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***HUMAN MESENCHYMAL STEM CELLS AS A MODEL OF STUDY FOR NEW BIOMATERIALS
IN BONE TISSUE REGENERATION***

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ABSTRACT

The reconstruction of large bone segments remains a critical clinical problem in the case of extensive bone loss due to traumatic or pathological events. Due to its ideal biocompatibility and osteogenic properties, autologous bone is still considered the gold standard for bone replacement applications, however its use has limitations such as supply amount and unpredictable healing kinetics. These limitations and recent progress in biotechnology have driven the development of materials/scaffolds for bone repair. Biocompatibility issues and materials/scaffolds features need to be investigated and the usefulness of human cell cultures for biocompatibility testing is confirmed by experimental studies. In-vitro models employing human cells to study the interactions between the cell system and the biomaterial/device allow for a reasonable prediction of its performance in vivo. To study coral derived Hydroxyl-Apatite (HA) osteoconductivity and cyto-toxicity we employed an engineered osteoblast-like cellular model named Saos-eGFP. Although the efficacy of this cellular model, the need of an in-vitro tool resembling a more physiological-like cellular response, led to the development of an adult human mesenchymal stem cell (hMSCs) model. This model was employed to assay porous stoichiometric HA and non-stoichiometric Mg-HA bone substitutes. Our studies showed how human cells may be employed to analyze osteoconductivity/inductivity and cyto-toxicity, and specifically how hMSCs allow a more insightful understanding of the earliest phase of cell-biomaterial interaction, addressing the question of which type of biomaterials could be better for bone regeneration applications.

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1 INTRODUCTION

1.1 Tissue engineering and regenerative medicine

The dream is as old as humankind. Injury, disease, and congenital malformation have always been part of the human experience. If only damaged bodies could be restored, life could go on for loved ones as though tragedy had not intervened. In recorded history, this possibility first was manifested through myth and magic, as in the Greek legend of Prometheus and eternal liver regeneration. With the introduction of the scientific method came new understanding of the natural world. The methodical unraveling of the secrets of biology was coupled with the scientific understanding of disease and trauma. Artificial or prosthetic materials for replacing limbs, teeth, and other tissues resulted in the partial restoration of lost function (Langer et al., 1993). Also, the concept of using one tissue as a replacement for another was developed. In the 16th century, Tagliacozzi of Bologna, Italy, reported in his work *Decusorum Chirurgia per Insitionem* a description of a nose replacement that he constructed from a forearm flap (Soto-Miranda et al., 2006). With the 19th-century scientific understanding of the germ theory of disease and the introduction of sterile technique, modern surgery emerged. The advent of anesthesia by the mid-19th century enabled the rapid evolution of many surgical techniques. Maintenance of life without regard to the crippling effects of tissue loss or the psychosocial impact of disfigurement, however, was not an acceptable end goal. Techniques that resulted in the restoration of function through structural replacement became integral to the advancement of human therapy. Now whole fields of reconstructive surgery have emerged to improve the quality of life by replacing missing function through rebuilding body structures. In our current era,

modern techniques of transplanting tissue and organs from one individual into another have been revolutionary and lifesaving (Lysaght et al., 2000). As with any successful undertaking, new problems have emerged. Techniques using implantable foreign body materials have produced dislodgment, infection at the foreign body/tissue interface, fracture, and migration over time. Techniques moving tissue from one position to another have produced biologic changes because of the abnormal interaction of the tissue at its new location (Wymenga et al., 1990; Lamas Pelayo et al., 2008; Schoen et al., 1992). Transplantation from one individual into another, although very successful, has severe constraints. The major problem is accessing enough tissue and organs for all of the patients who need them (Nerem et al., 2000). Also, problems with the immune system produce chronic rejection and destruction over time. Creating an imbalance of immune surveillance from immunosuppression can cause new tumor formation. The constraints have produced a need for new solutions to provide needed tissue. It is within this context that the field of tissue engineering has emerged (Vacanti et al., 1999; Vacanti et al., 2006). In essence, new and functional living tissue is fabricated using living cells, which are usually associated, in one way or another, with a matrix or scaffolding to guide tissue development. Cells may be obtained from adult tissues but new sources of cells, including many types of stem cells, have been identified in the past several years, igniting new interest in the field. In fact, the emergence of stem cell biology has led to a new term, regenerative medicine (Lanza et al., 2000; Lavik et al., 2004). Conceptually, the application of this new discipline to human health care can be thought of as a refinement of previously defined principles of medicine. The physician has historically treated certain disease processes by supporting nutrition, minimizing hostile factors, and optimizing the environment so that the body can heal itself. In the field of tissue engineering, the same thing is accomplished on a cellular level. The

harmful tissue is eliminated; the cells necessary for repair are then introduced in a configuration optimizing survival of the cells in an environment that will permit the body to heal itself. As a field, tissue engineering has been defined only since the mid-1980s. To date, much of the progress in this field has been related to the development of model systems, which have suggested a variety of approaches (Epari et al., 2010; Lammers et al., 2010; Haycock, 2011; Gjorevski et al., 2010; Poss, 2010; Elliott et al., 2011). Tissue engineering can draw on the knowledge gained in the fields of cell and stem cell biology, biochemistry, and molecular biology and apply it to the engineering of new tissues. Likewise, advances in materials science, chemical engineering, and bioengineering allow the rational application of engineering principles to living systems. Yet another branch of related knowledge is the area of human therapy as applied by surgeons and physicians. In addition, the fields of genetic engineering, cloning, and stem cell biology may ultimately develop hand in hand with the field of tissue engineering in the treatment of human disease, each discipline depending on developments in the others (Lanza et al., 2002; Caldorera-Moore et al., 2009; Stroka et al., 2010; Sefcik et al., 2010; Vazin et al., 2010; Romano et al., 2011; Azuaje et al., 2011). The scientific challenge in tissue engineering lies both in understanding cells and their mass transfer requirements and the fabrication of materials to provide scaffolding and templates (Stock et al., 2001). In the last decade new functionalized materials which may interact with the physiological environment influencing its behavior, has been developed. Cell therapies have been also employed as described in (Langer et al., 2004; Dutta et al., 2009; Richardson et al., 2010; Jung et al., 2011). Recently the term “regenerative medicine” has been introduced. Thus, we define clinical tissue engineering as “the use of a synthetic or natural biodegradable material, which has been seeded with living cells when necessary, to regenerate the form and/or function of a

damaged or diseased tissue or organ in a human patient.” We see clinical tissue engineering as a set of tools that can be used to perform regenerative medicine (Lanza et al., 2000). Technical as well as economic hurdles must be overcome before therapies based on tissue engineering will be able to reach the millions of patients who might benefit from them (Lysaght et al., 2004). One long-recognized challenge is the development of methods to enable engineering of tissues with complex three-dimensional architecture. A particular aspect of this problem is to overcome the mass transport limit by enabling provision of sufficient oxygen and nutrients to engineered tissue prior to vascularization and enhancing the formation of new blood vessels after implantation. The use of angiogenic factors, improved scaffold materials, printing technologies, and accelerated in vitro maturation of engineered tissues in bioreactors may help to address this problem (Martin et al., 2004). Of particular interest is the invention of novel scaffold materials designed to serve an instructive role in the development of engineered tissues (Rosso et al., 2005). A second key challenge centers on a fundamental dichotomy in strategies for sourcing of cells for engineered tissues — the use of autologous cells versus allogeneic or even xenogeneic cells. On the one hand, it appears most cost effective and efficient for manufacturing, regulatory approval, and wide delivery to end users to employ a minimal number of cell donors, unrelated to recipient patients, to generate an off-the-shelf product. On the other hand, grafts can be generated from autologous cells obtained from a biopsy of each individual patient. Such grafts present no risk of immune rejection because of genetic mismatches, thereby avoiding the need for immunosuppressive drug therapy. Thus, the autologous approach, though likely more laborious and costly, appears to have a major advantage (Moller et al., 1999; Nerem, 2006; Naughton et al., 2002). Nonetheless, there are many tissue-engineering applications for which appropriate autologous donor cells may not be

available. Therefore, new sources of cells for regenerative medicine are being sought and assessed, mainly from among progenitor and stem cell populations. The understanding of normal developmental and wound-healing gene programs and cell behavior, can be used to our advantage in the rational design of living tissues. (Zhang et al., 2005; Wang et al, 2010; Gonez et al., 2010; Chen et al., 2011; Wetsel et al., 2011; Chambers et al., 2011)

1.2 Stem Cells: origin and biology

1.2.1 Origin and definition

Stem cells are defined functionally as cells that have the capacity to self-renew as well as the ability to generate differentiated cells (Weissman et al., 2001; Smith, 2001). More explicitly, stem cells can generate daughter cells identical to their mother (self-renewal) as well as produce progeny with more restricted potential (differentiated cells). This simple and broad definition may be satisfactory for embryonic or fetal stem cells that do not perdure for the lifetime of an organism. But this definition breaks down in trying to discriminate between transient adult progenitor cells that have a reduced capacity for self-renewal and adult stem cells. It is therefore important when describing adult stem cells to further restrict this definition to cells that self-renew throughout the life span of the animal (van der Kooy and Weiss, 2000). Another parameter that should be considered is potency, the stem cell capacity to generate multiple differentiated cell types (multipotent), or only one type of differentiated cell (unipotent). Thus, a more complete description of a stem cell includes a consideration of replication capacity, clonality, and potency. Most somatic cells cultured in vitro display a finite number of (less than 80) population doublings prior to replicative arrest or senescence, and this can

be contrasted with the seemingly unlimited proliferative capacity of stem cells in culture (Houck et al., 1971; Hayflick, 1973; Hayflick, 1974; Sherr and DePinho, 2000; Shay and Wright, 2000). Therefore, it is reasonable to say that a cell that can undergo more than twice this number of population doublings (160) without oncogenic transformation can be termed “capable of extensive proliferation.” In a few cases, this criteria has been met, most notably with embryonic stem (ES) cells derived from either humans or mice as well as with adult neural stem cells (NSCs) (Morrison et al., 1997; Smith, 2001). An incomplete understanding of the factors required for self-renewal *ex vivo* for many adult stem cells precludes establishing similar proliferative limits *in vitro*. In some cases, a rigorous assessment of the capacity for self-renewal of certain adult stem cells can be obtained by single-cell or serial transfer into acceptable hosts, an excellent example of which is adult hematopoietic stem cells (HSCs) (Iscoe and Nawa, 1997; Allsopp and Weissman, 2002). Strictly related to self-renewal, is the idea that stem cells are clonogenic entities: single cells with the capacity to create more stem cells. Self-renewal can be defined as making a complete phenocopy of stem cells through mitosis, which means that at least one daughter cell generated by mitosis possesses the same capacity of self-renewal and differentiation. In stem cell self-renewal, symmetric cell division generates two stem cells; asymmetric cell division results in one stem cell and either one differentiated progeny or a stem cell with a restricted capacity for differentiation. Self-renewal by symmetric cell division is often observed in transient stem cells appearing in early embryonic development to increase body size. In contrast, self-renewal by asymmetric cell division can be found in permanent stem cells in embryos in later developmental stages and in adults to maintain the homeostasis of the established body plan (Merok and Sherley, 2001). The terms totipotent, pluripotent, and multipotent are commonly used to describe stem cell developmental capacity.

Totipotent is a term that some use to identify a stem cell that can give rise to every extraembryonic, somatic, or germ cell known in mammalian development. Pluripotent cells, on the other hand, are cells that can give rise to all cells of the body including germ cells, and some of the extraembryonic tissues that function to support development in mammals, but cannot give rise on their own to a new organism. Multipotent stem cells are cells that can give rise to several types of mature cells. There are also examples of stem cell populations that are bipotent, or unipotent (Prelle et al., 2002).

Embryonic development represents a state of flux in which multiplication, commitment, differentiation, and death constantly reshape and redefine the relationship between various cell populations until the body plan emerges and the major organ systems have developed (Nagy et al., 2003). Nevertheless some understanding of cell commitment in the embryo is critical to understanding of the concept of pluripotentiality, and to understanding of the developmental potential of stem cells derived from embryonic tissues.

The unfertilized oocyte may be regarded as a pluripotent cell, since artificial activation of the mammalian egg can give rise to a parthenogenetic conceptus that will develop up to the early postimplantation stages (Rougier, 2001), and ES cells derived from parthenogenetic embryos have been described in mice and primates. However, there are limitations to the development of parthenotes imposed by genomic imprinting and the resulting requirement for both a maternal and paternal genome for normal development (Allen et al., 1994). Thus, the oocyte is not truly totipotent in the sense that the zygote is.

The zygote and early blastomeres of the mouse embryo are totipotent, in the sense that we have defined the term, up to the eight-cell stage of development. Thereafter, the early stages of mammalian development are devoted largely to the formation of the extraembryonic tissues, tissues derived from the zygote that support development, including the trophoblast, yolk sac, amnion, and allantois. It is around the eight-cell stage that the formation of these extraembryonic tissues begins, with the preparation for the first commitment event in mammalian development, the formation of the trophectoderm (Rossant et al., 2003). Compaction of the embryo leads to an asymmetric division of its constituent cells to produce either inner or outer cells, leading to segregation of the future trophectoderm cells to the outer layer of the developing blastocyst (Johnson and McConnell, 2004). The outer cells lose pluripotency and become committed to the trophectoderm fate. Stem cell lines may be established from the trophoblast in the mouse, but these cell lines participate only in the development of the placenta (Rossant, 2001).

The cells on the inside of the blastocyst constitute the inner cell mass, precursor of all body tissues plus extraembryonic endoderm and mesoderm. Not long after it has been formed, the inner cell mass soon loses the ability to generate a trophoblast. The next commitment event in the development is the formation of the primitive endoderm, the precursor of the yolk sac. Following differentiation of the extraembryonic endoderm and implantation, the inner cell mass develops into an epithelium known as the epiblast. The epiblast retains pluripotentiality, since cell lineage tracing experiments show that it will contribute to multiple tissues at this stage, and it can give rise to teratomas when transplanted into ectopic sites (Stevens, 1980). This capacity to form teratomas disappears after about 7.5 days of development in the mouse. ES cells can also be derived from the mouse epiblast. However, epiblast cells, unlike inner cell mass cells,

cannot colonize a host blastocyst. Whether this reflects a limitation of their developmental capacity, or a limitation of the assay in that these cells may not be able physically to integrate into the host embryo, is uncertain.

Beginning shortly after implantation, pluripotent cells of the epiblast receive signals from the surrounding extraembryonic endoderm and extraembryonic ectoderm that specify cell fate in a regionally and temporally controlled fashion (Rossant and Tam, 2004). These signals help to define the future anteroposterior axis of the embryo (Bachiller et al. 2000). Subsequently the process of gastrulation results in the commitment of most cells in the embryo to particular fates. Thus, after gastrulation, cells become restricted in their developmental capacity. Movement through the primitive streak commits cells to an endodermal or a mesodermal fate, depending on the time of their emergence (Rossant, 2004).

One embryonic cell population escapes some of these restrictions on development fate. Primordial germ cells, the precursors of oogonia and prospermatogonia (Saitou et al., 2002; Adams and McLaren, 2002), are induced in the proximal epiblast by signals from the surrounding extraembryonic ectoderm and extraembryonic endoderm. Once formed, primordial germ cells proliferate as they migrate through the hindgut to the genital ridge (McLaren, 2003). However, stem cells have long been known to exist within the adult animal in various organs and tissues. A general feature of multipotent stem cells is that under homeostatic conditions, they remain quiescent. Upon entering the cell cycle, stem cells may divide symmetrically to give rise to two identical stem cells. Alternatively, stem cells may divide asymmetrically, resulting in one identical daughter stem cell (for self renewal) and one daughter progenitor cell, or two different daughter progenitor cells. The progenitor daughter cell then undergoes cellular proliferation and progressive differentiation, leading to an expansion of committed progenitor populations (Stocum,

2001). The presence of multipotential stem cells within the adult animal may be an evolved compensatory mechanism of cell replacement in response to injury and/or an ongoing required mechanism for replacing cells in tissues that have a high cell turnover, such as the skin and blood (Stocum et al., 2008). Recent studies have shown that certain tissue stem cells sometimes display the capacity to differentiate into multiple cell types, including cells outside their lineage of origin, i.e. they demonstrate the property of plasticity. The mechanism of tissue stem cell plasticity is not yet well understood. Different theories advanced to account for plasticity include transdifferentiation of a stem cell to unrelated cell type, de-differentiation of a mature cell to a more primitive stem cell followed by subsequent differentiation into another cell, or perhaps for some cases, fusion between two different cells. Evidence for the presence of plastic stem cells in mature tissues extends to bone marrow, brain, liver, skin and muscle (Theise, 2010). For example, facultative tissue stem cells found within the liver, known as oval cells, are capable of differentiation into hepatocytes as well as bile duct epithelium (Tanaka et al. 2011). Multipotent precursor cells from the pancreas have also been clonally isolated, and these cells may differentiate into various pancreatic cells as well as neural and glial cells (Seaberg, 2004).

