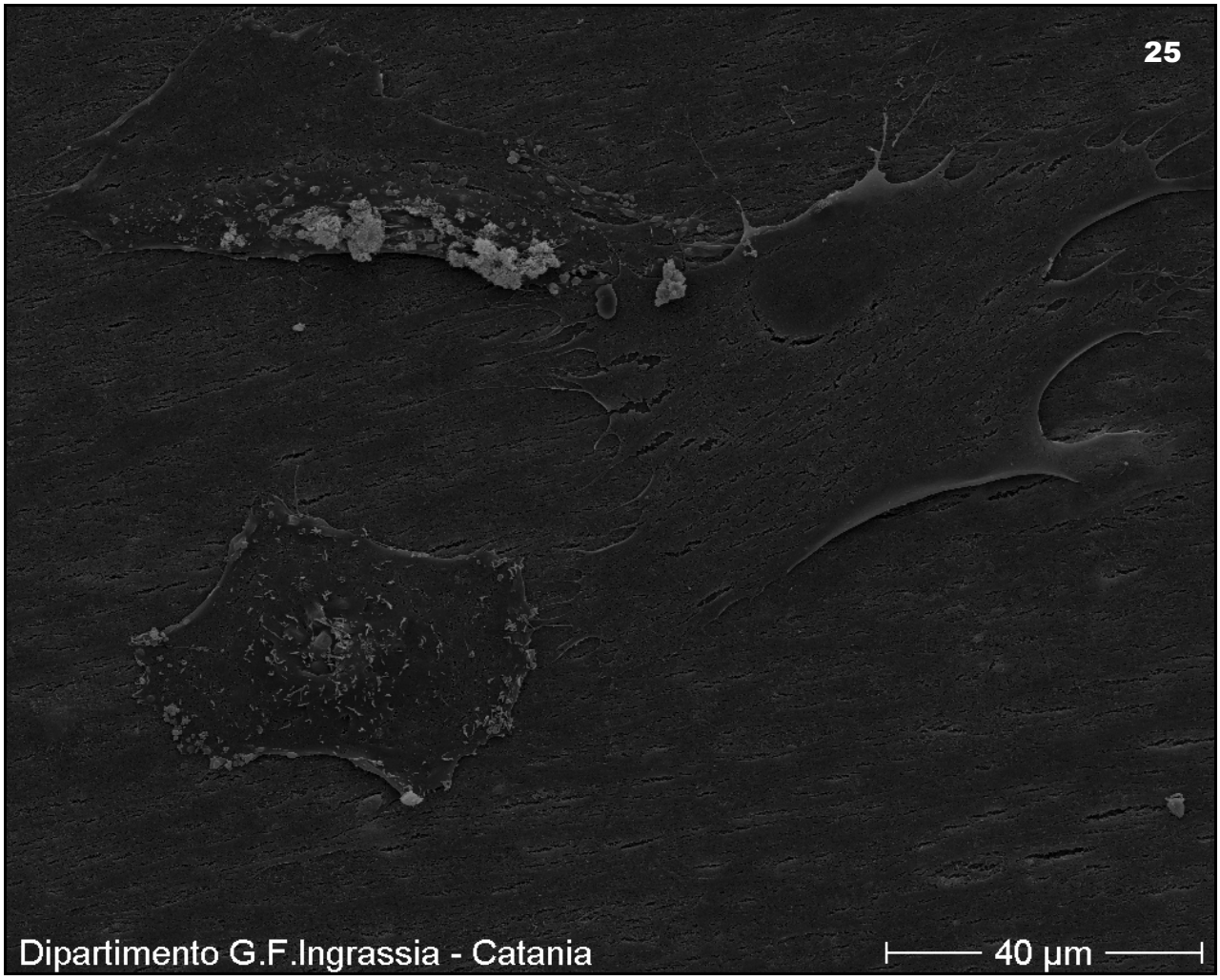
The morphology of hBMSCs under osteogenic treatment was examined by Scanning Electron Microscopy analysis at 3 hours, 6 and 15 days.

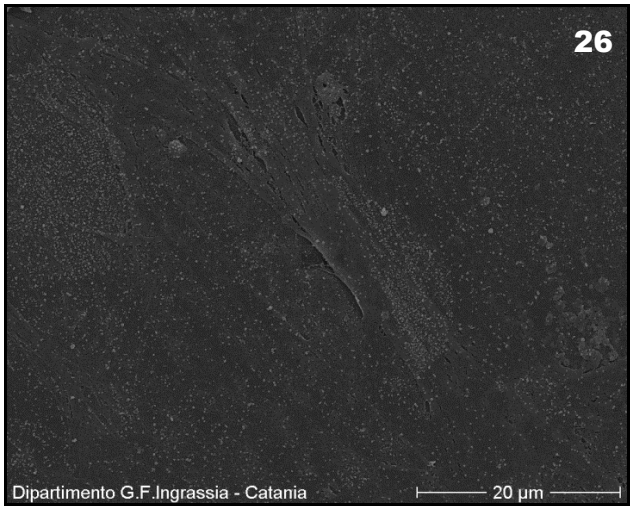
Cells maintained in osteogenic medium for 3 hours (Fig. 25) showed some similarity with those used as reference, however, during the treatment, hBMSCs seemed to be larger in size and also displayed various shapes: some were polygonal, others roundish and flat, well adherent to the ECM. Furthermore, cell plasma membrane had short and thick prolongations in the peripheral areas, and unlike the sample maintained in normal medium, numerous vesicles were variously dispersed on the cell surface.

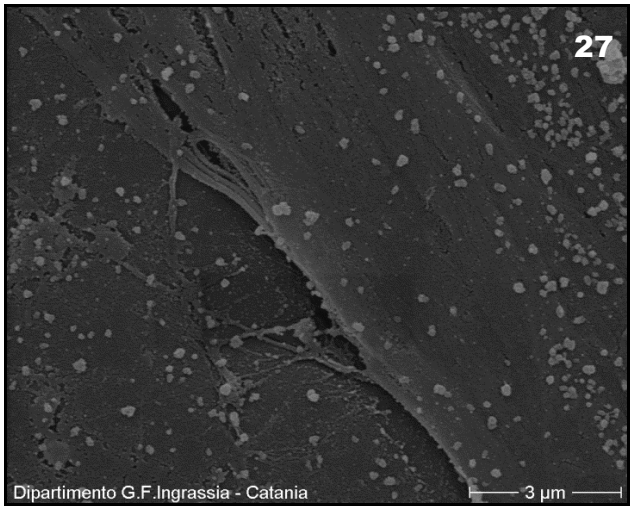
At day 6 the ECM coated surface appeared covered by cells whose plasma membrane presented very numerous small granulations that were also distributed on the underlying matrix coatings (Fig 26) and, at a more careful observation, they seemed to be in continuity with the ECM coating components (Fig 27).

With the progress of experimental time some effects of the osteogenic medium, as differentiating agent, become more pronounced. Indeed, Bone Marrow Mesenchymal Cells covered the entire ECM surface maintaining their individuality and their oriented arrangement (Fig 28), they lost the fibroblastic like conformation and appeared polygonal in shape and had a very spread cytoplasmic body which was apparently in close continuity with that of the other cells, sometimes making difficult to distinguish the cellular boundaries (Fig 29). Moreover, hBMSCs plasma membrane showed numerous prolongations and, especially, compared to the previously samples, the secretory granules on cell surfaces underwent an increase (Fig 30). The surrounding extracellular matrix appeared uniformly covered by amorphous material which seems to have been poured by cell vesicles (Fig 31).