1.2.2 Adult Stem cell niches

Given these definitions, different tissues showed to host adult stem cells. Stem cell number, division, self-renewal, and differentiation are likely to be regulated by the integration of intrinsic factors and extrinsic cues provided by the surrounding microenvironment, now known as the stem cell niche (Schofield, 1983). The concept of the stem cell niche arose from observations that many adult stem cells, such as hematopoietic stem cells, lose the potential for continued self-renewal when removed from their normal cellular environment and the idea from developmental biology that

different signaling microenvironments can direct daughter cells to adopt different fates. In the stem cell niche hypothesis, signals from the local microenvironment, or niche, specify stem cell self-renewal (Arai et al., 2005; Yamashita et al., 2005; Rizvi and Wong, 2005). If space within the niche were limited such that only one daughter cell could remain in the niche, the other daughter cell would be placed outside of the niche, where it might initiate differentiation due to lack of self-renewal factors. However, if space within the niche is available or an adjacent is empty, both daughters of a stem cell division can retain stem cell identity. Therefore, the stem cell niche hypothesis predicts that the number of stem cells can be limited by the availability of niches with the necessary signals for self-renewal and survival. As a consequence, the niche provides a mechanism to control and limit stem cell numbers (Ohlstein et al., 2004). The existence of a stem cell niche has been proposed for several adult stem cell systems. The precise spatial organization of the stem cells with respect to surrounding support cells plays an important role in the ability of the niche to adequately provide proliferative and antiapoptotic signals and to exclude factors that promote differentiation. In each case, stem cells are in intimate contact with surrounding support cells that serve as a source of critical signals controlling stem cell behavior. Adhesion between stem cells and either an underlying basement membrane or the support cells themselves appears to play an important role in holding the stem cells within the niche and close to self-renewal signals. In addition, the niche could provide polarity cues to orient stem cells within the niche so that, upon division, one cell is displaced outside of the niche into an alternate environment that encourages differentiation (Li and Xie, 2005). Specialized niches have been proposed to regulate the behavior of stem cells in several mammalian tissues maintained by stem cell populations, including the male germ line, the hematopoietic system, the epidermis, and the intestinal epithelium, neural system. Many of these

niches share specifically signaling molecules secreted from the surrounding microenvironment and cell adhesion molecules required for anchoring stem cells within the niche (Ogawa et al., 2005; Adams and Scadden, 2006; Blanpain and Fuchs, 2006; Larsson and Scadden, 2006; Simon-Assmann et al., 2007).

1.2.2.1 Foetal annexes

A pluripotential subpopulation of progenitor cells in the amniotic fluid can be isolated through positive selection for cells expressing the membrane receptor c-kit, which binds to the ligand stem cell factor. The progenitor cells derived from human amniotic fluid are pluripotent and have been shown to differentiate into osteogenic, adipogenic, myogenic, neurogenic, endothelial, and hepatic phenotypes in vitro. Each differentiation has been performed through proof of phenotypic and biochemical changes consistent with the differentiated tissue type (Parolini et al., 2009). Hematopoietic stem and progenitor cells from umbilical cord blood have been used to transplant more than 5,000 recipients with various malignant or genetic disorders since the first transplant, performed in October 1988. (Broxmeyer et al., 1992; Wagner et al., 1992). Cord blood transplantation can be used, especially in children, to treat a multiplicity of malignant and nonmalignant disorders currently treatable by bone marrow transplantation. One obvious advantage of cord blood as a source of transplantable stem cells is the lower incidence of graft-vs.-host disease compared to that of bone marrow, allowing the use of cord blood with a greater HLA-disparity than is usually acceptable for bone marrow transplantation (Broxmeyer et al, 2003; Christopherson and Broxmeyer, 2004; Broxmeyer 2005).

1.2.2.2 Ectoderm

The adult mammalian brain has vastly reduced regenerative potential compared to the developing brain. Nevertheless, cell proliferation occurs in the adult brain, as does neurogenesis, and cells that give rise to neurons and glia *in vivo* and *in vitro* have been identified in the adult CNS. Although studies dating back to the 1970s have documented neurogenesis in the adult mammalian brain (Kaplan and Hinds, 1977), the isolation of cells with stem cell properties, that is, multipotent and self-renewing, from adult neural tissue did not occur until much later. In the early 1990s, reports demonstrated that cultured cells from the adult rodent striatum produced both neurons and glia, effectively documenting multipotentiality (Reynolds and Weiss, 1992). Since that time, numerous reports have identified cells from adult animals with the potential to produce neurons and glia when grown in culture or transplanted into other brain regions (Alvarez-Buylla et al., 2000; Lenington et al., 2003; Kim and Szele, 2008). Collectively, these evidence support the view that new neurons (i.e., cells with morphological, ultrastructural, biochemical, and electrophysiological characteristics of neurons) are added to the dentate gyrus and olfactory bulb of adult mammals. Evidence for adult neurogenesis in other brain regions, including the neocortex, striatum, olfactory tubercle, amygdala, and substantia nigra, exists, but these findings await further investigation (Fowler et al., 2008; Mackay-Sim, 2010; Ohira et al., 2010; Vukovic et al., 2011; Sun et al., 2012). Numerous neurological conditions are associated with the loss of neural cells, perhaps most notably Alzheimer's disease and Parkinson's disease. The possibility that trauma and neurodegenerative disorders can be treated either by transplanting neural stem cells or by stimulating endogenous neurogenesis in existing populations of adult neural stem cells has theoretically powerful clinical potential.

The processes involved with repair of damaged dermal tissue require the activation of numerous cell types with subsequent migration, proliferation, and reconstitution of biological structures (Cohen et al., 1992). These dynamic cell–cell and cell–matrix interactions have largely been viewed in terms of activation of terminally differentiated cells. In this paradigm, the resident cells, largely quiescent, retain the capacity to enter the cell cycle given the appropriate environmental cues and repopulate the damaged area. However, recent studies show that for some of these cell types, activation of a stem cell compartment clearly occurs. Cell proliferation to replace lost epidermal cells occurs within two compartments: a stem cell compartment and a transient-amplifying cell compartment. The stem cell compartment consists of islands of cells located in the basal cell layer adjacent to the epidermal–dermal basement membrane (Jones and Watt, 1993; Jones et al., 1995). Epidermal stem cells have been also identified in human hair follicles in the mid region of the follicle (Rochat et al., 1994; Oshima et al., 2001; Tumbar, 2012).

The ocular surface epithelium comprises corneal, limbal and conjunctival epithelium. The corneal epithelium is under constant cell-turnover, where loss of the terminally differentiated cells located superficially results in replacement of the cells by the basal epithelial cells (Thoft et al., 1989; Tseng, 1989; Dua and Azuara-Blanco, 2000). Current evidence now supports the concept that corneal epithelial cells arise from specific progenitor cells located in the basal cell layer of the limbus, which are involved in renewal and regeneration of the corneal epithelium (Dua, 1995; Lehrer et al., 1998; Lavker et al., 1998). Cotsarelis et al. provided supporting evidence that the limbus was the site of corneal stem cells (Cotsarelis et al., 1989). This small subpopulation of limbal basal epithelial cells that are normally slow-cycling during the resting state, have been shown to have a significantly higher reserve capacity and proliferative response to

wounding and stimulation by tumor promoters, as compared with the peripheral cornea or central cornea. Pelligrini et al. also showed that goblet cells were found in cultures of transient amplifying cells, occurring late in the life of a single conjunctival clone (Pellegrini et al., 1999). The fornical conjunctiva has been shown to be the site that is enriched in conjunctival stem cells in rabbit and mice models (Wei et al. 1995). The retina is the innermost neural layer of eye, consisting of an inner neurosensory layer and an outer pigmented layer, the retinal pigment epithelium (RPE). The pigmented ciliary body and the peripheral margin of the retina in the adult mammalian eyes are believed to harbor neural progenitors that display stem cell properties and have the capacity to give rise to retinal neurons. They have been shown to be multipotential and have the ability to self-renew (Reh and Levine, 1998; Ahmad et al., 1999). Preliminary studies of retinal stem cell transplantation attempted in animals have shown some promising results. Retinal stem cells transplanted into the subretinal space of rats were able to survive and differentiate into cells of photoreceptor lineage (Chacko et al., 2000; Kurimoto et al., 2001). Clinical trials are in the early stages, and data on safety and efficacy are widely anticipated. Positive outcomes from these stem cell-based clinical studies would radically change the way that blinding disorders are approached in the clinic (Stern and Temple, 2011).

1.2.2.3 Endoderm

For years the very existence of the liver stem cell was questioned (Sell, 1990). Hepatic stem cells would potentially prove a valuable source from which to generate human hepatocytes for drug development (Guillouzo et al., 1997) or use in bioartificial liver support systems (Strain and Neuberger, 2002). The ability of the liver to regenerate

fully is a unique feature of what is a structurally and functionally highly complex organ (Grisham, 1962; Bucher and Farmer, 1998; Fausto, 2001). The process is precisely regulated resulting, under normal conditions, in full restoration of tissue mass and cellular architecture. Stem cells do, however, fulfill an important role when liver damage is severe and parenchymal hepatocytes, which are normally involved in the liver regeneration, are effectively eradicated or, for some reason, are prevented from growth initiation. Farber (1956) was the first to describe the cellular response in the rat liver to toxins such as acetylaminofluorene (AAF) or diethylnitrosamine, and to coin the term oval cell. These cells are small, characterized by oval nuclei and scant cytoplasm, and are suggested to arise from a stem cell pool (Fausto et al., 1993; Thorgeirsson, 1996). Following activation and growth expansion, they migrate from the margins of the portal tracts, in particular canals of Hering that connect the bile canaliculus and the biliary tree, into the parenchyma, where they differentiate into hepatocytes (Paku et al., 2001). They can also differentiate into BEC or abnormal ductular proliferative cells (Germain et al., 1988; Sirica et al., 1990; Lenzi et al., 1992; Dabeva and Shafritz, 1993; Golding et al., 1995). The indication, therefore, is that oval cells represent the activated progeny of the stem cell. (Fausto, 2004; Lee et al., 2006; Yovchev et al., 2008; Wu, 2009). Thus, oval cells may be attractive cell therapeutics. A recent study reports on the isolation of a novel progenitor cell population from unprocessed (that is, no prior exposure to chemicals and no injury) adult rat liver called liver-derived progenitor cells (LDPCs) that can be generated from unprocessed adult liver, which makes them potentially useful for clinical applications, especially for cell transplantation in the treatment of liver diseases (Sahin et al., 2008).

In general, adult pancreatic β cells are known to have a poor growth capacity (Lazarow, 1952) and, in pathogenic states where islet destruction occurs, such as Insulin-

dependent or type I, diabetes mellitus (IDDM), regeneration is not detected. However, after partial pancreatectomy of young rats, new islet cells can arise from ductal epithelium, recapitulating islet formation during embryogenesis and suggesting that stem cells in the adult pancreas can be activated during regeneration (Setalo et al., 1972; Bonner-Weir et al., 1981; Bonner-Weir et al., 1983; Bonner-Weir et al., 1993). Studies have shown that after treatment of newborn rats with streptozotocin, the pancreas is able to repair itself, and new islet formation is observed (Portha et al., 1974, 1979; Cantenys et al., 1981; Dutrillaux et al., 1982). New islet cells are thought to arise through regeneration of islets through division of existing terminally differentiated islet cells or the differentiation of new islet cells from stem cells residing in the pancreatic ducts (Cardinale et al., 2010). In recent years, several promising approaches to the generation of new β -cells have been developed including directed differentiation of pluripotent cells such as embryonic stem (ES) cells or induced pluripotent stem (iPS) cells, or reprogramming of mature tissue cells. In adults, many cell types within the pancreas have been studied as possible candidates for beta-cell stem/progenitor cells. These include acinar cells, centroacinar cells, duct cells, alpha- and delta-cells (Guz et al., 2001; Suzuki et al., 2003; Lardon et al., 2004; Xu et al., 2008; Zhou et al., 2008; Houbracken and Bouwens, 2010). Despite remaining open questions about the mechanisms of alpha- to beta-cell conversion, it has been demonstrated that the process occurs and that it can be rapid and robust. (Borowiak, 2010; Chung and Levine, 2010).

1.2.2.4 Mesoderm

Endothelial cells (ECs) are widely considered to be the active cellular component in new vessel formation by both angiogenesis and vasculogenesis, followed by contractile

cells such as pericytes and smooth muscle cells. The best known sources providing these cell types or their progenitors are ECs lining the vessel lumen and bone marrow. As easily evident, the vessel wall was recognized as being a passive player to a great extent except ECs of the vascular intima. Particularly, the vascular adventitia has been considered as a passive layer rather than an active part of the vessel wall. But results provided during the last few years have led to a revision of this classical view because of an apparent stem cell niche function of the vascular adventitia (Ergun et al., 2011).

In recent years, study of the muscle stem cell (MuSC) has been revitalized because of several provocative reports of potential plasticity of function within the heterogeneous MuSC population that can participate in processes ranging widely from hematopoiesis to osteogenesis, adipogenesis, and myogenesis (Shadrach and Wagers, 2011). The canonical MuSC in adult animals, designated satellite cell, was anatomically defined in 1961 by transmission electron microscopy (TEM) studies of the peripheral region of muscle fibers in the tibialis anticus muscle of the frog (Cooper and Konigsberg, 1961). The discovery of the satellite cell heralded the birth of the field of muscle regeneration. Satellite cells have a high ratio of nucleus to cytoplasm and are intimately juxtaposed to muscle fibers; they are resident in their own membrane-enclosed compartment, between the sarcolemma of the myofiber and the surrounding basal laminal membrane (Goldring et al., 2002). During the study of MuSCs and their role in muscle regeneration, an apparent functional and biochemical heterogeneity within the population has emerged. Differences between MuSCs isolated at different ages from distinct myofiber types, fast and slow, and from muscles with distinctive embryonic origins, the limbs versus the masseter, are characterized by distinct physiologic and biochemical phenotypes. The purpose of this complexity remains unclear. The observed heterogeneity within the MuSC population and the lack of conclusive evidence that satellite cells are the only

MuSC raise the possibility that other cells associated with muscle tissue also give rise to MuSCs. During the past 30 years, several reports have suggested that alternative sources of MuSC may include the thymus, dermis, vasculature, synovial membrane, and the bone marrow. A number of investigators have demonstrated that following a marrow transplant, bone marrow-derived cells (BMDCs) are present in diverse tissues in mice and humans, where they express characteristic tissue-specific proteins. These tissues include heart and skeletal muscle (Ferrari et al., 1998; Grounds et al., 2002; LaBarge and Blau 2002). These combined results suggest that repair of tissues, including skeletal muscle, may derive from cells other than tissue specific stem cells that derive from bone marrow. Taken together, these data provide strong evidence that cells within bone marrow can act as precursors to MuSCs, but their precise nature and origin remain to be determined. In this scenario, there may be a progenitor of satellite cells residing outside of the satellite cell niche. Such a progenitor could be a multipotent stem cell able to give rise to several differentiated cell types including satellite cells. Candidates for this resident “stem cell of satellite cells” include endothelial associated cells (De Angelis et al. 1999), interstitial cells (Tamaki et al. 2002; Poleskaya et al. 2003; Kuang et al. 2006), and side population (SP) cells (Gussoni et al. 1999; Asakura and Rudnicki 2002). Mesonagioblasts, stem cells derived from the developing vasculature (Minasi et al. 2002), are able to rescue α -sarcoglycan $2/2$ adult muscle (Galvez et al. 2006) and also occupy the satellite cell niche after transplantation (Sampaolesi et al. 2003). Satellite cells become dysfunctional not only because of exhaustion of their replicative capacity but also because of changes in their microenvironment imposed on them because of disease and aging. Complex mechanisms are likely to dictate how the milieu of factors in a MuSC niche might affect the recruitment and contribution of circulating cells to the MuSC population (Collins et

al., 2005). Thus, the challenge for the next few years will be to understand the role played during skeletal muscle regeneration by circulating cells, vascular associated cells, and cells from other non muscle tissues. Even if cells that originate from these sources do not play a significant role in normal muscle repair, their further study will illuminate the range of possible outcomes for those cells. Perhaps an understanding of the factors that govern their behavior will lead to information that is ultimately useful for developing new biomedical treatments of myopathies (Conboy 2003, Conboy 2005).

In mammals, the best understood niche is that harboring bone marrow hematopoietic stem cells (HSCs). Recent studies have expanded the number of cell types contributing to the HSC niche. Perivascular mesenchymal stem cells and macrophages now join the previously identified sinusoidal endothelial cells, sympathetic nerve fibers, and cells of the osteoblastic lineage to form similar, but distinct, niches that harbor dormant and self-renewing HSCs during homeostasis and mediate stem cell mobilization in response to granulocyte colony-stimulating factor (Ehninger and Trumpp, 2011).

Blood cells are continuously produced throughout our lifetime from rare pluripotent bone marrow stem cells, called hematopoietic stem cells (HSCs). HSCs are endowed with two characteristics: they give rise to additional HSCs through self-renewal and also undergo differentiation to progenitor cells that become variously committed to different hematopoietic lineages (Weissman 2000). Operationally, HSCs are best described as those cells capable of reconstituting the hematopoietic system of a recipient individual. Indeed, this defining *in vivo* property forms the basis of bone marrow transplantation, which was first developed as a lifesaving clinical procedure nearly a half century ago. Hematopoiesis takes place through the step-wise differentiation of multipotent HSC to generate a hierarchy of progenitor populations with progressively restricted developmental potential, none of which can dedifferentiate or show self-renewal

capacity. This leads to the production of multiple lineages of mature effector cells. The same methodology used for the prospective isolation of mouse HSC (testing the downstream production of various purified marrow derived populations) has been utilized to identify and isolate HSC-derived lineage restricted progenitors. Downstream of the multipotent progenitor (MPP), a lineage potential decision is made, and differentiation occurs via either the common myeloid progenitor (CMP) or the common lymphocyte progenitor (CLP), each of which has been isolated to homogeneity, and each expresses a distinct gene expression profile (Metcalf, 1989). and is a clonal precursor of a limited subset of progeny. The CD34 molecule, identified in 1984 (Strauss et al., 1984), is recognized as a major positive marker for human hematopoietic stem and progenitor cells. CD34 is expressed on a heterogenous population of cells which make up approximately 0.5–5% of human hematopoietic cells in human fetal liver, cord blood, and bone marrow (Krause et al., 1996).⁴¹ The function of CD34 on hematopoietic cells, however, is not well understood. On non-hematopoietic tissues, CD34 is expressed on endothelial cells of small vessels and is a ligand for L-selectin (CD62L) (Fina et al., 1990). In murine models, CD34 plays a role in adhesion to the stromal microenvironment (Healy et al., 1995), although CD34 mutant mice show no significant abnormalities, and consequently it does not appear to be essential for hematopoiesis. A small fraction (1–10% of CD34⁺ cells or 0.05–0.1% of human fetal bone marrow) contains multipotent hematopoietic precursors which are lineage negative for cell markers found on mature cells (CD3, CD4, CD8, CD19, CE20, CD56, CD11b, CD14 and CD15) and contains cells which, in vitro, will derive lymphoid (B and NK cells), and myeloid differentiation (Baum et al., 1992). HSC, while predominantly residing in the marrow, are in a constant state of migration between the marrow and the bloodstream. In mice, approximately 100 HSC are in transient flux (with less than 5

minutes of residence time in the blood) between the marrow and the circulation at any one time, which equates to approximately 30,000 HSC circulating through the blood each day. Parabiosis experiments (in which two mice are sutured together such that their vascular system undergoes anastomosis) have demonstrated that HSC from one partner mouse will leave the marrow, circulate through the blood and home to the marrow of the paired mouse (Wright et al., 2001; Abkowitz et al., 2003). The presence of HSC in the blood was initially discovered when clinical researchers described increased hematopoietic activity in the blood of patients treated with cytotoxic drugs (classically cyclophosphamide) (Richman et al., 1976). Cytotoxic drugs alone, or in combination with cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF subsequently, was shown to mobilize hematopoietic cells into the bloodstream (Siena et al., 1989). HSC were demonstrated to be present in high numbers in mobilized peripheral blood (MPB) as transplantation into lethally irradiated recipients led to rapid and sustained hematopoiesis, initially in murine models, and later as translated to the marrow transplant ward. Mobilization appears to occur through HSC proliferation and division (which occurs only within the marrow), followed by emigration of HSC into the blood spleen and liver (Morrison et al., 1997). The role of hematopoietic stem cells is central to the biological basis of bone marrow transplantation. Mobilized peripheral blood harvests are now used in the majority (>80%) of autologous transplants and in recent years for increasing numbers of allogeneic transplants. Cord blood, like mobilized peripheral blood contains a high frequency of HSC and hematopoietic progenitor cells (Broxmeyer et al., 1992) and has become an appealing alternative source of HSC for patients undergoing (Ringden and Le Blanc, 2005).

The presence of mesenchymal progenitor cells within the bone marrow has been documented from the late nineteenth century by the works of (Goujon, 1869), who was the first to show the osteogenic potential in heterotopic transplants of rabbit marrow, and which was later confirmed in transplantation experiments by (Baikow, 1870). Danis (Danis, 1960) showed that whole marrow itself was osteogenic, and not just an inductive factor or chemoattractant for osteogenic cells, by placing marrow within a diffusion chamber (two semipermeable membranes separated by a plastic ring), implanting this within a host animal, and observing the contents by histological methods. Similar confirmatory experiments were conducted by other groups (Petrakova et al., 1963; Friedenstein et al., 1966; Bruder et al., 1990), wherein the formation of cartilage and bone within the diffusion chamber was observed, demonstrating that bone marrow had, at a minimum, the potential to form bone and cartilage. Friedenstein and co-workers (Friedenstein, 1973) showed that the osteogenic potential of bone marrow was a feature of a specific subgroup of cells termed the CFU-f, which made up a very small percentage of the total marrow cell population. Friedenstein (Friedenstein, 1980) later showed that CFU-fs were formed from single cells and that some of these CFU-fs were able to form both bone and the microenvironment necessary for the formation of hematopoietic elements. These cells that form a hematopoietic microenvironment (Friedenstein et al., 1974; Trentin, 1976) can be considered a third phenotype for which the bone marrow has the potential to differentiate into, although other laboratories have categorized bone marrow stromal cells (hematopoietic supportive cells) as early osteogenic progenitors, a debate that has yet to be resolved. The first formal presentation of the concept of a stem cell residing in bone marrow was in a publication by Owen (Owen, 1978), wherein the marrow stroma was hypothesized to consist of a lineage analogous to the hematopoietic lineage (Till and McCulloch, 1980). Owen

(Owen, 1985) expanded on this hypothesis and proposed a model for the stromal lineage that contained “stem cell,” “committed progenitor,” and “maturing cell” compartments, and included a lineage diagram for “stromal stem cells” that included “reticular,” “fibroblastic,” “adipocytic,” and “osteogenic” cells as end-stage phenotypes. In 1991, Caplan (Caplan, 1991) proposed the existence of an mesenchymal stem cell (MSC) having the capacity to differentiate into multiple mesenchymal phenotypes including adipose, tendon, ligament, muscle, and dermis (Caplan, 1994). MSCs have an essential role for the differentiation of all the mesenchymal tissues of an organism. During limb development, the primitive limb bud first forms a cartilage anlage from condensing mesenchymal cells. Bone then forms around the cartilage anlage at the middiaphysis and expands bidirectionally toward the distal and proximal epiphyses. Just at the time that bone is forming at the middiaphysis, the cartilage beneath the bone becomes hypertrophic, and soon after the initial bone matrix has mineralized, the cartilage of the middiaphysis is invaded by vasculature (Pechak et al., 1986). The invading vasculature infiltrates the region once occupied by hypertrophic cartilage and eventually the entire cartilage anlage is replaced by vasculature and marrow elements. Hematopoietic stem cells migrate to the nascent bone marrow from their preceding embryonic location within the liver. The MSCs in bone marrow can arrive by three different mechanisms: (1) They can enter along with the vasculature; (2) they can migrate into the space after vascularization along the vessel paths, i.e., from the periosteum, which has documented multipotentiality (Nakahara et al., 1992; Yoo and Johnstone, 1998); or (3) they can arrive via the blood proper, indicating the existence of a circulating MSCs. This mechanism of MSC migration or circulation has important implications for adult tissue repair in that it may be possible to deliver reparative MSCs via the circulation as opposed to only localized applications, especially if MSC docking

sites exist on the endothelium lining the vascular network. Pericytes are cells that are closely associated with capillaries (Rouget, 1873), which express smooth muscle markers (Meyrick et al., 1981; Herman and D'Amore, 1985) and have the potential to differentiate into osteoblasts (Brighton et al., 1992). Although often categorized as being a cell type distinct from that of smooth muscle cells, some data indicate that pericytes have the potential to differentiate into smooth muscle cells (Meyrick et al., 1981). The pericytes of the periosteum are one candidate cell type for the origin of marrow MSCs because they are in the correct anatomical region for migration into the nascent marrow space, and also because pericytes have been shown to have the potential to differentiate into chondrocytes and adipocytes (Rhodin, 1968; Doherty et al., 1998). The role of MSCs within the marrow of an adult organism can be as varied as the phenotypes that MSCs can express. MSCs within marrow also function as a reservoir of stem cells for the repair of bone fractures and for the natural turnover of bone. The sequence of cellular events in fracture repair includes the formation of a clot, migration of macrophages and "mesenchymal" cells into the site, formation of cartilage in the fracture callus, and the bridging of the fracture via endochondral ossification (Ham and harris, 1971). The MSCs are the cells that form both the cartilage callus and the bone that bridges the repair site. MSCs also play an osteogenic role in normal bone remodeling or turnover. A more controversial role for MSCs has to do with the expression of the adipocyte phenotype. A number of laboratories have documented the ability of MSCs to differentiate into adipocytes, but the precise role of adipocytes within the marrow remains unknown. There is evidence that rat bone marrow adipocytes produce an uncoupling protein that is consistent with marrow adipocytes functioning in the regulation of heat (Marko et al., 1995). Another theory is that adipocytes serve as an expandable and retractable space-regulatory cell within the

confines of the bone marrow (Tavassoli, 1974). MSCs were first described as rapidly adherent, non-phagocytic clonogenic cells capable of extended proliferation in vitro (Friedenstein et al., 1968; Friedenstein et al., 1970; Castro-Malaspina et al., 1980). Assays of colony forming units (CFU-F) from aspirates of human bone marrow yields colony numbers between 1–20/105 mononuclear cells plated (Simmons and Torok-Storb, 1991; Waller et al., 1995). The CFU-F in adult human BM is feeder cell-independent (Kuznetsov et al., 1997). A combination of plastic adherence and in vitro culture was first used to establish stromal cell cultures and the contaminating hematopoietic cells were subsequently removed by negative selection using antibodies to CD45, CD34, and CD11b (Baddoo et al., 2003). The stromal cells obtained were shown to exhibit osteogenic, adipose, and chondrogenic differentiation potential in vitro and expressed Sca-1, CD29, CD44, and CD106, but not markers of hematopoietic or vascular endothelial cells. Stromal progenitors in human fetal BM express CD34 but are distinguished from the majority of hematopoietic progenitors by their lack of CD38 and HLA-DR. Subsequent studies demonstrated that stromal progenitors were restricted to a subpopulation of CD34⁺CD38[–]HLA-DR[–] that lacked expression of CD50 (Gronthos et al., 1994). The STRO-1 antibody, which is non-reactive with the hematopoietic progenitors, results in a 10- to 20-fold enrichment of CFU-F in fresh aspirates of human bone among the heterogeneous population of adult human bone marrow mononuclear cells. In accordance with the previously described properties of the unfractionated STRO-1⁺ population, STRO-1 bright VCAM-1⁺ cells assayed at the clonal level, exhibited differentiation into cells with the characteristics of adipose, cartilage and bone cells in vitro and formed human bone tissue following transplantation into immune deficient SCID mice (Gronthos et al., 1994). Collectively, these data strongly suggest that primitive stromal precursors, including putative stromal stem cells with the capacity

for differentiation into multiple mesenchymal lineages, are restricted to the STRO-1+ fraction in adult human bone marrow (Gronthos et al., 2003; Dennis et al., 2002). MSC enrichment could also be accomplished using several markers, including Thy-1, CD49a, CD10, Muc18/CD146, and in accordance with their response to growth factors, antibodies to receptors for PDGF and EGF (Simmons et al., 1994; Filshie et al., 1998). Additional antibodies that identify human bone marrow have been described but have not yet been able to be verified by other groups (Haynesworth et al., 1992). After harvest of bone marrow, culture adherent cells are expanded and subcultured to increase the number of pluripotent cells. MSCs derived from human bone marrow have been shown to retain their undifferentiated phenotype through an average of 38 doublings, resulting in over a billion-fold expansion (Bruder et al., 1997). The first and most obvious use of MSCs is in the area of bone regeneration in sites where the body cannot organize this activity, such as in nonunions. Critical size defects in nonunion models have shown that culture-expanded marrow MSCs in a porous, calcium phosphate, ceramic delivery vehicle are capable of regenerating structurally sound bone, where whole marrow or the vehicle alone cannot satisfactorily accomplish this repair. These preclinical models include the use of human MSCs in a femoral nonunion model in an immuno-compromised rat that will not reject the human cells (Goldberg and Caplan, 2004). MSCs represent a useful, easily obtained, characterized cell population to explore mesenchymal tissue regeneration, and there is good evidence to suggest the cells can be used allogeneically. The hMSCs do express small amounts of the major histocompatibility complex (MHC) class I molecule but express little or no MHC class II or B7 costimulatory molecules. In vitro experiments with lymphocytes from unrelated donors suggest that hMSCs do not elicit proliferation of T cells and may actually suppress a mixed lymphocyte reaction, suggesting the potential for allogeneic use of

hMSCs. The lack of a pronounced immunological response to implanted allogeneic hMSCs and the ability to produce large numbers of cells from a small marrow aspirate open the potential to use donor-derived cells for multiple recipients. Thus, MSCs are considered a readily accepted source of stem cells because such cells have already demonstrated efficacy in multiple types of cellular therapeutic strategies, including applications in treating children with osteogenesis imperfecta (Horwitz et al., 2002), hematopoietic recovery (Koc et al., 2000), and bone tissue regeneration strategies (Petite et al., 2000). Recent studies, showed that MSCs can be isolated from several tissue other than bone marrow, including peripheral blood (Zvaifler et al., 2000), cord blood (Erices et al., 2000), cord Wharton's jelly (Sarugaser et al., 2005), adipose tissue (Zuk et al., 2002), amniotic fluid (In't Anker et al., 2003), compact bone (Guo et al., 2006), periosteum (Nakahara et al., 1991; De Bari et al., 2001a; De Bari et al., 2006), synovial membrane (De Bari et al., 2001b; De Bari et al., 2003) and synovial fluid (Jones et al., 2004), articular cartilage (Dowthwaite et al., 2004) and foetal tissues (Campagnoli et al., 2001; Miao et al., 2006). Cells derived from different tissues show phenotypic heterogeneity and different growth abilities, but they also show similarities, with the potential to differentiate into the classical mesenchymal lineages and the expression of common surface markers (Baksh et al., 2007). However, there is increasing evidence that marked differences exist in the biology of MSCs that are dependent on the tissue of origin, which appears to be the main source of variation in the biological properties of MSCs (De Bari et al., 2008). Within each tissue source, single-cell-derived clonal MSC populations are known to be highly heterogeneous in their proliferative and differentiation potential (Phinney and Prockop, 2007; De Bari et al., 2008). The resulting variability limits standardization of MSC-based bone repair strategies and impedes the comparison of clinical study outcomes. Understanding the in

vivo MSC niches and their molecular regulation in health and disease is therefore of the utmost importance for the development of novel pharmacological approaches to tissue repair by targeting endogenous stem cells and niches and their regulatory reparative signalling networks.