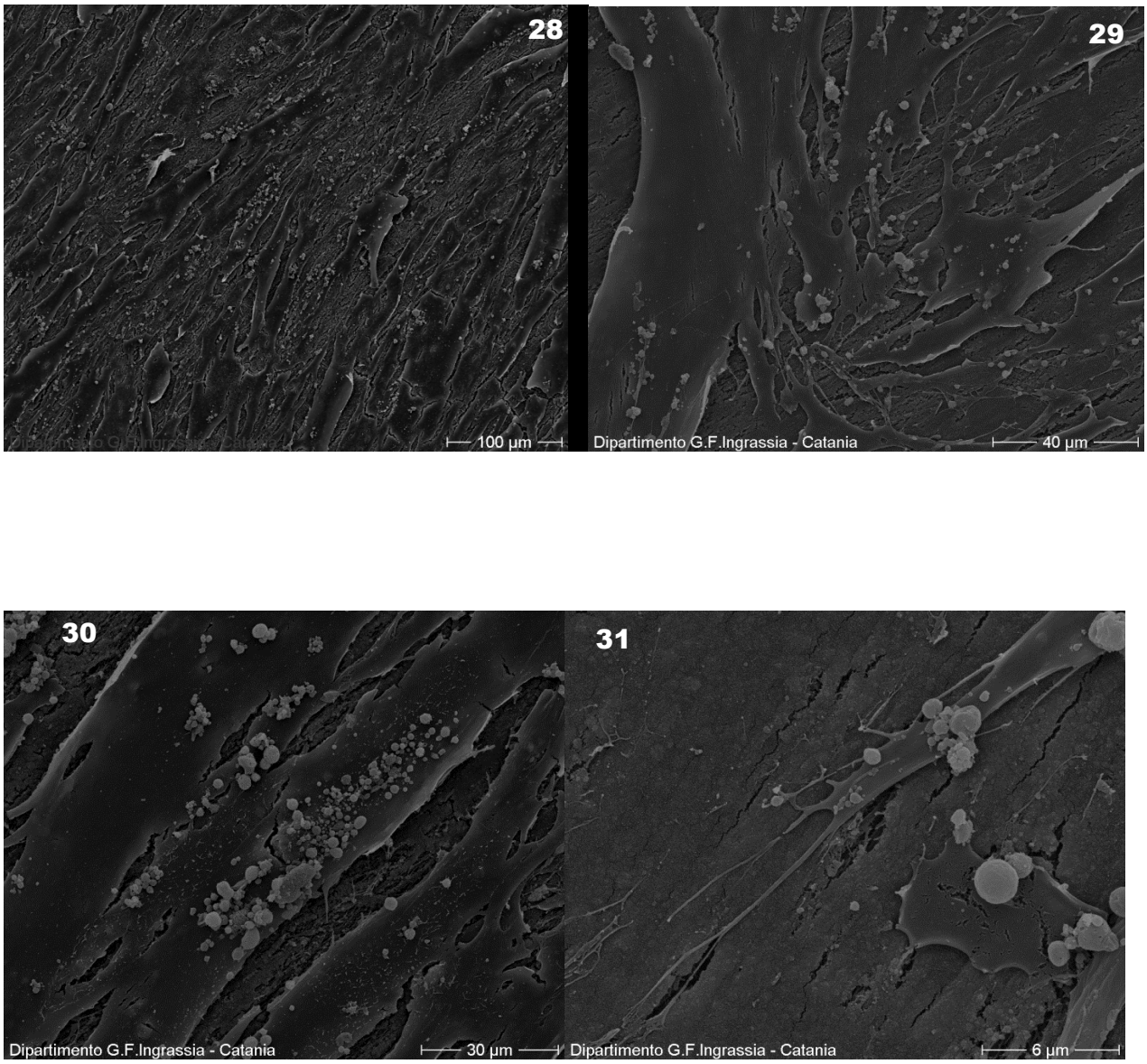
Micrographies, obtained at the end of experimental time, confirmed images acquired by light microscopy; indeed hBMSCs, when maintained on plastic, were grouped into small cellular islets, with cells covered by small granulation or secretory granules. They also appeared reduced in number with respect to all the samples examined (micrographies not shown).







Figg. 25-27 –hBMSCs cultured on extracellular matrix coatings under osteogenic treatment. **25.** Cells after 3 hours showed greater sizes and numerous protrusions (green circles) variously dispersed on their surface (800x). **26.** ECM coatings surface at day 6 covered by small granulations (2000 x). **27.** High magnification revealed hBMSCs embedded under ECM coatings composition, day 6 (10000 x).



Figg. 28-31 – SEM images of hBMSCs cultured on extracellular matrix coatings under osteogenic treatment at day 15. **28.** Polygonal cells in a disorderly arrangement (250 x). **29.** Cell bodies disposed in close continuity (800 x). **30.** hBMSCs surfaces covered by exocytose vesicles (1000 x). **31.** The ECM surrounding cells appeared uniformly covered by small granules (5000 x).

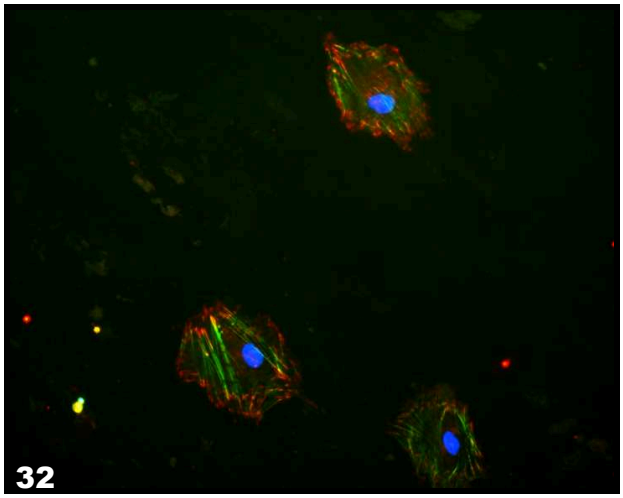
3.2.3 Integrin α5and Microfilaments: cell adhesion to substrate

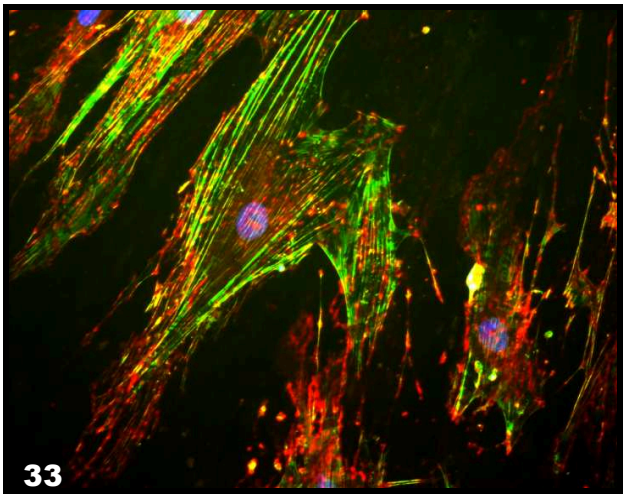
The Integrin α5 in Bone Marrow Mesenchymal cells, analyzed after 3 hours and 6 days under discontinuous treatment with osteogenic medium, underwent an increase both in ECM coated and uncoated substrates with respect to samples in normal medium.