In 2001–2002, several studies demonstrated that while attempting to select and culture mesenchymal stem cells (MSCs) from human and subsequently mouse and rat bone marrow (BM), was accidentally identified a rare population of cells that has characteristics unlike most adult somatic stem cells in that they appear to proliferate without senescence and have pluripotent differentiation ability in vitro and in vivo (Sohni and Verfaillie, 2011). Multipotent Adult Progenitor Cells (MAPCs) can be cultured from human, mouse, and rat bone marrow (BM). Unlike MSC, MAPCs do not express major histocompatibility (MHC)-class I antigens, do not express or express only low levels of the CD44 antigen, and are CD105 (also endoglin, or SH2) negative. Unlike hematopoietic stem cells (HSC), MAPCs do not express CD45, CD34, and cKit antigens, but like HSC, MAPCs express Thy1, AC133 (human MAPC), and Sca1 (mouse), albeit at low levels. In the mouse, MAPCs express low levels of stage-specific embryonic antigen (SSEA)-1 and low levels of the transcription factors Oct4 and Rex1, known to be important for maintaining embryonic stem (ES) cells undifferentiated and known to be down-regulated when ES cells undergo somatic cell commitment and differentiation. Rodent MAPC differentiate into cells of all the three germ layers (Jiang et al., 2002; Ulloa-Montoya et al., 2007). MAPC differentiate robustly to endothelium and smooth muscle making up the components of blood vessel. Aranguren et al. demonstrated that MAPC can be differentiated to both venous and arterial endothelial cells (Aranguren et al., 2007) which was confirmed further by Liu et al. and Xu et al. (Liu et al., 2007; Xu et al., 2008). In a functional analysis study, Aranguren et al. also

demonstrated that undifferentiated MAPC could restore muscle function as well as blood flow in a limb ischemia model in part by direct contribution to smooth muscle and endothelium, as well as skeletal muscle, and in part by elaborating trophic factors that enhance endogenous angiogenesis and myogenesis (Aranguren et al., 2008). Ross et al. demonstrated that MAPC when treated with TGF- β and PDGFbb under serum free conditions can form smooth muscle cells (SMC) (Ross et al., 2006). Serafini et al. demonstrated that when mouse MAPC were injected into lethally irradiated mice, reconstitution of the hematopoietic system could be seen, with generation of HSC that could repopulate secondary recipients (Serafini et al., (2007). Maes et al. demonstrated that like MSC, MAPC can generate osteoblasts, which is enhanced by placenta-like growth factor (Maes et al., 2006). MAPC have been instrumental in development of robust protocol (Roelandt et al., 2010) for differentiation to functional hepatocyte-like cells. This protocol is not only applicable for differentiation of MAPC to liver cells, but also can be adapted for mouse and human ESC (Roelandt et al., 2010). Roelandt et al. have demonstrated that by mimicking early embryonic developmental cues that MAPC and ESC can be differentiated to functional hepatocyte-like cells. Jiang et al. in 2002 demonstrated that rat and mouse MAPC can differentiate to the neuroectodermal lineage, generating neurons with electrophysiological properties similar to CNS neurons (Jiang et al., 2002). More recently, differentiation toward neural stem cell-like cells has been achieved using a modified Conti et al. protocol (Conti et al., 2005), although maturation to neurons and astrocytes has not yet been achieved (Ulloa-Montoya et al., 2007). Human MAPC can be isolated and expanded long-term (more than 70 population doublings), and unlike MSC can robustly generate endothelial cells (Reyes et al., 2002; Aranguren et al., 2007). Compared with rodent MAPC human MAPC are cultured without LIF, but like rodent MAPC with FCS, EGF and PDGF-BB. Like MSC they

have extensive immunomodulatory properties *in vitro* and *in vivo* (Kovacsovics-Bankowski et al., 2009; Van't Hof et al., 2007). When grafted *in vivo* in ischemia models, human MAPC like rodent MAPC, significantly increase angiogenesis and endogenous stem cell proliferation, leading to improved CNS (Walker et al., 2010), cardiac (Van't Hof et al., 2007) and skeletal muscle function (Aranguren et al., 2008).

1.3 Molecular bases of pluripotency

In many cases, the culture of adult stem cells has been complicated by the lack of knowledge of their cellular environment or niche. In addition, they might require complicated three-dimensional arrangements of specific stromal cells in order to proliferate. In contrast, Embryonic Stem (ES) cells are relatively easy to be technically handled. The establishment of the first murine embryonic stem lines was achieved by culturing early embryos on a layer of mitotically inactivated mouse fibroblasts. Without such a “feeder” layer, cultured embryonic cells would not remain pluripotent, suggesting that fibroblasts either promote self-renewal or suppress differentiation, or both (Evans and Kaufman, 1981; Martin, 1981). Fibroblasts maintain pluripotency of ES cells by secreting a factor, which was identified as leukemia inhibiting factor (LIF), also known as differentiation inhibiting activity (DIA) (Williams et al., 1988). LIF is a member of the interleukin-6 family of cytokines, including IL-6, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1). The IL-6 family cytokines are structurally and functionally related. They act on a variety of cells (i.e., they are pleiotropic) and can mediate proliferation or differentiation or both according to the target cell types. The receptors involved in the IL-6 family cytokine signaling cascade belong to the cytokine receptor class I family. The extracellular domain of all

members of cytokine receptor class I family is composed of a variable number of fibronectin type III modules. Two of the fibronectin modules are conserved among all members of the family and constitute the cytokine-binding module. The cytoplasmic domain of the receptor contains three conserved motifs, called box 1, box 2, and box 3 in a membrane proximal to distal order, and lacks intrinsic kinase activity. These three subdomains are responsible for transmitting the extracellular signal into the cytoplasm. Binding of the IL-6 family cytokine to their cognate receptors leads to homodimerization of gp130 or heterodimerization of gp30 with the cytokine cognate receptor. For example, IL-6/IL6R and IL-11/IL-11-R complexes induce gp130 homodimerization. Described receptor complexes share gp130 as the common component critical for signal transduction, which explains the observed redundancy in cytokine functions (Taga and Kishimoto, 1997; Ernst and Jenkins, 2004). Although gp130 is widely expressed in various tissues, ligand-specific receptor components display a more restricted expression. LIFR, OSMR, and CTNFR are expressed in ES cells; consequently, CT-1, OSM, and CNTF and LIF are interchangeable in preventing ES cell differentiation and supporting ES cell derivation and maintenance of ES cells in culture (Boeuf et al., 1997). Homo- or heterodimerization of gp130 results in the activation of receptor-associated kinases of the Janus family Jak1, Jak2 and Tyk2. These tyrosine kinases constitutively interact with the conserved regions box 1 and box 2 of gp130. Activated Jaks phosphorylate specific tyrosines on the intracellular domain of gp130, creating docking sites for the recruitment of SH2 proteins to the activated receptor complex. When gp130 is phosphorylated, several signaling pathways are activated, involving STAT1 and STAT3, the SH2-domain containing tyrosine phosphatase (SHP2), ERK1 and ERK2 (extracellular signal receptor kinases or mitogen-activated kinases (MAPK), growth-factor receptor-bound protein (Grb) 2,

Grb2-associated binder protein (Gab) 1, and phosphatidylinositol-3 kinase (PI3K) (Stahl et al., 1995; Heinrich et al., 1998). Identification of STAT3 as a key determinant of ES renewal came from the elegant studies (Niwa et al., 1998; Matsuda et al., 1999). STAT 1 and 3 are only two of the downstream effector molecules induced by cytokine signaling via gp130. LIF treatment of ES cells increases MAP kinase activity and induces phosphorylation of ERK1 and ERK2. The bridging factor between cytokine receptor and MAP kinase is the widely expressed tyrosine phosphatase SHP-2. Enhanced ES self-renewal is observed when either a catalytically inactive SHP-2 is overexpressed or ERK phosphorylation is chemically blocked. These results indicate that SHP-2 and ERK activation is not required for the maintenance of self-renewal signaling, but rather they inhibit it (Burdon et al., 1999; Jirmanova et al., 2002; Humphrey et al., 2004). Elucidation of the molecular basis of a pluripotency is based primarily on the identification of the key transcription factors involved in regulating gene expression at the pluripotent “state.” In a very simplistic model, genes that promote and maintain an undifferentiated cellular state would be expressed in pluripotent cells, while those activated during stem cell differentiation would be repressed. Differentiation stage at which the gene undergoes zygotic activation result in high Oct4 protein levels in the nuclei of all blastomeres until compaction. Oct4 is also expressed in undifferentiated mouse embryonic stem (ES), embryonic germ (EG), and embryonic carcinoma (EC) cell lines. ES and EC cell treatment with the differentiation-inducing agent retinoic acid (RA) induce rapid Oct4 down-regulation in both cell types. Oct4 is not expressed in differentiated tissues. Oct4 is also highly expressed in ICM cells relative to TE cells in discarded human embryos (Niwa et al., 2000; Pesce and Scholer, 2001). Recent reports have highlighted the importance of another player on the stage of pluripotency. Nanog, a divergent homeobox factor, is expressed in vivo in the

interior cells of compacted morulae, in the ICM or epiblast of a preimplantation blastocyst, and in postmigratory germ cells. In vitro Nanog is a marker of all pluripotent cell lines. Nanog-deficient embryos die soon after implantation due to a failure in the specification of the pluripotent epiblast, which is diverted to endodermal fate. Similarly, Nanog deletion in ES cells causes differentiation into parietal/visceral endoderm lineages. Nanog overexpression renders ES cells independent from LIF/STAT3 stimulation for selfrenewal. LIF and Nanog have an additive effect on the propagation of undifferentiated versus differentiated colonies. The differentiation potential of ES cells overexpressing Nanog is both reduced and retarded, but removal of Nanog transgene reverses the cells' status to that of the parental stem cell. Nanog expression does not seem to be regulated by Oct4, but the two homeo-factors work in concert in order to maintain a pluripotent phenotype (Mitsui et al., 2003; Chambers et al., 2003). Epigenetic modifications, including CpG methylation and histone modifications, regulate gene transcription and are also important in the maintenance of pluripotency. The methylation pattern of Oct3/4 and Nanog is highly comparable with their expression pattern, and the methylation would suppress their transcription in differentiated cells (Gidekel and Bergman, 2002). In vitro fusion with ES cells or treatment with demethylating agent, 5-azacytidine, could erase, at least in part, epigenetic status of differentiated cells (Tada et al., 2001; Tsuji-Takayama et al., 2004). In addition to the factors discussed, it is highly likely that other factors and pathways are also involved, including miRNA (Suh et al., 2004). Recently and surprisingly, was discovered that pluripotency can be regained by numerous differentiated somatic cell types through overexpressing transcription-factor-encoding genes. Yamanaka and colleagues showed (Takahashi and Yamanaka, 2006; Yamanaka, 2007) that a combination of only four transcription factors could generate ES-cell-like pluripotent

cells from mouse fibroblasts. The authors identified OCT4, SOX2, KLF4 and c-MYC as key factors that sufficed to induce pluripotency in fibroblasts and they named these pluripotent cells iPS cells. These cells, are the strongest example so far of the plasticity of cells in response to a disruption in the stoichiometry of their transcriptional regulators. Human iPS cells can be used to generate cells for tissue repair or replacement while avoiding the ethical and immunological issues that are inherent in the use of ES cells. Furthermore, because these cells can be derived from a patient's own cells, they give researchers the ability to model human diseases and screen drug candidates in vitro in an unprecedented manner (Inoue and Yamanaka, 2011; Okita and Yamanaka, 2011).

1.4 From Stem Cells to Functional Tissue Architecture

The precise coordination of cell growth and differentiation that is necessary to create functional tissue architecture requires that each cell correctly interpret multiple temporal and spatial cues from its microenvironment. Great focus has been placed on the identification of signals that determine long-range patterning, such as the anterior–posterior axes of the embryonic body plan or the proximal–distal axes in limbs (Wolpert, 1996). At this level, concentration gradients of soluble regulator molecules – morphogens – are thought to convey the positional information that regulates each cell's fate (Gurdon and Bourillot, 2001). The cellular response to these signals would, in turn, control overall organ position, size, and shape. To generate structures that exhibit localized bending, budding, or branching, one cell or a small group of cells has to divide to expand tissue mass, while its immediate neighbors must remain quiescent and differentiated. This spatial heterogeneity of cell fate control is the basis for creating and

preserving micro-anatomical patterns that are characteristic of all tissues. Thus, individual stem cells that are responsible for replenishing lost cells and maintaining stable tissue form have to continuously monitor their environment and make their cell fate decisions based on local cues in addition to global information. Advances in cell and molecular biology as well as tissue engineering have shed light on the nature of these local cues. A central notion is that “solid-state” signals play a regulatory role in the cell fate decision, in addition to soluble growth factors and cytokines. These structural signals include adhesive cues from cell binding to extra-cellular matrix (ECM) and to other cells as well as mechanical cues that come from physical distortion of the cells or their ECM. In other words, both molecularly encoded information (e.g., the specific 3D structure of receptor ligands) and “nonspecific” physical factors devoid of genetic information impinge on the cell’s internal signaling network to govern when and where it is appropriate to divide, differentiate, or die. Much of the work done in the field, has been devoted to elucidating the molecular targets and establishing the biochemical link between input signal and cellular response, based on the concept of instructive regulation (Brivanlou and Darnell, 2002). Extra-cellular informations include signals that depend on appropriate mechanical signals from the cytoskeleton (Huang and Ingber, 2002). In other words, non-specific cues, such as mechanical distortion of cells, can harness the same molecular machinery responsible for cell cycle regulation as molecular growth factors. Rules of cell behavior are well known in developmental biology, which takes a supracellular, rather than subcellular, view of cell fate decisions. From this perspective, regulation of cell behavior is a selective rather than an instructive process (Wolpert, 1994). In a given tissue context, cells have only a limited number of behavioral programs or cell fates available to choose from. The regulatory cues simply serve to activate endogenous mechanisms that select among

these different fates within the cell's behavioral repertoire. Thus, exit from the proliferative state is often directly coupled to entry into the differentiation program; the cell does not need a specific instruction for how to differentiate. For instance, removal of serum triggers differentiation of precursor cells into different types of mature postmitotic cells, such as myocytes, adipocytes or neurons (Harrison et al., 1985; Olson, 1992; Brüstle et al., 1999). Adult cell differentiation models similarly reveal the intrinsic robustness of cell fates, underscoring the model of selective regulation. A frequent observation is that differentiation can be induced by a multitude of reagents that do not necessarily need to be specific differentiation factors. For instance, various nonspecific chemicals that do not act instructively by binding high-affinity receptors, such as dimethylsulfoxide, ethanol, methanol, sodium butyrate, or ions, can induce the same terminal differentiation as do physiological signals. This has been described for the differentiation of various cells, including myeloid, neuronal, and cartilage precursor cells (Boyd and Metcalf, 1984; Spremulli and Dexter, 1984; Langdon and Hickman, 1987; Messing, 1993; Newmark et al., 1994; Yu and Quinn, 1994; Waclavicek et al., 2001). Conversely, in cell fate control by physiological chemicals that act on specific pathways, such as retinoic acid (RA) or cyclic adenosine monophosphate (cAMP), the molecules do not actually specify the destination of differentiation but, instead, can cause differentiation in many different tissues. These differentiating agents carry no specific instructions, as the destination cell fate is "programed" in the state of the precursor cell. Thus, the product of the switch depends on the existing cell state and on environmental factors (Voorhees et al; 1973; Deshpande and Siddiqui, 1976; Waymire et al., 1978; Breitman et al., 1980; Giuffre et al., 1988; Linney et al., 1992; Jiang et al., 1997; McCaffery and Drager, 2000; Hansen et al., 2000). This idea of selection of preexisting, intrinsically robust fates was actually captured in Waddington's epigenetic