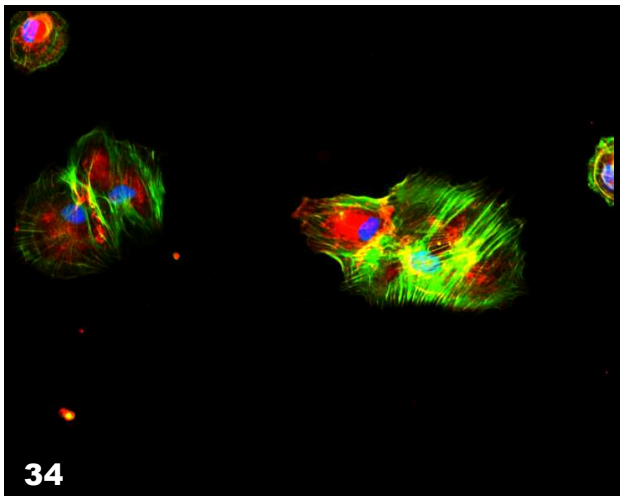
During the early hours of cell adhesion on extracellular matrix coatings, Integrin α5 displayed a more peripheral location with a dot-like organization and microfilaments began to be organized in bundles whose terminus end corresponded with Integrin α5 position (Fig 32).

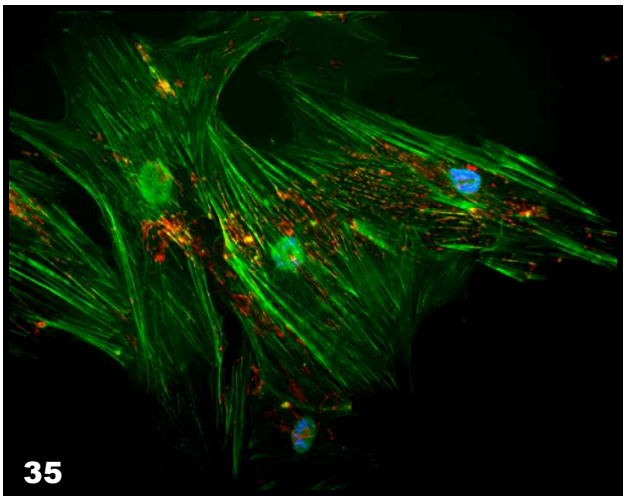
After 6 day, α5 molecules were located with evident rod-like clusters on the whole cell adhesion surface, particularly evident in the peripheral cellular area. Furthermore, microfilaments were well organized in thick bundles through the whole cytoplasmatic body with a directionality reflecting that of extracellular matrix components (Fig 33). During osteogenic treatment, there was a clear correlation between cell shape and the cytoskeleton organization, underlined by size differences between differentiated cells and those in growth medium.

The Integrin α5 in MSCs grown on uncoated surfaces exhibited after 3 hours of treatment a dot-like organization and it was particularly concentrated in the central area of the cells with microfilaments arranged in very thick and short bundles (Fig 34). Cells, in the course of experimental time, underwent a increase in distension as demonstrated by extended actin bundles. Integrin α5, although the cellular size change, remained located in the perinuclear region in a dot like conformation; then holding the position it had yet during early hours of seeding (Fig 35).









Figg. 32-35 – Integrin α5 and Microfilaments analysis in hBMSCs on ECM coatings and TCP under treatment with osteogenic medium. Double immunofluorescence technique (20x). **32.** Cells on ECM coatings after 3 hours, Integrin α5 peripheral location and dot-like organization. **33.** Cells on ECM coatings after 6 day, Integrin α5 formed evident rod-like clusters in the whole cell surface. Microfilaments displayed thick bundles oriented following the ECM compounds visible in the background **34.** hBMSCs on TCP after 3 hours, Integrin α5 central localization. **35.** hBMSCs on TCP at day 6, Integrin α5 retained a central location and a dot-like organization.

3.2.4 Histochemistry

a) *Alkaline Phosphatase Activity and Staining Measurement*

The histochemical analysis of ALP enzymatic activity visibly changed during discontinuous treatment with osteogenic medium.

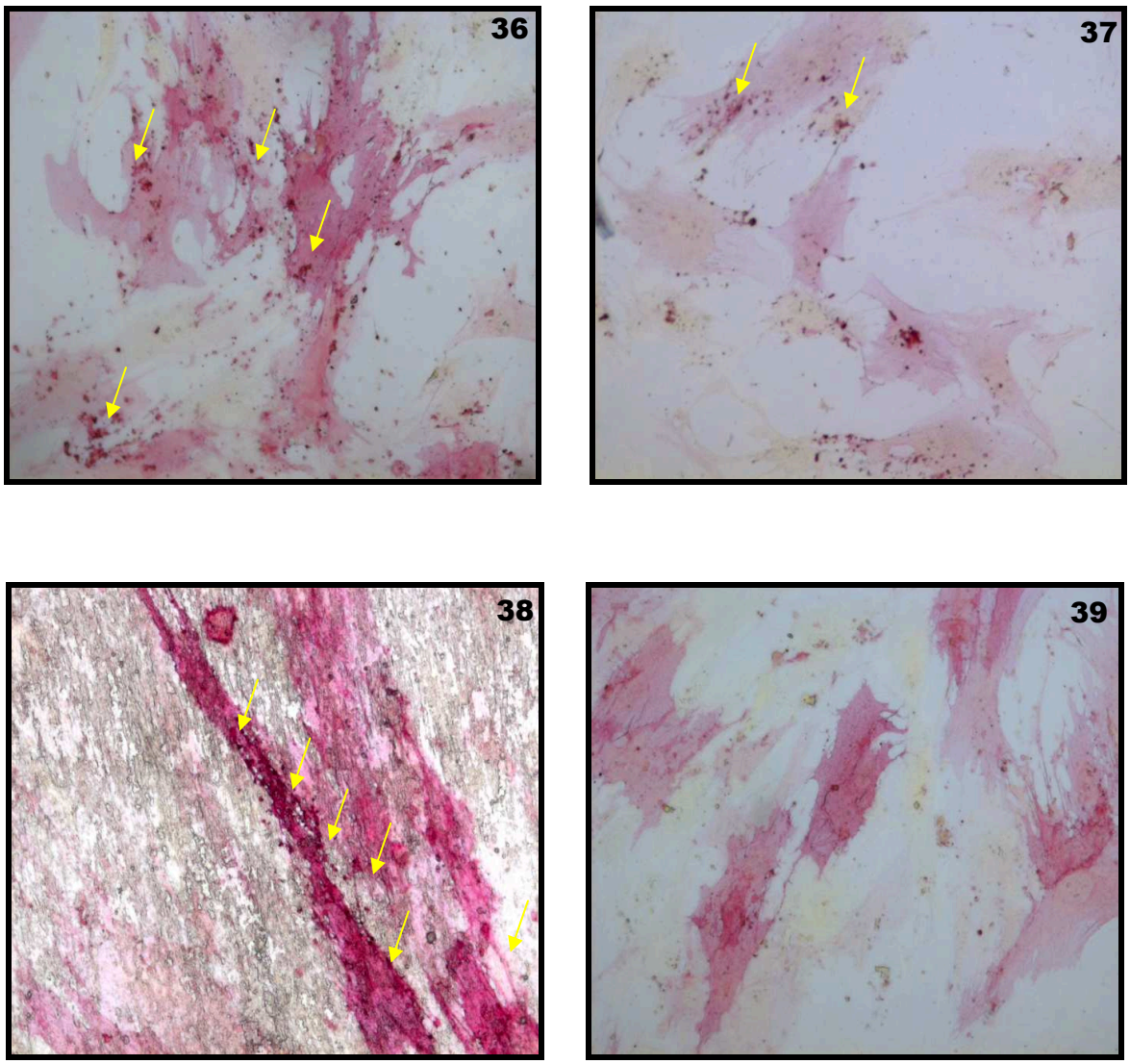
The ALP positivity in hBMSCs grown on ECM coatings and exposed to dexamethasone was markedly detected after already 6 days (Fig. 36) with respect to reference samples (Fig. 17); indeed cell cytoplasm appeared very strong pink colored and there were a lot of red granulations visible both on the cell body and on cell prolongations. Although cells maintained on TCP (Fig. 37) showed a greater number of stained elements and ALP vesicles compared to the correspondent samples analyzed in growth medium (Fig. 18), cells on ECM coatings gave a more strong response to the staining.