landscape, a model for channeling in development that was first proposed more than 60 years ago (Stern, 2000). The robustness of developmental processes in generating particular macroscopic 3D structures suggests that during the formation of such complex structures by coordinated cell fate switches, the cells have to continuously reinterpret signals from the physical world, such as mechanical forces to which the tissue is exposed. This feedback control is essential in allowing the organism to form the higher-level structures that are subjected to the laws of macroscopic mechanics (Chicurel et al., 1998; Huang and Ingber, 2000). In this scenario, the cell–ECM interaction has two components: a chemical component, which is mediated by the ligand-induced activation of integrins and associated biochemical signaling cascades, and a mechanical component, which is mediated by integrin’s role in physically resisting cell-generated tractional forces (Ingber and Jamieson, 1985; Ingber and Folkman, 1989). This latter structural effect of ECM binding to integrins is responsible for the rearrangement of the actin cytoskeleton and ensuing cell shape changes that are observed in response to cell adhesion to the ECM. Cell distortion may represent a fundamental control element for switching between cell fates. For example, when cells are forced onto even smaller size islands so that they almost round up, they undergo apoptosis even though they still remain attached to the ECM and receive growth factor stimulation, which normally would induce proliferation (Chen et al., 1997). Interestingly, cells cultured on an intermediate island size, such that they neither divide nor die, can be triggered to differentiate. Endothelial cells cultured under these conditions form 3D capillary tubes (Dike et al., 1999) and hepatocytes secrete blood proteins, such as albumin, at high levels (Singhvi et al., 1994). There is another solid-state factor that contributes significantly to control of growth and differentiation in the tissue microenvironment: cell-cell adhesion (Aplin et al., 1998). This environmental

input again has two components: one being physical (nonspecific) and the other molecular (specific). Physical cell–cell contacts modulate cytoskeleton changes and influence cell shape by modulating transfer of physical forces across the cell surface from other cells, much like integrins do for forces transmitted from ECM. Thus, restriction of cell spreading by high levels of cell–cell contact formation may influence cell fate much like prevention of cell spreading by experimental ECM islands (Folkman and Moscona, 1978). In addition, this contact inhibition or, more precisely, cell density–dependent inhibition is also mediated by specific cell-surface receptors that engage in high-affinity interactions and signal transduction. One of the best-studied classes of cell–cell adhesion molecules is the cadherin family of homophilic receptors. E-cadherin and N-cadherin have been shown to have a role in signal transduction in addition to mediating cell–cell adhesion in epithelium and neuronal cells, respectively (St Croix et al., 1998; Levenberg et al., 1999; Huang et al., 1998). In contrast to growth control, cell–cell interactions mediated by specific molecular binding events play a dominant role in the control of differentiation. Specifically, in asymmetric stem cell division (Wolpert, 1988), in which only one daughter cell switches into differentiation, distinct molecular signals on neighboring cells, such as the Notch signaling system, ensure the disparate cell fates of the two progenies. Besides the existence of distinct, mutually exclusive, robust cell fates, genome-scale characterization of cellular signaling reveals the existence of a cellular regulatory network that replaces the traditional pathway paradigm (Marcotte, 2001). Gene expression levels and activity values across all the proteins encoded, directly map into the cellular phenotype, or network state. With recent insights into the detailed dynamics of cell fate regulation in mammalian cells, a finer picture has emerged: cell fate switching, such as among growth, differentiation into distinct cellular phenotypes, and commitment to apoptosis, similarly obeys constrained

dynamics that are governed by underlying interactions of the cellular regulatory network, establishing a link between the observed selective nature of cell fate regulation and the molecular network of signal transduction. Regulatory inputs from the cell's environment, such as soluble factors, insoluble ECM, or mechanical forces are perturbations to the network that may cause cell fate switching (Huang, 2002). Thus, the challenge for the future is to understand how this complex network of interactions gives rise to a phenotypic landscape like that described by Waddington, and the associated prevalence of rule-like cell behavior.

1.5 Biomaterials and Tissue Engineering

The use of biomaterials did not become practical until the advent of an aseptic surgical technique developed by Dr. J. Lister in the 1860s. Earlier surgical procedures, whether they involved biomaterials or not, were generally unsuccessful as a result of infection (Norton et al., 2008). Problems of infection tend to be exacerbated in the presence of biomaterials, since the implant can provide a region inaccessible to the body's immunologically competent cells. The earliest successful implants, as well as a large fraction of modern ones, were in the skeletal system. Bone plates were introduced in the early 1900s to aid in the fixation of long bone fractures (Lane, 2009). Many of these early plates broke as a result of unsophisticated mechanical design; they were too thin and had stress concentrating corners. Also, materials such as vanadium steel which was chosen for its good mechanical properties, corroded rapidly in the body and caused adverse effects on the healing processes. Better designs and materials soon followed. Following the introduction of stainless steels and cobalt chromium alloys in the 1930s, greater success was achieved in fracture fixation, and the first joint replacement

surgeries were performed (Woo et al., 1984). As for polymers, it was found that warplane pilots in World War II who were injured by fragments of plastic (polymethyl methacrylate) aircraft canopy, did not suffer adverse chronic reactions from the presence of the fragments in the body. Polymethyl methacrylate (PMMA) became widely used after that time for corneal replacement and for replacements of sections of damaged skull bones. Following further advances in materials and in surgical technique, blood vessel replacements were tried in the 1950s; and heart valve replacements and cemented joint replacements in the 1960s. Recent years have seen many further advances (Nerem and Seliktar, 2001; Kuehn et al., 2005; Sales et al., 2005; Albes, 2010). The success of a biomaterials in the body depends on factors such as the material properties, design, and biocompatibility of the material used, as well as other factors not under control of the engineer, including the technique used by the surgeon, the health and condition of the patient, and the activities of the patient. Biocompatibility involves the acceptance of an artificial implant by the surrounding tissues and by the body as a whole. Biocompatible materials do not irritate the surrounding structures, do not provoke an abnormal inflammatory response, do not incite allergic or immunologic reactions, and do not cause cancer (Park, 2000).

1.5.1 Evaluation of biomaterials

The usefulness of mammalian cell cultures for biocompatibility testing is confirmed by experimental studies which found a good correlation between in vitro and in vivo tests. The rationale is that in vitro models employing human cells to study the interactions between the cell system and the biomaterial/device allow for a reasonable prediction of the performance in vivo of the biomaterial/device. It has to be acknowledged that

isolated cell systems are more sensitive to toxic materials than body tissues. Nevertheless, there is an increasing demand for reliable in vitro methods for two main reasons: (1) cellular mechanisms of toxicity can be described using biochemical assays; (2) valid alternatives to animal models are needed. Using such testing methods the materials can be screened according to their grade of toxicity and discarded, if this is the case, prior to further testing. A number of characteristics and functions of cells can be verified after challenge with biomaterials: morphology (Tamada and Ikada, 1994), membrane integrity (Bordenave et al., 1993), cytoskeleton (Naji and Harmand, 1990), surface molecules (Van Kooten et al., 1997), viability, proliferation (Rudolph et al., 1992), protein synthesis (Fronzoza et al., 1993), oxidative response (Shanbhag et al., 1992), motility (Remes and Williams, 1990), secretion (Papatheofanis and Barmada, 1991), response to growth factors and cytokines (Kieswetter et al., 1996), cell-cell interactions and gene expression (Sun et al., 1997; Chou et al., 1998). Adhesion of cells onto biomaterials is considered a positive phenomenon for implant outcome: such process is known to influence cell activities. Cell adhesion onto foreign surfaces is inspected mainly by cell count, while electron microscopic techniques or computer-assisted measurement are used for the assessment of spreading and morphology (Jansen et al., 1991; Knabe et al., 1997). Tamada (Tamada and Ikada, 1994) showed that not only the rate of adhesion and spreading of cells is conditioned by the presence of a collagen layer onto polymer surface, but the functions of cells are influenced. Ertel et al. (Ertel et al., 1994) were able to demonstrate that the bare surface of some polymers, including polydimethylsiloxane (PDMS), polyethylene (PE), poly(methyl methacrylate) (PMMA) and polyurethanes, was able to cause cell death, unless it was covered by a proteinaceous layer. The ability of surface topography to determine orientation and alignment of cells and to modify bone cell response has been demonstrated in several

studies (den Braber et al., 1995; Boyan et al., 1998). The 24 hours period is the endpoint commonly used for cytotoxicity testing, but in many instances the exposure may be prolonged or shortened. Tomas et al. tested bone-marrow derived cells for 7, 14, 21 days to look for proliferation and osteogenic differentiation in vitro (Tomas et al., 1997). On the other hand, the duration of contact with materials may be limited to 1–2 hours, as recommended for some types of dental materials which will be applied to oral mucosa for a few minutes (International Organization for Standardization (1995). The finding of clear toxicity for a material/device after a 24 hours testing in vitro could lead to its discard, due to high sensitivity of cell culture systems, although in vivo it is applied for few minutes. If this material/device has unique properties and optimum performance in vivo with short exposure, the benefit from its use could be higher than the risk for the patient (Ciapetti et al., 1998). The determination of the safety of a material and, subsequently, its efficacy as a device, will, of necessity, require implantation in animals after in vitro cytotoxicity assay. Such studies, if performed in the correct animal model, have proven to be directly applicable to humans. The phenomena of life-sustaining processes and the adjustments to disease are similar, if not identical, certainly throughout the class Mammalia, if not others. Most biological events, wound healing in particular, are also comparable. Only the magnitude and timing of specific features may differ. The American College of Laboratory Animal Medicine has sponsored several reference texts on spontaneous animal models of human disease (Andrews et al., 1979a; Andrews et al., 1979b), and the Armed Forces Institute of Pathology has developed a series of publications that summarize nearly 300 animal models (Armed Forces Institute of Pathology, 1972-1998). Several other publications list newly developed animal models, some of which may be applicable to

biomaterial technology (Fisher et al., 2002; Carpenter and Hankenson, 2004; Kruyt et al., 2004).

1.5.2 Cell culture systems for tissue engineering

One approach to tissue engineering involves the in vitro culture of cells on biomaterial scaffolds to generate functional engineered tissues for in vivo applications, such as the repair of damaged articular cartilage or myocardium. Scaffolds are defined as 3D porous solid biomaterials designed to perform some or all of the following functions: promote cell–biomaterial interactions, cell adhesion, and ECM deposition; permit sufficient transport of gases, nutrients, and regulatory factors to allow cell survival, proliferation, and differentiation; biodegrade at a controllable rate that approximates the rate of tissue regeneration under the culture conditions of interest; and provoke a minimal degree of inflammation or toxicity in vivo (Langer and Tirrell, 2004; Muschler et al., 2004; Hollister, 2005; Lutolf and Aubbell, 2005). Cell seeding of a biomaterial scaffold is the first step in tissue engineering and plays a critical role in determining subsequent tissue formation (Freed et al., 1994; Kim et al., 1998). The spinner flask system improved the efficiency and spatial uniformity of cell seeding throughout porous solid 3D scaffolds (e.g., fiber-based textiles and porogen-leached sponges) as compared with controls seeded statically (Vunjak-Novakovic et al., 1998). Convective mixing, flow, and mass transport are required to supply the oxygen, nutrients, and regulatory factors that are in turn required for the in vitro cultivation of large tissue constructs. Oxygen is the factor that generally limits cell survival and tissue growth, due to its relatively low stability slow diffusion rate and high consumption rate (Martin and Vermette, 2005). Regulatory factors can be used as culture medium supplements to

selectively induce differentiation and to enhance the growth, composition, and mechanical function of engineered tissues. The most fundamental, biologically inspired, approach to engineering tissues is to direct the organization of metabolically active cells into three-dimensional (3D) spatial arrangements (via hydrogels or porous scaffolds) and to establish the environmental conditions such that the cells are induced to reconstruct immature but functional tissues. In both in vivo (development/regeneration) and in vitro (tissue engineering) settings, the cues with which cells are presented are the principal determinants of the phenotypic nature of the resultant tissues. Bioreactors are the primary tools for mimicking the natural environments and provide cell-based constructs with physiologically relevant stimuli that facilitate and orchestrate the conversion of a collection of cells into a specific tissue phenotype. Tissue-engineering bioreactors are designed to precisely regulate the cellular microenvironment in order to facilitate construct uniformity and overall cell viability. Key requirements of the reactors include (i) increased cell-seeding efficiency into the 3D scaffold, (ii) improved mass transfer, (iii) adequate gas exchange to the bulk culture media, (iv) regular replenishment of spent medium, (v) temperature/pH control, and (vi) physiological stimuli (Vunjak-Novakovic et al., 2002; Depprich, R., et al., 2008; Gandaglia, A., et al., 2011).

1.5.3 Polymeric Scaffolds

The incorporation of polymeric scaffolds in tissue regeneration occurred in the early 1980s, and it continues to play a vital role in tissue engineering (Hutmacher , 2001). There are two main classes of polymers, based upon their source: natural or synthetic. Polymeric scaffolds may be used to support a variety of cells for numerous tissues

within the body. Again, there are two major classes of natural polymers used as scaffolds: polypeptides and polysaccharides. Natural polymers are typically biocompatible and enzymatically biodegradable. The main advantage for using natural polymers is that they contain bio-functional molecules that aid the attachment, proliferation, and differentiation of cells. However, disadvantages of natural polymers do exist. Depending upon the application, the previously mentioned enzymatic degradation may inhibit function. Further, the rate of this degradation may not be easily controlled. Since the enzymatic activity varies between hosts, so will the degradation rate. Therefore it may be difficult to determine the lifespan of natural polymers in vivo. Additionally, natural polymers are often weak in terms of mechanical strength but cross-linking these polymers have shown to enhance their structural stability. Agarose, Alginate, Hyaluronic acid and Chitosan are naturally occurring polysaccharides and are widely employed as scaffolds for cell culture. Agarose gels have been found to maintain chondrocytes, the predominant cell type in cartilage, in culture for 2 to 6 weeks. Furthermore, agarose hydrogels embedded with chondrocytes allow the expression of type II collagen and proteoglycans and have been investigated as a matrix for nerve regeneration (Dillon et al., 1998; Hung et al., 2003). Besides, a variety of different cells have been found to maintain their morphology and function in alginate scaffolds (Glicklis et al., 2000; Mosahebi et al., 2001; Wang et al., 2003). Toward hyaluronic acid, currently, an ester derivative of it has been used as a tissue engineering scaffold for culture of adipose precursor cells, mesenchymal progenitor cells and chondrocytes (Aigner et al., 1998; Halbleib et al., 2003). Also chitosan has been successfully employed as porous scaffold. It allowed growth of hepatocytes, osteoblasts and chondrocyte (Yagi et al., 1997; Nettles et al., 2002).

Collagen belongs to natural polypeptides class. It has features that allow its employment as a scaffold for cells within the epithelium, chondrocytes, hepatocytes. Furthermore, corneal keratocytes cultured on collagen sponges synthesized proteoglycans, indicating corneal extracellular matrix formation (Nehrer et al., 1998; Orwin and Hubel, 2000; Risbud et al., 2003). Gelatin, a water soluble collagen derivative acquired by denaturing the triple-helix structure of collagen into single-strand molecules, has been used to support cells for orthopaedic applications and human adipose derived stem cell attachment and differentiation into a variety of cell lineages (Payne et al., 2002; Awad et al., 2004). Polymers that are chemically synthesized offer several notable advantages over natural-origin polymers. A major advantage of synthetic polymers is that they can be tailored to suit specific functions and thus exhibit controllable properties. The most common synthetic polymers are polyesters. Poly(glycolic) acid polymers have been investigated as a scaffold to support various types of cell growth such as chondrocytes, myofibroblasts and endothelial cells, hepatocytes and for retinal pigment epithelium engineering (Freed et al., 1993; Shinoka et al., 1996; Kaihara et al., 2000; Lu et al., 2001). In recent investigations, results have shown that the hydrophobic surface of Poly(L-lactic) acid (PLLA) has resulted in decreased adhesion of cells compared to other types of polymers, such as PGA. However, PLLA has been found to support various nerve stem cells, human bladder smooth muscle cells and condrocytes (Ishaug-Riley et al., 1999; Pariente et al., 2002; Park et al., 2004). Poly(D,L-lactic acid-co-glycolic acid) (PLGA), Poly(ϵ -caprolactone) (PCL), and other synthetic polymers are under investigation to understand the possible role in tissue engineering application. Most polymeric scaffolds are designed to provide temporary support and, therefore to be biodegradable. Natural polymers mainly undergo surface degradation, since enzymes are generally too large to penetrate into the bulk of the scaffold. However, synthetic

polymers can degrade by surface, bulk, or both, depending on its composition (von Burkersroda et al., 2002).

1.5.4 Ca-P Scaffolds for Bone Repair

In reconstructive surgery, repair and regeneration of large bone defects is a major challenge. The reconstruction of large bone segments remains a critical clinical problem in the case of extensive bone loss due to pathological events such as trauma, inflammation, degeneration and surgical treatment of tumours (Jenis, 2006; De Long et al., 2007). For this purpose, several approaches have been used in attempts to fill defects and assist in subsequent regeneration, including autogenous and xenogenous bone grafting and synthetic biomaterials (Gazdag et al.; 1995). Due to its ideal biocompatibility and osteogenic properties, autogenous bone taken from a secondary surgical site has been widely utilised and is still considered the gold standard (Summers and Eisenstein, 1989; Aaboe et al., 1995). Its use has limitations, however, such as supply amount and unpredictable healing kinetics. In addition to this, donor site pain and potential post-surgical infections are common complications associated with such a procedure. These limitations and considerable recent progress in biotechnology have driven the development of synthetic materials/scaffolds engineered specifically for bone replacement applications (Devin et al., 1996). Over the past 10 years, attention has focused on the development of optimized synthetic or semi-synthetic substitutes for autogenous bone grafting. 1 commonly used grafting materials include allografts – such as demineralised bone matrix particles, de-proteinised cancellous chips – and synthetic alloplasts – such as calcium sulphate pellets and porous calcium phosphate materials. Synthetic materials are currently used in only 10% of orthopaedic procedures

worldwide, yet the progress and evaluation of these products demonstrates the prospect of rapid evolution. The class of ceramics used for the repair and replacement of diseased and damaged parts of musculoskeletal systems is termed bioceramics. Bioceramics have become a diverse class of biomaterials, generally consisting of three basic types: bioinert high-strength ceramics, bioactive ceramics that form direct chemical bonds with bone or even with the soft tissue of a living organism, and various bioresorbable ceramics that actively participate in the metabolic processes of an organism with predictable results. Alumina (Al_2O_3), zirconia (ZrO_2) and carbon are termed bioinert. Bioglass and glass ceramics are bioactive. calcium phosphate ceramics are considered to be bioresorbable and are currently the most promising biomaterials for medical applications. They are mainly used as implants in orthopaedics, oral-maxillofacial surgery, neurosurgery and for dental implants. Each potential substitute, including tissue engineering approaches with delivery of osteogenic cells or osteoinductive macromolecules, or both, is based on an appropriate scaffold biomaterial that is biocompatible, allows bone ingrowth and shows subsequent degradation of the material. Bone tissue is a living organ composed of an organic and an inorganic component. The inorganic bone mineral is composed of specific phases of calcium phosphate (Ca-P), especially carbonate rich hydroxyapatite (HA). Biological HA also contains other impurity ions as Cl, Mg, Na, K, and F and trace elements like Sr and Zn (LeGeros, 2002). Ca-P materials show high similarity to natural tissue, characterized by a high biocompatibility, the ability of direct bone bonding and osteoconduction, osteoinduction and a variable resorbability. Biomechanical properties are a considerable concern in the use of Ca-P ceramics. Compressive strength of cortical and trabecular bone varies, depending on the bone density, from 130 to 180 MPa and 5 to 50 MPa respectively. For tensile strength these values fluctuate from 60 to 160 MPa and 3 to 15 MPa

respectively. Dense sintered Ca-P ceramics may reach compressive strengths much higher than cortical bone (300–900 MPa), whereas tensile strengths similar and higher than cortical bone (40–300 MPa) have been reported (Ravaglioli and Krajewski, 1992). Due to the inferior biomechanical properties, Ca-P ceramics are less suitable for clinical application under weight-bearing conditions compared to, for example, metallic or polymeric biomaterials. Obviously, mechanical properties of porous ceramics deteriorate even further with an increasing porosity. Nevertheless, compressive strengths similar to trabecular bone have been reported (Le Huec, 1995). Cortical bone has pores ranging from 1 to 100 μm (volumetric porosity 5 to 10%), whereas trabecular bone has pores of 200 to 400 μm (volumetric porosity 70 to 90%). Porosity in bone provides space for nutrients supply in cortical bone and marrow cavity in trabecular bone. Microporosity covers pores sizes smaller than 5 μm : too small for penetration and ingrowth of cells, but sufficient for penetration of fluids. Crystalline Ca-P materials intrinsically exhibit, depending on crystal size and structure, a nano- or microporous structure. It has even been reported that microstructure plays a crucial role in osteoinductive properties of ceramics (Yuan et al., 1999). Pores larger than 10 μm can be considered as macropores. Various methods can be used to induce macroporosity in Ca-P material. Obviously, interconnection of pores is essential for tissue growth throughout the scaffold. Tsuruga and Kuboki have investigated the influences of pore size and geometry in induced bone formation in ceramics. They reported that for porous sintered HA blocks with pore sizes ranging from 106–212 μm to 500–600 μm , the highest amount of bone was produced in implants with pore sizes of 300 to 400 μm (Tsuruga et al., 1997). Macroporous dimensions are also reported to play a role in osteoinductive behavior of Ca-P ceramics. In a series of experiments in primates, Ripamonti et al. reported that implant geometry is of critical importance for cell shape,

cell locomotion and cell differentiation (Ripamonti, 1996). Numerous Ca-P products are currently marketed for bone regenerative purposes. Most frequently used Ca-P products in dentistry and surgery are Ca-P coatings applied to prostheses. Several techniques, such as plasma spraying and RF magnetron sputtering can provide a thin bio-active Ca-P coating on the inert metal prosthesis surface (Jansen et al., 1993). Other Ca-P products are marketed as bone filler and are available in various forms as granules, blocks, and injectable cements. Both granules and blocks are available with a dense or porous structure and several resorption rates. Most synthetic products are composed of β -TCP, HA or a combination of both, so called bi-phasic ceramics. These Ca-P biomaterials have outstanding properties: bioactivity (ability to form bone apatitelike material or carbonate hydroxyapatite on their surfaces), ability to promote cellular function and expression leading to formation of a uniquely strong bone-Ca-P biomaterial interface; and osteoconductivity (ability to provide the appropriate scaffold or template for bone formation). In addition, Ca-P biomaterials with appropriate three-dimensional geometry are able to bind and concentrate endogenous bone morphogenetic proteins in circulation, and may become osteoinductive (capable of osteogenesis), and can be effective carriers of bone cell seeds (LeGeros, 2002).

Over the last decade, considerable advances in the engineering of biomaterials that elicit specific cellular responses have been attained by exploiting biomolecular recognition. ECMs comprise a complex, insoluble, three-dimensional mixture of secreted macromolecules, including collagens and non-collagenous proteins, such as elastin and fibronectin, glycosaminoglycans, and proteoglycans that are present between cells. ECMs function to provide structure and order in the extracellular space and regulate multiple functions associated with the establishment, maintenance, and remodeling of differentiated tissues (Reichardt, 1999). ECM components also interact with growth and

differentiation factors, chemotropic agents, and other soluble factors that regulate cell cycle progression and differentiation to control their availability and activity. By immobilizing and ordering these ligands, ECMs control the spatial and temporal profiles of these signals and generate gradients necessary for vectorial responses. Artificial analogues of ECM proteins incorporating structural motifs to reconstitute secondary structures (e.g., coiled coil, α -helix) and convey controlled mechanical properties have been engineered via both synthetic routes and recombinant DNA technology (van Hest and Tirrell, 2001). These artificial proteins provide opportunities to generate novel hybrid macromolecules with additional or new functionalities and enhanced cost-efficiency, while overcoming limitations associated with natural ECMs, such as a restricted range in mechanical properties, processability, batch-to-batch variability, and the potential of pathogen transmission. Although considerable progress has been attained in mimicking particular characteristics of ECMs, next-generation, bio-inspired materials must incorporate multiple characteristics from biological matrices. Advances in materials engineering should provide routes for integrating multiple ligands, ligand gradients, nanoscale clustering, and dynamic, environment-responsive interfacial, and bulk properties.

1.6 Aims of the study

1.6.1 Novel Engineered Human Fluorescent Osteoblasts for Scaffolds Bioassays

Investigations into bone substitutes have been addressed to different biomaterials including coral-derived hydroxylapatite (HA). One of the main goals of this biomaterial, which is similar in its chemical composition to human bone, is to modulate cellular

responses which control the interaction with the scaffold. Specifically, it may induce spontaneous three dimensional self-organisation in the new tissue, as it has been observed in the physiological environment (Hubbell, 1999; Reichardt, 1999). Indeed, cell behavior and phenotype are governed by responses to different types of signals that include mechanical forces, electrical stimuli, and various physical cues (Danen and Sonnenberg, 2003). In addition, cells sense and respond to a variety of signals including soluble growth factors, differentiation factors, cytokines, and ion gradients (Sandberg and Aro, 1993; Kon and Cho, 2001; Lieberman and Daluiski, 2002). Materials employed as scaffolds must possess specific features, such as cyto- and bio-compatibility, osteo-inductivity and -conductivity, as well as the right mechanical strength to provide structural support during tissue growth and remodelling. Besides those of a bovine origin, natural HA biomaterials can also be derived from coral exoskeletons (genus *Porites* and *Goniopora*). Hydrothermal treatment (260°C; 15.000 PSI) of the calcium carbonate exoskeletal microstructure of these corals results in conversion into hydroxylapatite (Roy and Linnehan, 1974). Different hydrothermal coral treatments have resulted in only partial conversion of its calcium carbonate to HA (Vaccaro et al., 2002). As a result, its HA/CaCO₃ composite is resorbed faster than pure HA. Like natural bone, this HA may contain minor elements such as Mg, Sr, F, and CO₃ and has a completely interconnected porosity which is similar to trabecular bone. Coral-derived HA biomaterial in block and granule forms, as well as other types of HA materials, have been well characterized for many parameters including their cytocompatibility using different cellular models (Fricain et al., 2002; Shamsuria et al., 2004; Barron et al., 2007). These cellular models however are time-consuming, with high costs and tedious to be used. The first step of this study was to investigate whether a new cellular model known as Saos-eGFP, derived from the osteoblast-like cell line

Saos-2, can be employed for the in-vitro bioactivity of coral-derived HA biomaterial in block and granule forms. Saos-eGFP is a genetically engineered cell line obtained from Saos-2 osteosarcoma cells which express the enhanced Green Fluorescent Protein (eGFP) (Morelli et al., 2007; Tognon et al., 2008). Saos-eGFP cellular model allowed us to assay (i) cell adhesion, (ii) cell proliferation and (iii) colony capability, by evaluating their emitted fluorescence. We also evaluated the (iv) morphology of the cells seeded on the biomaterial surface by employing electron microscopy. In addition, (v) histological analyses of bone grown in vivo after scaffold implantation are presented.

1.6.2 In vitro study of three hydroxyapatite based bone graft substitutes with human mesenchymal stem cells.

Our investigations into bone substitutes are also focused on synthetic composite materials pursuing a biomimetic strategy. This challenge will enable interactive responses to be created by building intelligent interfaces capable of inducing spontaneous self-organization, like those found in the physiological environment (Tampieri et al., 2005; Liu et al., 2004). This function is affected by the chemical nature of the scaffolding material used and physical structures at various size scales. For these reasons, scaffolds for osteogenesis should mimic bone morphology and structure in order to optimize integration into the surrounding tissue. The micro- and macro-architecture of the scaffold is known to be highly dependent on the production process (Martinetti et al., 2004; Jenis and Erulkar, 2006). A well-characterized biomaterial is hydroxylapatite (HA), which is currently used in clinical applications in different forms. HA is suitable for substituting or integrating diseased or damaged bone tissues since it resembles the mineralized bone phase and supplies fundamental ions for the newly

forming bone during resorption (Schmitz et al., 1999; Dorozhkin and Epple, 2002; LeGeros, 2002; Landi et al., 2008). Biological apatites contain cationic and anionic substitutions in HA crystal structure sites, Mg^{2+} ion being the most abundant, typically amounting to around 6 mol%, in cartilage and bone tissue during the initial phases of osteogenesis (Landi et al., 2008). Magnesium is certainly one of the most important bivalent ions associated with biological apatites: enamel, dentin and bone contain 0.44, 1.23 and 0.72 wt.% Mg, respectively (Kim et al., 2003; Bigi et al., 1998). For biomedical purposes, carbonated apatite, magnesium-doped HA (Mg-HA) and fluorapatite are the materials eliciting the most interest. This is due to their close similarity to bony apatite and decreased solubility in aqueous solutions, respectively, compared to stoichiometric HA (Landi et al., 2006). Non-stoichiometric apatites containing both Mg^{2+} and CO_3^{2-} ions are highly resorbable (Suchanek et al., 2002) and therefore preferred to their stoichiometric counterparts in terms of remodeling kinetics. Several types of HA-derived biomaterials have already been characterized in terms of their biological features (Padilla et al., 2006; Wang et al., 2007). Bio-engineered scaffolds used in orthopedic clinical applications give different tissue response after implantation. The aim of this study was to compare three types of bone substitute (chip, microgranule, nanopowder) routinely used for orthopedic surgery applications and differing by chemical composition and/or physical structure. We focused our attention on Sintlife® and Engipore® biomaterials, which belong to the magnesium-doped non-stoichiometric hydroxyapatite and stoichiometric hydroxyapatite class of bioceramics, respectively. The cell response elicited by biomaterials was investigated with human bone marrow-derived mesenchymal stem cells (hMSCs) which showed osteogenic differentiation potential in vitro and were involved in physiological osteogenesis in vivo (Lennon and Caplan, 2006). These hMSCs were employed to evaluate whether Mg^{2+}

ions affect cell growth and their possible role together with biomaterial structural organization in modulating osteoconductivity.

2 MATERIALS AND METHODS

2.1 *Saos-eGFP cell line*

An engineered human osteoblast-like cell line, known as Saos-eGFP, was obtained from parental Saos-2 cells as described elsewhere (Morelli et al., 2007; Tognon et al., 2008). In brief, Saos-eGFP cells were cultured in DMEM-F12 (BioWhittaker, Milan, Italy) supplemented with 10% fetal bovine serum (BioWhittaker, Milan, Italy), 500 μ /ml penicillin/streptomycin (Sigma, Milan, Italy), and maintained in a humidified atmosphere at 37°C containing 5% CO₂. The antibiotic Genetycin (500 ug/ml), known as G418 (Invitrogen, Milan, Italy) was employed to keep in selection Saos-2 cells, engineered with the recombinant plasmid vector expressing the enhanced green fluorescent protein (eGFP) which carries the resistance gene (Neo) to the antibiotic (Fig. 1). A known number of Saos2-eGFP (5x10³, 10x10⁴, 20x10⁴, 40x10⁴, 80x10⁴, 16x10⁵) was seeded and cultured for 24 hours in 24-well plates (\varnothing = 10 mm). A calibration curve was obtained by reporting the number of cells present in each sample, which emitted fluorescence, on a graph (excitation λ =488 nm, emission λ =508 nm) (Fig. 2).

2.2 *Isolation and growth of human mesenchymal stem cells (hMSCs)*

Iliac crest bone marrow aspirates (10-mL) were obtained from orthopedic patients who underwent bone marrow harvesting under general anesthesia. Specimens were obtained according to the tenets of the Declaration of Helsinki and the ethical committee of the Istituto Ortopedico “Rizzoli”, Bologna, Italy. All donors provided informed consent for the biopsy. A mononuclear fraction was isolated by Ficoll-mediated (Histopaque, # 10771, Sigma Company, Milan, Italy) discontinuous density gradient centrifugation and

polystyrene adherence capacity. Briefly, the bone marrow aspirate was diluted in Hanks Balanced Salt Solution (HBSS) to make the volume up to 25 mL. This cell solution was then added to a 50-mL centrifuge tube, gently overlaid on 20 mL of Histopaque. The layer at the interface of the Ficoll and HBSS was collected after 30 min of centrifugation at 1,800 g at room temperature.