Furthermore, the alkaline phosphatase positivity was still more pronounced at day 15 in ECM coatings (Fig. 38) with respect to cells on TCP (Fig. 39) even if the microscope observation was disturbed due to the presence of slightly granulations which covered the entire sample.

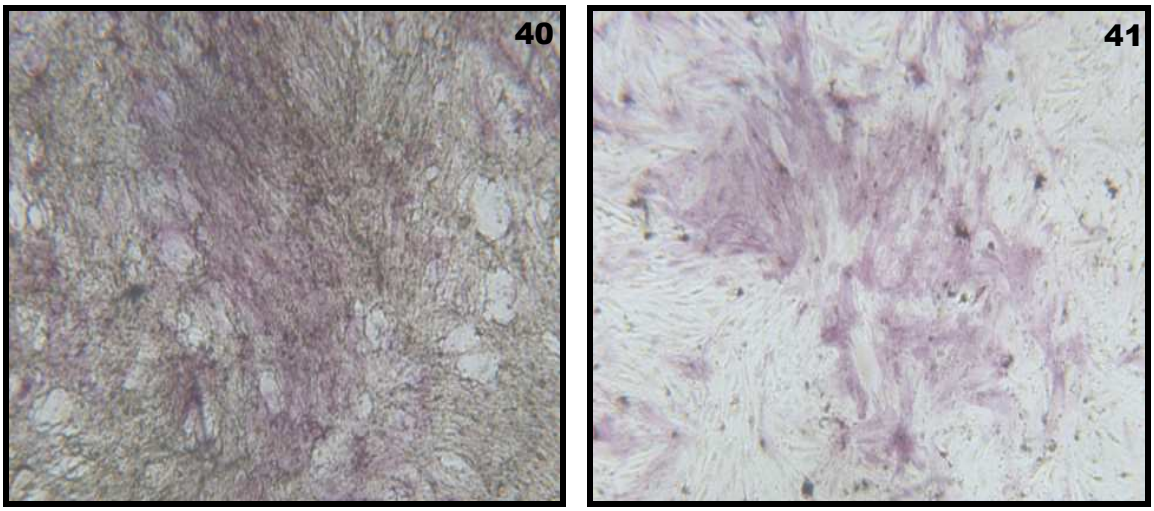
Histochemical reaction accomplished on ECM coatings at day 21 appeared masked by the augmented granulation on sample surface (Fig. 40). On the other hand, cells grown on tissue culture plates showed a lower number of positive elements with respect to all the examined samples (Fig. 41) and no evident granulation was detected.

The measurements of ALP staining (Graphic IV) was accomplished especially to understand the previous images obtained by light microscopy, with particular attention to the cells in ECM coatings at 15 and 21 days, which were covered by granulations. Analysis at day 6 of culture under osteogenic treatment showed a slightly increment in optical density in both ECM coatings and tissue culture plates compared to samples in growth medium (Graphic II).

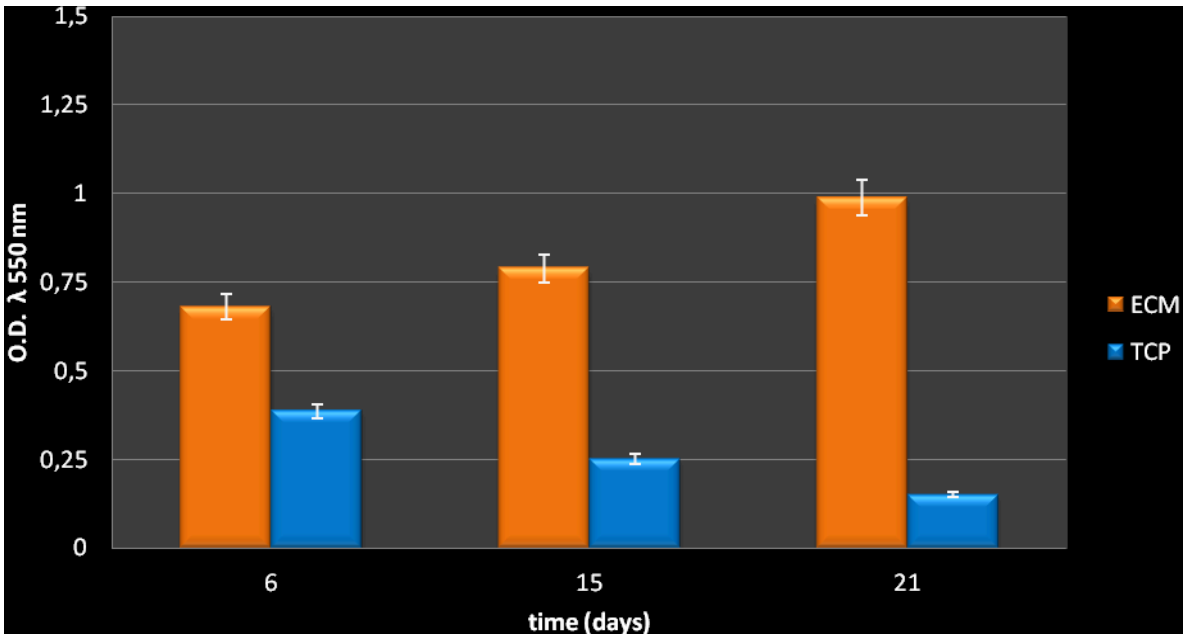
Bone marrow mesenchymal cells on ECM coatings under osteogenic treatment displayed a little increase at day 15 and moreover at day 21; whereas cells on TCP underwent a reduction for all the experimental time.



Figg. 36-39 –ALP staining of hBMSCs cultured on ECM coatings and TCP in osteogenic medium observed by Light microscopy (20x). **36.** Cells on ECM substrates after 6 day of expansion. **37.** hBMSCs cultured on TCP at day 6. **38.** Cells on ECM coatings at day 15 showed a typical oriented disposition. **39.** hBMSCs stained on TCP after 15 days displayed a greater ALP positivity.



Figg. 40-41 –ALP staining of hBMSCs on ECM coatings and TCP at day 21. Light microscopy images (8x) **40.** Cells on ECM, positive stained elements under the granulation. **41.** hBMSCs on TCP showed intense staining.



Graphic IV – Alkaline phosphatase staining measurement in hBMSCs on extracellular matrix coating and plastic culture plates under osteogenic treatment at different time points. Spectrophotometric analysis.