The interface cell layer was transferred to a 50-mL tube in 10 ml PBS. Cell suspension was centrifuged at 1,000 g for 10 min at room temperature. The pellet was suspended and cells were seeded in a T75 flask and cultured with α MEM (Lonza, Milan, Italy) supplemented with 20% fetal bovine serum (FBS), antibiotics and incubated at 37°C with 5 % CO₂ in a humidified atmosphere. At 2 day intervals, the medium was replaced, while non-adherent cells were removed. After 6 days adherent fibroblast-like colonies of hMSCs were clearly visible. Cultures were re-fed weekly and grown to 50-60% confluence in 2-3 weeks; then the hMSCs were passed (0.25% trypsin and 1 mM EDTA) and expanded in new plastic vessels.

2.3 hMSCs Self-Renewal

Primary cell cultures, obtained from the bone marrow of (n=3) patients, were seeded at 1×10^3 cells/cm² (N₀) and cultured in T75 flasks as described above. Cell monolayers, at 50-60% confluence, were detached, cells were counted by a hemocytometer (N), and re-seeded at initial density. Cell-doublings (CD) and mean doubling time (DT) was calculated from cellular counts for each passage as previously described (Vidal et al., 2006):

$$CD = \frac{\ln\left(\frac{N}{N_0}\right)}{\ln 2} \quad (1)$$

where CD is the cell-doubling number, N the number of counted cells, N_0 the number of seeded cells. The average mean doubling time was estimated at each passage according to the following formula:

$$DT = \frac{CT}{CD} \quad (2)$$

where DT is the average doubling time and CT the cell culture time.

2.4 Flow cytometric analysis (FCA) of hMSCs

Cell culture at the first passage isolated from (n=3) patients as described in the MM section, was characterized by flow cytometric analysis (FCA) for specific surface antigens, in accordance with the International Society for Cell Therapy (Dominici et al., 2006). Each sample analyzed by FCA contained 1×10^5 cells. The hMSCs were incubated for 30 min at room temperature with specific phycoerythrin (PE) or fluorescein isothiocyanate (FITC) conjugated-antibodies. The follow markers were assayed: FITC anti-human Stro-1 (Santa Cruz, #sc-47733), PE anti-human CD29 (integrin $\beta 1$, Biosciences clone IgG1 MEM-101A), PE anti-mouse/human CD44 (H-CAM, Biosciences clone IgG2b IM7), FITC anti-human CD45 (Bioscience, clone IgG1 HI30), FITC anti-human CD71 (Biosciences clone IgG1 OKT9), FITC anti-human

CD73 (Ecto-5'-nucleotidase, Biosciences clone AD2), FITC anti-human CD90 (Thy-1, clone 5E10) and PE anti-human CD105 (Endoglin, Bioscience clone IgG1 SN6), PE anti-human CD235a (glycophorin A, Bioscience clone IgG2b HIR2). The hMSCs were analyzed using FCA (Becton Dickinson, Milan, Italy) after PBS washes (Buhring et al., 2007).

2.5 Differentiation of hMSCs

Cell culture at the first passage was induced to differentiate to osteoblasts, adipocytes and chondroblasts using standard in vitro tissue culture-differentiating conditions. For osteogenic differentiation the hMSCs were cultured with complete medium (see above) supplemented with 5 mM β -glycerol phosphate (Sigma # G9891), 50 μ g/mL ascorbate-2-phosphate (Sigma # 49752), and 1 nM dexamethasone (Sigma # D2915). The hMSC culture was re-fed every 3 days for 4 weeks. Afterwards, the monolayer was washed with PBS, formalin 10% fixed for 10 minutes at room temperature and then incubated for 30 minutes with 1% (w/v) Alizarin Red Staining (Sigma, #A5533) in distilled water adjusted to pH 4.1 with 0.5 N ammonium hydroxide (Sigma, #318612). Then, the monolayer was washed with distilled water and observed under an optical inverted microscope.

For adipogenic differentiation, the hMSCs were cultured with complete medium (see above) supplemented with Isobutylmethylxanthine (Sigma, # I7018) 0.5 μ M in methanol, Indomethacin (Sigma, # I7378), 50 μ M in methanol and 0.5 μ M dexamethasone (Sigma # D2915). The hMSC culture was re-fed every 3 days for 4 weeks. Afterwards, the monolayer was washed with PBS, 10% formalin fixed for 10 minutes at room temperature and then incubated for 30 minutes with 3:2 Oil Red O

(Sigma, #O0625) 1% (w/v) in isopropyl alcohol and PBS. Then, the monolayer was washed with distilled water and observed under an optical inverted microscope.

For chondrogenic differentiation, the hMSCs were pelletized and cultured in a 15 ml tube with complete medium (see above) supplemented with 50 mg/ml ITS (Sigma, # I3146), 0.1 μ M dexamethasone, 50 μ g/ml ascorbate-2-phosphate, 40 μ g/ml proline (Sigma, #81709), 100 μ g/ml pyruvate (Sigma, # P8574), 10 ng/ml TGF- β 3 (Biovision, #4344-5), at 37°C 5% CO₂ and re-fed every 3 days for 4 weeks. Afterwards, the pellet was washed with PBS, 10% formalin fixed for 10 minutes at room temperature and embedded in paraffin. 10- μ m sections were stained with Safranin O (Sigma, # S2255) according to standard histological procedures and observed with an optical inverted microscope.

2.6 Marine coral exoskeleton biomaterial

The assayed biomaterial, which is commercially available, is made with marine coral exoskeleton material, hydrothermally converted to hydroxylapatite (HA). HA scaffolds are indicated for use as cancellous bone substitutes or as augmentation material for repairing bone defects, in combination with autologous bone, allografts, blood or bone marrow. This HA material is routinely used in oral-maxillo-facial surgery clinical practice for bone regeneration. In our cyto-compatibility assays the biomaterial was employed both in small blocks (10x10x10 mm) and granules (1 - 4 mm).

2.7 Stoichiometric and non-stoichiometric HA-derived biomaterials

Porous stoichiometric HA bone substitute, known as Engipore® (Fin-Ceramica Faenza S.p.A., Faenza, Ravenna, Italy), was prepared with technology based on slurry expansion: a slurry with a high powder concentration was used and expanded in a known volume to achieve a total porosity of close to 80% in volume, which corresponds to a large surface area ($\approx 0,9 \text{ m}^2/\text{gr}$); its porosity is characterized by bi-modal porous structures and controlled morphology with pore size mainly in the 100-200 μm (32 vol.%) and 200-500 μm (40 vol.%) range (Fig. 3). According to X-ray diffraction analysis, the starting powder used for the production of hydroxyl-apatite (HA) scaffolding resulted as being single phase crystalline HA, with a purity of $\geq 95\%$. Engipore® biomaterial samples were cut into polygonal-shaped chips.

Non-stoichiometric Mg-HA biomaterial known as Sintlife® (Fin-Ceramica Faenza S.p.A., Faenza, Ravenna, Italy) composed of Mg-doped hydroxyl-apatite (MgHA) powder, was prepared at 40 °C in an air atmosphere by dropping it into a 3h 600 mL aqueous solution containing 88.8 g of H_3PO_4 (Aldrich, 85% pure) in basic suspension with 100 g $\text{Ca}(\text{OH})_2$ (Aldrich, 95% pure) and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Merck, A.C.S., ISO) in 1,000 mL of water. A powder with an Mg molar fraction of $X_{\text{Mg}}/(\text{Mg} + \text{Ca}) = 0.25$ was synthesized from the basic starting suspension. Each precipitation product was aged for 24 h at 25 °C, then washed and filtered 3 times, freeze dried, and finally sieved at 150 μm . The specific surface area of the doped powder was approximately 125 m^2/gr . The synthetic MgHA powder ($X_{\text{Mg}} = 0.15$ in the starting suspension, producing 5.7 mol% Mg-substituted HA) was granulated (granule size 400–600 μm) by spraying distilled water followed by sieving (Fig. 4). Sintlife® biomaterial samples were used in paste form, shaped into 2.5 x 2.5 mm cubes, or microgranules.

2.8 Scaffold cell loading

Proliferating Saos-eGFP cell monolayers, with 50÷60% confluence, were detached from the culture flasks and re-suspended in culture medium to obtain a cell suspension with a density of 10^4 cells/cm² surface area, in 1 ml, for each scaffold. A tissue culture polystyrene (TCPS) vessel was employed as control. The scaffold was then placed in wells ($\varnothing = 10$ mm) filled with the proper cell suspension and incubated for 2h. To maximize cell-scaffold interaction, cell suspension was subjected to pipetting every 15 min. After the incubation period, scaffolds were placed in empty wells. Fresh culture medium, 1 ml, was added to each scaffold.

At the first passage of 50÷60% confluence, the hMSCs were detached from the culture flasks and resuspended in culture medium to obtain a cell suspension. The number of seeding cells was determined by normalizing according to the surface area available for colonization per scaffold, with a density of 10^3 cells per cm². The biomaterials (Engipore® chips, Sintlife® microgranules, Sintlife® nanopowder) were placed in wells ($\varnothing = 10$ mm) to cover the surface area, then filled with 1 ml cell suspension for each sample, including tissue culture polystyrene (TCPS) as a control, and incubated for 2 hours. To maximize cell-scaffold interaction probability, the cell suspension was subjected to shaking every 15 min. After the incubation period, the cell suspension was removed and 1 ml fresh culture medium was added to each well.

2.9 Saos-eGFP adhesion and proliferation

Saos-eGFP cells were loaded onto scaffolds and TCPS (control), in 24-well culture plates ($\varnothing=10$ mm) and cultured as described above. In order to mimic the clinical application environment, the same volume of bone void filler was used for each type of scaffold. To analyze cell adhesion on each biomaterial and on the control, samples were incubated for 24 h at 37° C, 5% CO₂. Attached cells per scaffolds were detected by measuring the fluorescence (excitation $\lambda=488$ nm, emission $\lambda=508$ nm) emitted by the viable cells. Then, cells were re-fed with fresh culture medium and cultured at 37° C in a humidified atmosphere with 5% CO₂. Assays were carried out to evaluate cells attached on biomaterials and on the control, at 24 h, 48 h, and 96 h.

2.10 Saos-eGFP spreading

Saos-eGFP cells were loaded onto the scaffolds and TCPS, in 24-well culture plates ($\varnothing=10$ mm) and cultured as described above. Samples were incubated for 24 h, 48 h and 96 h at 37° C, 5% CO₂. Direct observation of the living cells on the biomaterial was carried out by fluorescence microscopy in order to determine their distribution, colonization and morphology on the biomaterials.

2.11 hMSCs viability assay

The hMSCs were loaded onto HA scaffolds and TCPS, which were used as a control, in 24-well culture plates ($\varnothing=10$ mm) and cultured as described above. The AlamarBlue

assay was employed to analyze cell viability on each biomaterial and on the control. Briefly, the culture medium was removed, each sample washed with PBS, then 1 ml of fresh medium with 5% AlamarBlue (Invitrogen) was added to each well. The sample was incubated for 2.5 hours at 37° C, 5% CO₂. Cell metabolic activity was detected by measuring the fluorescence at $\lambda=590$ nm (excitation $\lambda=540$ nm). Then, cells were re-fed with fresh culture medium and cultured at 37°C in a humidified atmosphere with 5% CO₂. The assay was carried out to evaluate the viability of cells attached and grown on the biomaterials and control at 72 hours, 144 hours and 216 hours.

2.12 Molecular assessment of hMSCs attachment

The hMSCs were loaded onto HA scaffolds, and TCPS as a control, in 24-well culture plates ($\varnothing=10$ mm) and cultured as described above. After 72 hours of incubation, cell lysates were assayed using the Calbiochem PhosphoDetect FAK (pTyr397) ELISA kit (Merck KGaA, Darmstadt, Germany) to detect and quantify the amount of focal adhesion kinase (FAK) protein phosphorylated at the tyrosin position 397 (Tyr397) following the manufacturer's instructions. Values were corrected for the protein concentration as determined by a colorimetric assay (BCA assay; Pierce, Rockford, IL).

2.13 Fluorescence microscopy detection of p-FAK (Tyr397) and hMSCs cytoskeleton architecture

To carry out fluorescence microscopy detection of the p-FAK (Tyr397), the hMSCs were grown on the biomaterials for 72 hours, washed twice with PBS and fixed for 15 min with 3% paraformaldehyde at room temperature. Afterwards, the cells were treated

for 10 min with 0.1% Triton X-100, washed twice with PBS and incubated for 1 hour at room temperature with a phospho-specific rabbit polyclonal p-FAK (Tyr 397) antibody (Santa Cruz Biotechnology, # sc-11765-R). After two washes in PBS/0.1% BSA, the cells were incubated for 1 hour at room temperature with the secondary goat anti-rabbit IgG-TR antibody (Santa Cruz Biotechnology, #sc-2780) diluted 1:100 in PBS/0.1% BSA in the presence of DAPI dye (Sigma, #D9542), 0.5 µg/ml in PBS, to stain the cellular nuclei. Afterwards, the cells were washed twice with PBS and mounted with glycerol 9:1 in PBS.

To evaluate the influence of biomaterials on the cytoskeleton organization of the hMSCs, cells were seeded onto biomaterials. After 72 hours, actin filaments were stained with rhodamine conjugated Phalloidin. The cells were washed with a detergent solution (PBS 1x, EGTA 2mM, MgCl₂ 2mM) and then fixed with paraformaldehyde 3.7% (Sigma, Milan, Italy) in PBS (10 min at room temperature). The cells were stained with DAPI (4',6-diamidino-2-phenylindole Sigma, Milan, Italy) 0.5 µg/ml in PBS and then incubated with TRITC (tetramethylrhodamine isothiocyanate) conjugated-Phalloidin (Sigma, Milan, Italy) 1µg/ml for 1 hour at 37°C.

Photographs were taken from an TE2000E fluorescence microscope, whereas digital images were captured using the ACT-1 software for the DXM1200F digital camera (Nikon S.p.A., Florence, Italy).

2.14 Scanning electron microscopy (SEM) analysis

For the SEM analysis, Saos-eGFP (10⁴ cells/well) were cultured on HA scaffolds for 48 h. Cells which were attached to the biomaterials were washed with PBS 1X solution and fixed for 1 h by 2.5% glutaraldehyde in a phosphate buffer and then for 4h with a 1%

osmium solution in a phosphate buffer. Specimens were coated with colloidal gold and analyzed using scanning electron microscopy (SEM, Cambridge UK, model Stereoscan S-360).

hMSCs were cultured on HA scaffolds for 72 hours and processed as described previously 19. Briefly, the cells attached to the biomaterials were washed with PBS 1X solution and fixed for 1 h by 2.5% glutaraldehyde in phosphate buffer and then for 4h with a 1% osmium solution in phosphate buffer. The specimens were coated with colloidal gold and analyzed using scanning electron microscopy (SEM, Cambridge UK, model Stereoscan S-360).

2.15 Histology

Granules were used for maxillary sinus augmentation in patients with bone resorption in the posterior segment of the upper jaw for implant-prosthetic rehabilitation. Blocks were used as interpositional materials in orthognathic surgery patients when the upper jaw was down grafted in order to increase the stability of the mobilized segment. Biopsies were taken in accordance with the local ethical committee and after patients' written consent. In one clinical case (patient 1), biomaterial granules were used for bone regeneration in a patient who had undergone maxillary sinus augmentation for pre-prosthetic surgical rehabilitation. In this patient, a bone biopsy was taken four months after the first surgical procedure, during implant surgery. In another clinical case, (patient 2), to correct dentoskeletal deformities, blocks of biomaterial were employed as interpositional grafts in a patient treated with LeFort I osteotomy. Bone biopsies were taken from this patient, during plate removal, one year after surgery. Tissues were fixed in 4% formaldehyde and dehydrated in a graded series of alcohols. Then, all specimens

were embedded in methylmethacrylate resin. Undecalcified sections were obtained using a water-cooled diamond saw. Slides were ground to a final thickness of about five μm , placed on glass slides, stained with ematossilin-eosin and viewed and photographed in a Leitz Orthoplan photomicroscope.