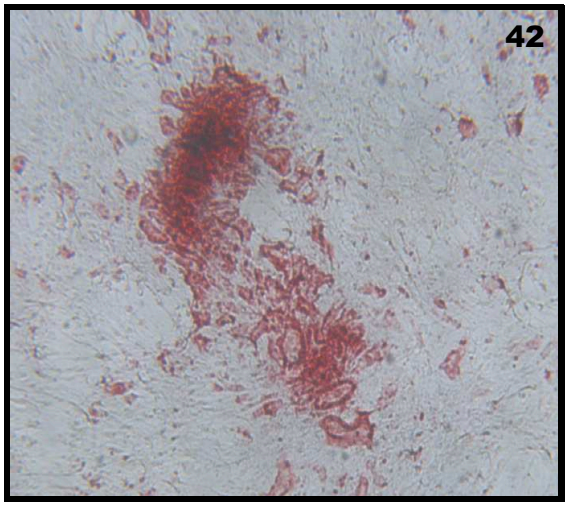
b) *Alizarin Red Staining and Measurement*

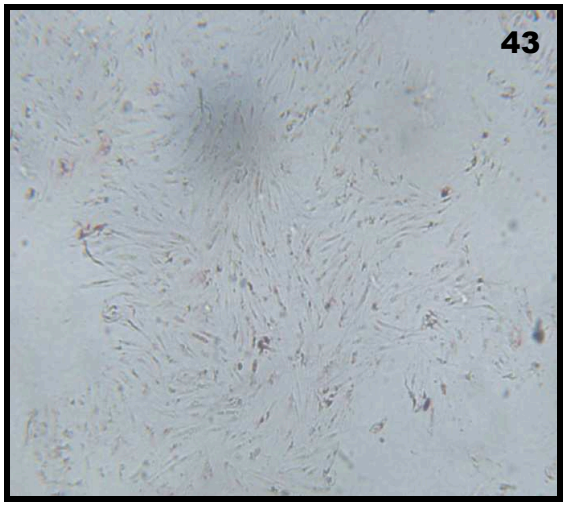
To estimate the Calcium deposition after 6, 15 and 21 days of exposure to osteogenic treatment, Bone Marrow Mesenchymal cells were stained with Alizarin Red (Figg. 42-45).

Mesenchymal cells on ECM coatings showed a little appreciable Calcium deposition at day 6 with respect to those grown on tissue culture plates. The situation evolves following discontinuous treatment with induction medium; indeed hBMSCs on extracellular matrix coatings showed the presence of more intensely stained clusters after 2 weeks of culture (Fig. 42) when compared to cells cultured on TCP, which had only small deposits (Fig. 43).

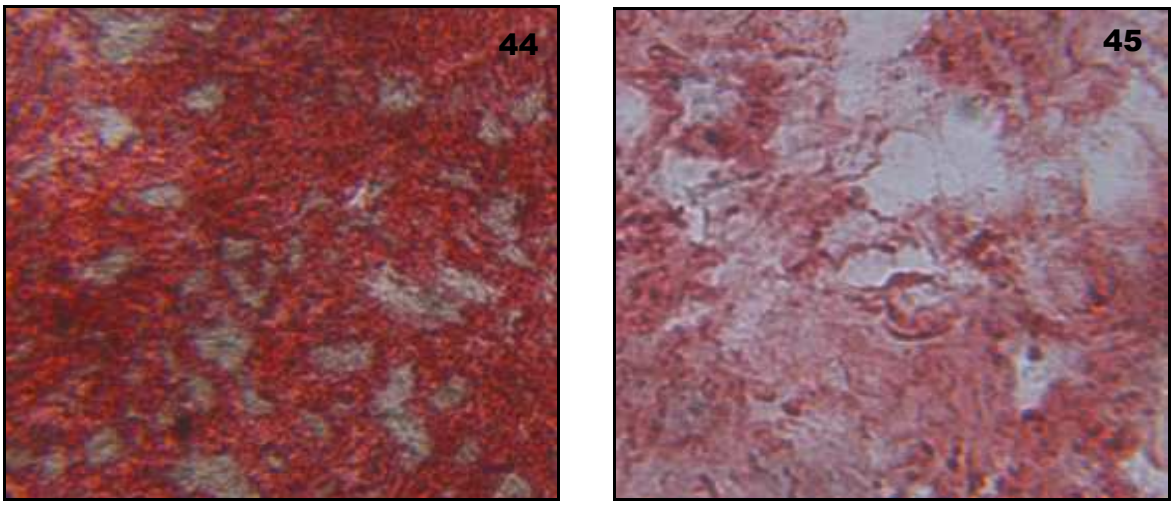
Furthermore, cells on matrix had the greatest Calcium deposition after 3 weeks of treatment, indeed the ECM coatings was totally covered by a strong red staining (Fig. 44); on the contrary hBMSCs cultured on TCP displayed an intense staining only after 3 weeks (Fig. 45).

Spectrophotometric analysis (Graphic V) confirmed the light microscopy observations. The graphic trend was consistent with an increase in Calcium deposits laid down by Mesenchymal cells cultured on ECM coatings after two and above all three weeks, compared to cells on TCP.

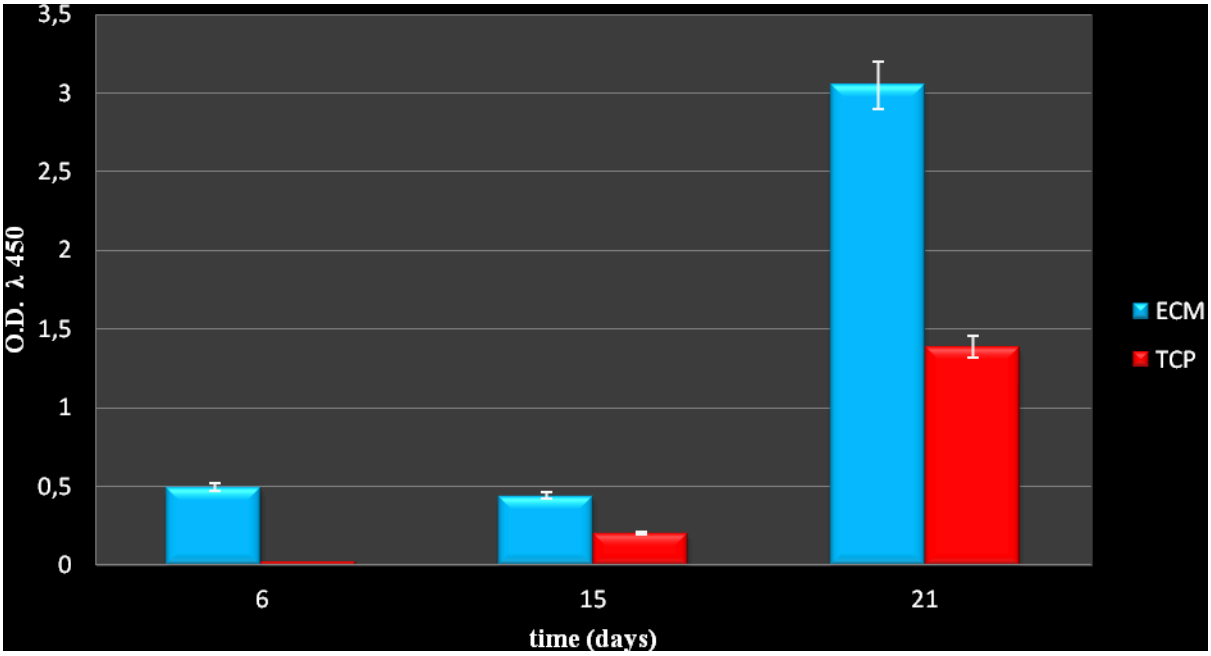




Figg. 42-43 – Calcium deposition in cells exposed to osteogenic medium at day 15. Light microscopy (8x). **42.** Cells on ECM substrates showed intensely stained clusters. **43.** hBMSCs cultured on TCP had few slightly positive elements.



Figg. 44-45 – Calcium deposition in hBMSCs exposed to osteogenic medium at day 21. Light microscopy (8x). **44.** Cells on ECM coatings exhibited the greatest calcium deposition. **45.** hBMSCs stained on TCP displayed intense positivity.



Graphic V – Alizarin red staining measurement in hBMSCs on extracellular matrix coating and plastic culture plates under osteogenic treatment at different time points. Spectrophotometric analysis.

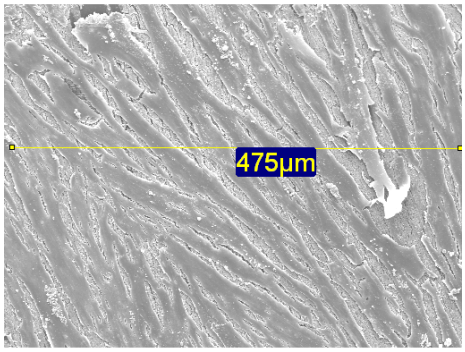
3.2.5 X-Ray Microanalysis

X-Ray analysis was carried out on cells grown on ECM coatings after 3 hours, 6 and 15 days under discontinuous treatment with osteogenic medium. The analysis were performed taking into consideration cell surfaces, the granules above cellular membranes and those present on the extracellular matrix coatings.

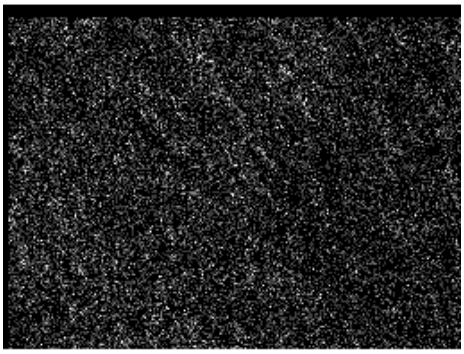
Samples at 3 hours and at 6 days did not show Calcium at detectable levels.

Instead hBMSCs seeded on ECM coatings after 15 days of exposure revealed the presence of Calcium in both the two fields examined and also in all the punctiform acquisitions. In particular Calcium represented the 0.40 in weight% of the elements in area 1 (Tab II) and 1.68 in weight% of the elements in area 2 (Tab III). Furthermore, in some spectra belonging to area II, appeared also Phosphorus when Ca^{2+} was more higher in weight%, and it was detected as 1.31 weight% in Tab II. These two elements, whose distribution inside each area was also showed in the element map are the principle constituents of hydroxyapatyte crystals [$Ca_{10}(PO_4)_6(OH)_2$]; thus indicating that after 15 days in osteogenic medium hBMSCs laid down Calcium and Phosphorus beginning the process of matrix mineralization.

In addition, as observed in the micrographies obtained by punctiform acquisition, X-Ray microanalysis detected Calcium on cells, but also in the exocytose vesicles and in the small granules that covered the surrounding extracellular matrix (data not shown).



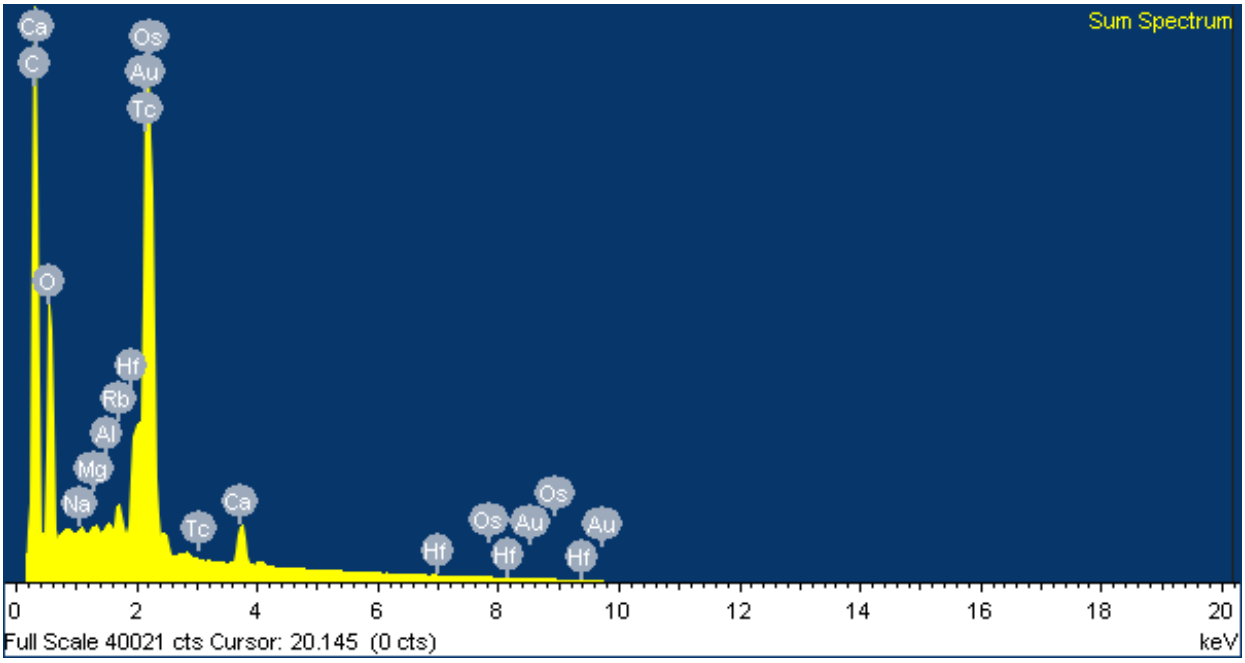
Electron Image 1

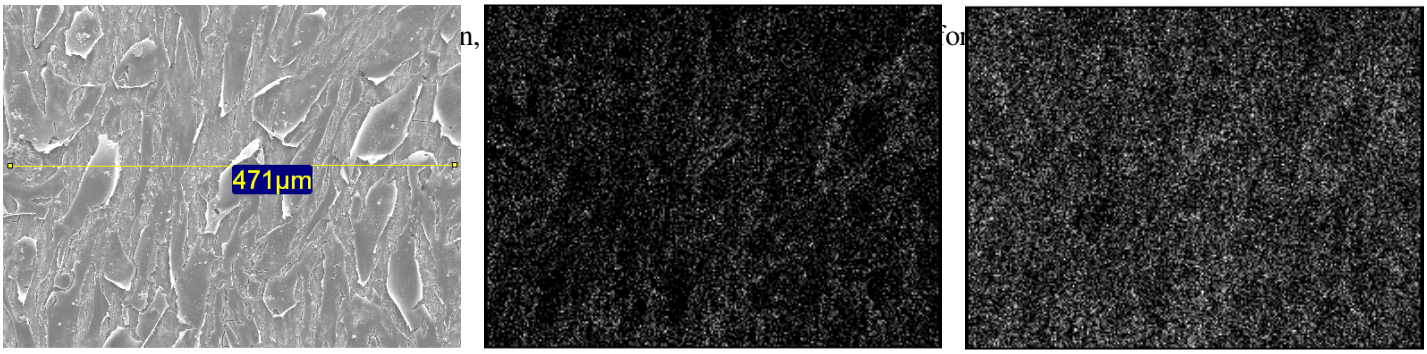


Ca Ka1

Spectrum	C	Na	Mg	Al	**Ca**	Rb	Tc	Hf	Os	O	Total
Sum Spectrum	26.42	0.08	0.06	0.04	**0.40**	0.16	0.08	0.00	1.77	71.00	100.00
Mean	26.42	0.08	0.06	0.04	**0.40**	0.16	0.08	0.00	1.77	71.00	100.00
Std. deviation	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00	0.00	
Max.	26.42	0.08	0.06	0.04	**0.40**	0.16	0.08	0.00	1.77	71.00	
Min.	26.42	0.08	0.06	0.04	**0.40**	0.16	0.08	0.00	1.77	71.00	

All results in weight%





Spectrum	C	Na	Mg	Al	**P**	**Ca**	Hg	Pb	O	Total
Sum Spectrum	24.83	0.15	0.11	0.10	**1.31**	**1.68**	0.50	0.35	69.26	100.00

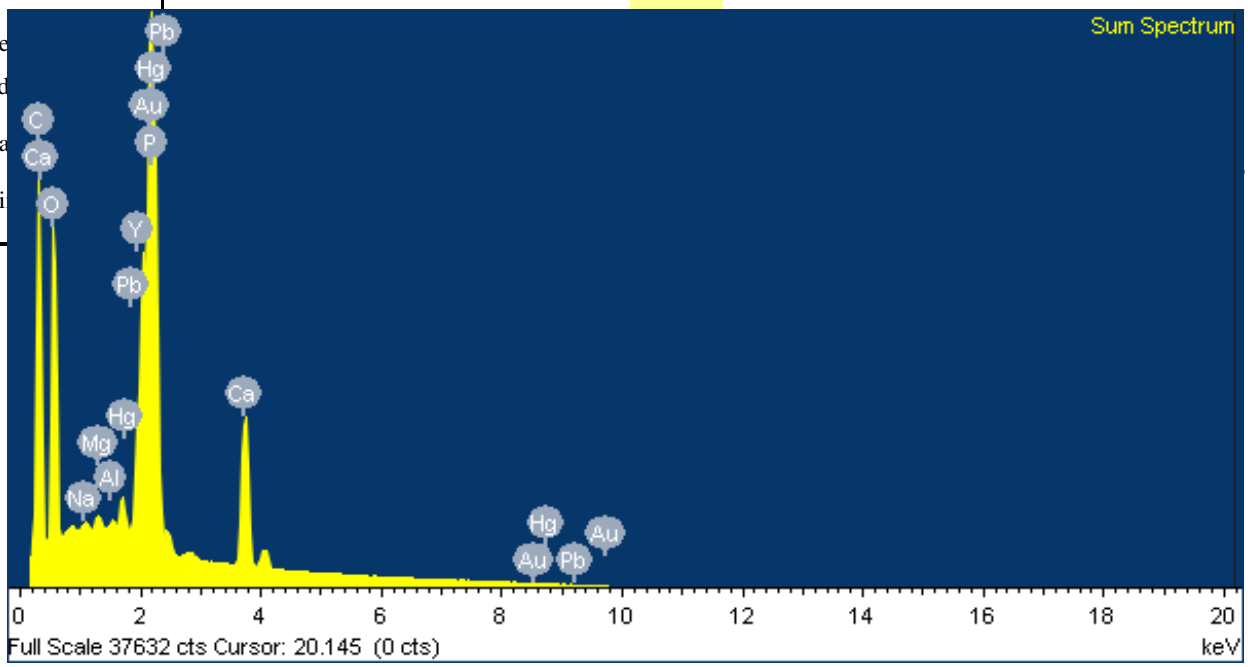


Table III – X-ray microanalysis. hBMSC grown on ECM after 15 days in osteogenic medium. Mapping element related to Calcium and Phosphorus detection, table of the elements (in bold values for Ca (3,8-4 Kev) and P (2.01 Kev)). and spectrum corresponding to area 2 (471 µm diameter).

3.2.6 Osteogenic markers and α5 adhesion molecule gene expression

Gene expression was assessed by reverse transcriptase- PCR in cells under discontinuous treatment with osteogenic medium at 1 and 6 days of culture and also in a reference sample obtained by cell suspension at time 0. All genes were expressed in hBMSCs used as reference; no change in the expression of all analyzed genes (ALP, RUNX2, α5 integrin subunit) was shown between the cells grown on plastic surfaces with or without extracellular matrix (data not shown).

4. DISCUSSION

This study aims to demonstrate that substrates coated with extracellular matrix obtained *in vitro* by bone marrow stem cells secretion and their following decellularization, could be an advantageous biological model to be used for the induction of osteoblastic phenotype in the presence of glucocorticoids, due to its high ability to provide an appropriate environment able to protect cells and elicit their biological responses.

Extracellular matrix coatings influenced hBMSCs responses in normal growth conditions; indeed our results showed that free cell ECM enhanced a remarkable continuous cell proliferation. On the other hand, survival of hBMSCs expanded on tissue culture plates underwent an increase until day 7 that was lower compared to cells expanded on the ECM. The positive effect of extracellular matrix on hBMSCs survival and proliferation was supported in literature; indeed Pei *et al.* (2011) founded that cell number, calculated by a counting hemocytometer, dramatically increased in free cell ECM compared to plastic. The Author demonstrated that the benefited cell survival was linked to a decreased level of intracellular ROS in hBMSCs expanded on ECM that plays an important role in cell adhesion, migration, and proliferation; whereas a high level of ROS has an inhibitory influence on cell proliferation by arresting cell cycle at the G1, S, and G2 phases through down regulation of cyclin D1 and D3 signaling. Furthermore another study sustaining the ECM influence on bone marrow stem cells self-renewal came from the observation of telomerase activity, considered in view of its involvement in cellular life span, that remained highly stable in cells maintained on the ECM while rapidly decreased in cells grown on tissue culture plastic (Lai Y *et al* 2010).

Thus extracellular matrix coatings seemed able to delay, unlike uncoated surfaces, cell spontaneous differentiation in osteoblastic line and this could underlying an important interrelationship between the extracellular matrix components and the preservation of cell stemness, as it was also demonstrated for human and murine BMSCs (Chen *et al.* 2007; Lai Y *et al* 2010). The maintenance of stem characteristics, when cells were cultured on the ECM coatings, supported the hypothesis that the marrow ECM provides important microenvironmental cues to cells *in vitro*.

Morphological investigations revealed that cells grown on extracellular matrix, differently from those maintained on tissue culture plates, changed from a initial roundish to an oriented fibroblastic-like shape characterized by a very evident cellular body. Furthermore human BMSCs during the culture began to synthesize a new extracellular matrix that connected cells each others. Cell disposition and activity on ECM coatings was probably ascribable to the innate ability of extracellular matrix components not only to provide structural support, but also to actively modulate cell phenotype (Guilak, F *et al.*, 2009; Fernandes H *et al.*, 2009). Cell free ECM influenced hBMSCs directional orientation along the matrix fibrils, whereas the cells expanded on uncoated

substrates showed random orientation; this data was in accordance with Lai *et al.*, 2010 and Pei *et al.*, 2011.

The distension and orientation of cells on ECM coated or uncoated surfaces was clearly mediated by cytoskeletal components, particularly microfilaments. Actin filaments in mesenchymal cells grown on extracellular matrix were strictly disposed delineating cell fibroblastic shape and they appeared slightly fluorescent and resolved. Cells on uncoated substrate were more spreading and actin bundles were observable above all along cellular edges. Interestingly Integrin α5 on ECM coatings followed cell morphological modifications: initially having a more central localization and after along the entire adhesive cell surface. Whereas adhesion molecules on uncoated surfaces were less detectable and not homogeneously distributed. This different pattern of adhesion and microfilament rearrangement could be explainable with the instructive role of adhesion targets present in the cell free extracellular matrix; indeed it was demonstrated that its molecular composition did not seem to be affected by permeabilization and decellularization procedures (Chen X-D *et al.*, 2007). Moreover the presence of Integrin α5 in hBMSCs on uncoated surface is probably do to fetal bovine serum fibronectin that is rapidly adsorbed on the substrate thus mediating cell adhesion, as demonstrated blocking αvβ3 or α5β1integrins (Decaris *et al.*, 2012).

On tissue culture plates, the Alkaline phosphatase, early osteoblast marker, progressively increased on tissue culture plates and this trend was consistent with the slight hBMSCs "osteoblastic imprinting" reported in literature (Bianco P and Robey PG, 2000; Satomura K *et al.*, 2000). In contrast, the less positivity of ALP in cells cultured on extracellular matrix substrates can be explained by the fact that the ECM maintained cells in a undifferentiated stage for more time than tissue culture plates delaying their spontaneous differentiation, as confirmed by results of cell viability assay. Alizarin red staining, accomplished to further understand cell commitment to osteogenic lineage, revealed that even though bone marrow mesenchymal stem cells have a slight osteoblastic imprinting as revealed by ALP results (both on ECM and above all on TCP), they failed to begin mineralization. Indeed no relevant Calcium deposits were observed by the Alizarin Red and this data was confirmed by X Ray microanalysis in which this element was not at detectable levels.

These results were in agreement with those reported in literature in which MSCs maintained in basal medium, without osteogenic supplements, showed increased levels of ALP, but did not express mineralized ECM neither other osteogenic markers such as Col1 (Hildebrandt C *et al.*, 2009).

Extracellular matrix proprieties influenced mesenchymal cells behavior also during osteogenic differentiation. It is known that Dexamethasone is able to induce osteogenic differentiation *in vitro* also in high concentration promoting the cell osteoblastic maturation with the disadvantage of a decrease in cell number (Walsh S *et al.*, 2001).

Cell survival and proliferation rate was only slightly reduced in cells seeded on extracellular matrix compared to samples maintained in growth medium, instead a pronounced decrease was detected in hBMSCs on uncoated substrates. These data suggested that ECM coatings, besides having a stimulatory effect on MSCs, played a protective role during cell differentiation. On the contrary, the disadvantageous effects of dexamethasone on cell proliferation were very clear in cell maintained on uncoated surfaces.

Mesenchymal cells morphology denoted the beginning of the mineralization process that was consequence of the acquisition of the osteoblastic phenotype. On extracellular matrix coatings, cells changed from the characteristic fibroblast-like phenotype, observed in normal medium, to polygonal shape and, in late experimental stages, appeared evenly covered by numerous small granules.

It is known that Integrin α5β1, the main fibronectin receptor, can be found in different adhesion structures (Larsen M *et al.*, 2006), and has been implicated in the control of various cell type differentiation (Gronthos S *et al.*, 2001; Keselowsky BG *et al.*, 2007), but its effect on human mesenchymal stem cells osteogenic phenotype is still unknown. However, recently transcriptome analysis confirmed that hMSCs osteoblast differentiation induced by dexamethasone is associated with up-regulation of Integrin α5 (Hamidouche Z *et al.*, 2009). Interestingly, during osteogenic induction Integrin α5 in Bone Marrow Mesenchymal cells increased in both substrates. In particular, α5 adhesion molecules in hBMSCs cultured on ECM coatings were organized on the whole cell adhesion surface, with rod-like clusters particularly evident in the peripheral cellular area, corresponding to the microfilaments end terminus, with a directionality reflecting that of extracellular matrix components. These α5 Integrin peripheral pattern was already detectable during early events of cell adhesion only on cell free ECM; whereas α5 molecules maintained mainly a central distribution when cultured on uncoated surfaces.

Alkaline phosphatase is normally detectable in treated Mesenchymal stem cells during the beginning of differentiation and after it decrease (Vater *et al.*, 2011). While ALP underwent an increase in mesenchymal cells cultured on ECM coatings with respect to normal medium, its value was in continuous decrease on uncoated surfaces. The different behavior of Alkaline phosphatase activity could be explained because human MSCs on extracellular matrix coatings preserved probably a certain degree of stemness giving rise to a new differentiating cell progeny.

The protective action exerted by the extracellular matrix on bone marrow mesenchymal cells to high concentration of dexamethasone used in osteogenic medium emerged mainly when compared to ALP activity in cells grown directly on uncoated surfaces; indeed the enzymatic activity drop observed in TCP indicate that hBMSCs have reached late stages of ALP activity and also that most of them have died, results according with those of viability assay. This result was supported by an increase in mineralization on TCP, as revealed by alizarin red staining, which indicate that cells at

three weeks have really reached late stages of osteogenic differentiation. In contrast mesenchymal stem cells on extracellular matrix coatings showed mineralization already at two weeks with a further increase at three weeks, thus exhibited an high osteogenic potential.

It is known that ALP stimulates the extracellular matrix mineralization promoting the precipitation of Calcium phosphates. X Ray microanalysis conducted both on cells and in the small granules that covered the surrounding extracellular matrix showed Calcium and Phosphorus, principle constituents of hydroxyapatyte crystals.

Since cell free ECM influenced human BMSCs behavior, modifying their phenotype, it was expected to show osteoblastic marker modulation. This hypothesis was not confirmed by RT-PCR results that showed no modulation in mRNA level between cells grown in ECM coatings and uncoated plastic dishes. Furthermore, all these gene were also expressed in the reference sample. Although Dexamethasone has been demonstrated to direct BMSCs osteoblastic differentiation *in vitro* at both early and late stages of maturation (Porter RM *et al.*, 2003), response to this agent was reported to be biphasic, concentration-dependent, and depending on exposure time (Aubin JE, 1998, 2001; Cheng *et al*,. 2000).

Data obtained by RT-PCR could be partly explained considering that osteogenic differentiation was induced with a discontinuous treatment; indeed osteogenic medium exposition was followed by a recovery phase (growth medium) in which cells could have maintained their transcript level.

In conclusion the combination of both an extracellular matrix coating and dexamethasone resulted in strong hBMSCs osteogenic differentiation as confirmed by SEM images (osteogenic phenotype and granulation) ALP activity (staining) and Calcium deposition displayed by alizarin red staining and X-Ray microanalysis results.

5. CONCLUSIONS

The behavior and multilineage differentiation potential of Mesenchymal stem cells is regulated by the relationship they establish with a tissue-specific environment or niche consisting of extracellular matrix proteins associated with growth factors. To reproduce the complex highly ordered nature of the ECM using synthetic materials or purified components can be a difficult task. So, I have realized a cell free extracellular matrix produced by bone marrow mesenchymal stem cells in order to reconstitute a relatively optimal substrate for cell maintenance in *vitro*. From the results obtained on culture systems, the ECM provides an ideal environment that promote cell adhesion (probably by providing all or most of the molecules involved in the cell attachment to the substrate) and cell proliferation, creating an ideal setting for the large-scale expansion of MSCs for their possible use in stem cell-based therapy. In addition, cell free extracellular matrix was able to enhance, in the presence of specific factors (osteogenic medium), the differentiative capabilities of the cells grown on it, exerting a protective role in the maintenance of hBMSCs proliferative activity, and these could provide the basis for an easier tissue-specific fate control of MSCs for therapeutic applications.

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