2.16 Statistical analysis

Data are expressed as the mean of the \pm standard deviation. Statistical analyses of cell-biology experiments, which were performed in triplicate, were carried out by R (Ihaka and Gentleman, 1996), one-way analysis of variance (ANOVA) with Dunnet post-test analysis (* $p < 0.05$ and ** $p < 0.01$). Flow cytometric data were analyzed by flowCore Bioconductor package (Gentleman et al., 2004; Hahne et al., 2009).

3. RESULTS

3.1 *Saos-eGFP adhesion and proliferation*

The results obtained showed a good adhesive capability of Saos-eGFP cells to the biomaterial. Indeed, Saos-eGFP cells attached both to block and granules of the biomaterial with a prevalence of 67% and 64%, respectively, compared to seeded cells. In the control experiment, 94% of the seeded cells were attached to the plastic well, showing a statistically significant difference ($p < 0.01$) as regards the two scaffolds (Fig. 5). Cells proliferated in each sample during the assay as in the control ($p < 0.01$). At 48 h, the fluorescence detected for the granules was lower than the fluorescence detected for the control and block ($p < 0.01$). Cells grown on blocks behaved differently, as shown by the data obtained at 96 incubation h. Indeed at this point, the fluorescence detected on these samples diminished with statistically significant differences as regards the control and granules ($p < 0.05$) (Fig. 6).

3.2 *Saos-eGFP spreading*

In the control experiment, the cell population was homogeneously distributed and appeared to have a normal morphology. Cells behaved similarly for both forms of the biomaterial. Indeed, the cells were able to colonize homogeneously both granules and blocks (Fig. 7).

3.3 Saos-eGFP Scanning Electron Microscopy analysis

The results obtained with the SEM analysis indicate no difference in the morphology of the cells grown on the biomaterial under analysis, both granules and blocks, when compared to the control. SEM images revealed the presence of several cells anchored to the surface of the biomaterial by cytoplasmic bridges, which were similar to pseudopodia (Fig. 8). In addition, a large amount of biomaterial debris was detected on the surface of the cell membrane. This debris was not present on the cells grown on glass, where the cell surface appeared homogeneous.

3.4 Histological evaluation

Histological analysis (patient 1) showed new bone formation with resorption of the biomaterial. Indeed, fibro-osseous tissue represented about 50% of the biopsy volume, while lamellar bone was 10% of the biopsy. Bone fatty marrow and vessels represented 40% of the biopsy. There was no evidence of inflammation or foreign body reactions. The histological evaluation (patient 2) showed, as expected, the residual biomaterial surrounded by mature compact bone. There was evidence of bone regrowth inside the biomaterial giving continuity between osteotomy sites with significant stability for the down grafted maxilla. This biomaterial represented 20% of the biopsy volume, while bone density was in the order of 40%. The remaining 40% of the biopsy was represented by fibro-osseous tissue. In this patient, a minor inflammation process with the presence of macrophages was revealed by histological analysis (Fig. 9).

3.5 hMSC culture and Flow cytometric analysis (FCA)

The first hMSC culture passage was evaluated by FCA for the presence of specific surface antigens. The surface antigen profile matched with expected markers, in agreement with the International Society for Cell Therapy guidelines.¹⁷ 50.61% of the first cell population passage expressed Stro-1 antigen. In the analyzed samples, the level of hMSC purity was >95% towards the expression of CD105 and CD90, 89.61% towards the expression of CD73, 9.51% and 6.63% towards the expression of hematopoietic antigens CD235 and CD45, respectively. Samples also expressed CD29 (99.03%), CD44 (98.18%) and CD71 (52.08%) (Fig. 10).

3.6 hMSC Self-Renewal

The bone marrow aspirates gave fibroblast-like adherent cells, capable of forming colonies when seeded at low density (1,000 cells/cm²). To verify the self-renewal capacity of the cell population obtained, we measured the number of cell doublings during 5 culture passages. Cell growth potential was maintained during 40 days of cultivation with a maximum of 12 cumulative cell doublings (Fig. 11). The average mean doubling time, obtained from the same samples, was 3.2 days.

3.7 Multipotent differentiation capacity of hMSCs

Human mesenchymal stem cells cultured as indicated in the MM section, will be able to differentiate into osteogenic lineage as shown by the deposition of calcium salts revealed by Alizarin red staining after 28 days of incubation. More than 30% of the monolayer surface was positive to ARS staining. The Oil Red O staining showed that

the hMSCs were able to differentiate into adipocytes characterized by the presence of cytosolic fat droplets, amounting to more than 30% of the monolayer. Chondrogenic differentiation was reached in the pellet treated as indicated in the MM section, after 28 days of incubation. Cartilage-bound lacunae were clearly visible in the cartilage construct which was obtained (Figure 12, a-c). The 30% cut off value associated with a satisfactory level of differentiation was in agreement with (Gregory and Prockop, 2007).

3.8 hMSCs viability assay

Purified hMSCs were loaded onto scaffolds as described in the MM section. The control (TCPS), which had the lowest surface area, increased its metabolic activity during the assay period. Furthermore, Engipore® samples increased their metabolic activity during the assay. Different behavior was shown by the Sintlife® biomaterials; indeed the granular form increased its metabolic activity while the paste form maintained the same metabolic activity during the 9 days of the assay (Fig. 13). A statistically significant ($p < 0.05$) difference in metabolic activity was found between the TCPS and the other samples and between the granular materials and the Sintlife in paste form.

3.9 Fluorescence microscopy detection of p-FAK (Tyr397)

The immuno-localization of pFAK-Tyr397 in hMSCs cultured on TCPS by immunostaining with polyclonal antibodies showed its cytosolic distribution, confirming protein activation toward cell adhesion (Fig. 14).

3.10 Molecular Assessment of hMCSs attachment

Quantifying pFAK relative amounts per scaffold is useful in evaluating the strength of cell adhesion to biomaterials and thus scaffold osteoconductivity. To quantify the relative amount of pFAK-Tyr397 per scaffold we performed a sandwich ELISA test (n=3). The results showed that TCPS had the highest relative amount of pFAK-Tyr397 normalized to the total protein amount per sample, with a statistically significant difference ($p < 0.01$) towards each biomaterial. A statistically significant difference was obtained between Engipore and Sintlife (granular form) and towards Sintlife in paste form ($p < 0.05$) (Fig. 15).

3.11 Determination of cytoskeleton architecture

The cytoskeleton architecture is well organized and its integrity is not influenced by the cultivation of cells on the different scaffolds. Actin fibers connect cell membranes and the cytoskeleton to the scaffold surface without any visible loss or structural displacement (Fig. 16).

3.12 hMSCs scanning electron microscopy analysis

During morphological analysis of the hMSCs seeded on the biomaterials, the cells appeared to be well attached to the scaffold with several cytoplasmic extroflexions. Cells grown on Engipore® scaffolds showed normal morphology and appeared to be well attached to the substrate with a pseudopodia in contact with the extra-cellular matrix. The amount of debris on the cell surface was low, according to its low release by these types of biomaterials. The same result was obtained with Sintlife® in the form

of granules. Different behavior was registered towards Sintlife® in paste form: cells attached to it showed a low number of shorter pseudopodia and a greater amount of debris on the cell surface. This result could be determined by the release of granular nano-powder by this type of scaffolding when placed in culture medium (Fig. 17).

4. DISCUSSION

Saos-eGFP cells were employed to determine mature osteoblastic cell morphology, spread and proliferation on coralline-HA biomaterials. Saos-eGFP on the biomaterial showed homogeneous spreading and a different rate of proliferation. This result demonstrates that composition, surface shape and biomaterial morphology, may influence the relationship between scaffold and cell. Indeed, Saos-eGFP cells which attached to the biomaterial, in the two forms assayed, showed a good adhesion ratio, although lower than for the control (TCPS). Biomaterial blocks appear to provide a better environment for adhesion compared to biomaterial granules, as shown by the percentage of adhered cells and by proliferation at 48 culture hours. Otherwise, the proliferation index determined for the biomaterial in blocks was lower than for granules at 96 incubation hours. Results showed that the granular form of the biomaterial has a greater capability to achieve Saos-eGFP cell proliferation. Direct observation at the fluorescence microscope showed that in the control experiment, the cell population was homogeneously distributed and appeared to have a normal morphology. Cells behaved similarly on both forms of the biomaterial until 96 cultivation hours. Indeed, cells were able to colonize homogeneously both granules and blocks forming colonies on the surface and on the trabecular structure of the biomaterials. SEM images revealed the presence of several cells anchored to the surface of the biomaterial by cytoplasmic bridges, which appeared similar to pseudopodia, confirming the capability of the biomaterial to achieve cell adhesion. The presence of debris, absent on cells grown on glass, observed at 48 and incubation hours, is probably an additional cause for the diminished number of cells grown on the block after that incubation period .

Histological evaluations indicated that both biomaterial granules and blocks showed osteoconductive properties, inducing new bone formation. From a clinical point of

view, the osteoconductive properties were confirmed by the stability of the endosseous implants inserted in the posterior segment of the maxilla and in down grafted maxilla.

The possibility of learning which biomaterial leaves cell morphology, spread and proliferation unaltered, would be an important step towards obtaining bone tissue regeneration by employing three dimensional scaffolds. This knowledge is fundamental to oral and maxillo-facial surgery, which uses different types of biomaterials, in particular for treating specific pathologies, such as jaw bone atrophies, periodontal defects and cystic lesions.

In this study, we have described the use of Saos-eGFP engineered cell lines employed for the preliminary in vitro characterization of cell morphology, spread and proliferation on biomaterials, and we have clinically demonstrated bone re-growth driven by the biomaterials tested herein. The coralline-HA biomaterials, tested with Saos-eGFP cells, showed good cyto-compatibility although some differences in proliferation activity was found as reported earlier (Fricain et al., 2002). This cellular model will allow in vivo testing only for those materials which have shown the minimal required in vitro properties for successful bone tissue regeneration: an absence of cyto-toxic effects and good adhesion, spread and proliferation capabilities. This study has demonstrated that human engineered osteoblast-like cells expressing the eGFP is a suitable cellular model to assay biomaterials. Saos-eGFP has the advantage to test biomaterials by measuring the emitted fluorescence instead to count each time the number of cells as usually performed with the traditional primary cell lines. In particular our cellular model reduces (i) the cost of the experiments; (ii) the time of the execution; (iii) while giving the same kind of results (Begley et al., 1993; Shamsuria et al., 2004; Barron et al., 2007) obtained with other cellular models, as demonstrate herein in assaying coral derived HA biomaterials.

Recent improvements in the knowledge of stem cell biology has allowed new cellular models to be set up based on the use of precursor cells. These cellular models can be employed to characterize the behavior in vitro of different biomaterials which are used as bone substitutes and three dimensional scaffolds. In our study, bone precursor cells from the bone marrow aspirates of orthopedic patients were used to analyze bone stem cell behavior when cultured in close contact with selected scaffolds. Human mesenchymal stem cells were employed to investigate whether magnesium-doped non-stoichiometric hydroxyapatite may elicit a cell response toward extracellular matrix interaction which is different from that of stoichiometric hydroxyapatite. The influence of the physical form of the biomaterial in modulating the osteoconductivity of biomaterials was investigated. The study focused on Sintlife® and Engipore®, biomaterials that are routinely employed in orthopedic surgery and belong to the magnesium-doped non-stoichiometric hydroxyapatite and stoichiometric hydroxyapatite class of bioceramics, respectively. The presence of magnesium ions in the human adult bone matrix suggested the development of new bioceramics that resemble bone chemical composition to facilitate the implant resorption rate, while retaining the typical bioactive properties of stoichiometric HA (Serre et al., 1998). The hMSCs, which were isolated from the bone marrow of the iliac crest of donors, are a cellular model for biomaterial in vitro assays. In this study, the standardization of hMSC isolation methods has been verified by cytofluorimetric analysis. Stro-1 surface antigen was found to be expressed in 50.61% of cells. This marker suggests that contaminant cells, such as fibroblasts, smooth muscle cells, and adipocytes, are present in the cell population isolated at the first passage. An earlier investigation reported more than 95% of STRO-1-positive cells in the BM were nucleated erythroid cells (Simmons and Torok-Storb,

1991). Interestingly, in our study only the 9.51% of cells were CD235 positive, the latter being an erythroid marker, and that cultures were also negative for the pan-leukocyte marker CD45 (6.63% positivity). In agreement with the guidelines of the International Society for Cellular Therapy, which define the minimal criteria for hMSC identification (Dominici et al., 2006), cell populations isolated herein expressed CD105 (99.58%), CD90 (99.61%) and CD73 (89.61%). These results suggest that the isolation method employed may be suitable for obtaining a cell population containing human mesenchymal stem cells although contaminant cells from the hematopoietic lineage are present, in the early stages of cultivation. Another parameter investigated, relating to stem cell characteristic growth kinetics, was self-renewal capacity. Isolated fibroblast-like adherent cells were capable of forming colonies and exhibited a self-renewal capacity during several culture passages. Observation of cell doublings revealed that the isolated cell population was able to grow for 5 culture passages without depletion of its self-renewal capacity. The mean doubling time calculated from the culture times and cell numbers in each sample (number of donors=3) at each expansion, is in agreement with an earlier report (Baksh et al., 2004). The biologic property that most uniquely identifies hMSCs is their capacity for tri-lineage mesenchymal differentiation. As shown by results obtained in differentiation assays, isolated cells may be induced to osteo- condro- and adipogenic lineage. We adopted these minimal criteria to standardize the human mesenchymal stem cell preparations from bone marrow aspirates.

A viability assay demonstrated that all the biomaterials assayed did not elicit any cytotoxic effects, although they induced different cellular growth kinetics. We observed that the TCPS control induced the best response in terms of cell proliferation, in agreement with the earlier study carried out with MC3T3-E1 preosteoblasts (Chou et al., 2005). We found interesting behavior towards cells cultured on biomaterial in nano-structured

paste form. Indeed, these cells did not proliferate and their metabolic activity was constant during the assay time frame. This result suggests that its structural organization is a less favorable support for cell anchoring and migration. Differently, magnesium-doped non-stoichiometric hydroxyapatite in microgranules form, induced cell behavior similar to that of stoichiometric hydroxyapatite. These observations, suggest that Mg^{2+} ions does not influence cell proliferation.

In order to gain more insight into the modulation of cell behavior induced by the different biomaterials, we focused our attention on FAK phosphorylation. This molecular event is involved in the assembling of focal adhesion and induces a cellular response that leads to cell survival, proliferation and differentiation via several cellular signal pathways. It is also responsible for maintaining cell adhesion to the substrate. FAK plays a central role in integrating adhesion and growth factor signals to direct Runx2 transcriptional activity, osteoblast-specific gene expression, and matrix mineralization (Geiger et al., 2001; Garcia, 2005). We demonstrated FAK activation by phosphorylation of Tyr³⁹⁷ with antigen immunolocalization in cells cultured on a TCPS support (Figure 14). Then we analyzed samples cultured on the different HA-derived biomaterials by ELISA assay for their content in pFAK-Tyr³⁹⁷. Stoichiometric hydroxyapatite (Engipore®) and non-stoichiometric Mg-HA (Sintlife®) in granular form, both showed a phosphorylated FAK relative amount that was higher than the biomaterial in nano-structured paste form. The control (TCPS) exhibited the highest level of phosphorylated FAK, as we expected, because of culture plastic surface modification typical of the production process. We did not find any differences between the two granular biomaterials apart from their different chemical composition. This would suggest that Mg^{2+} ions are not involved in modulating the adhesion process. These findings also suggest that nano-structured paste forms do not have a positive

effect on focal adhesion assembly. Although a rough surface has a greater ability to induce FAK activation in response to integrin binding (Hamilton and Brunette, 2007), biomaterial in paste form is characterized by a microstructure without the porosity required for cell hosting and fluid flow. In addition, debris is released from the biomaterial surface, probably limiting with cell adhesion.

Findings from the cytoskeleton and the SEM analysis of cells cultured on the different biomaterials indicated that while the cytoskeleton architecture appears to be well organized and its integrity is not, as reported in (Morelli et al., 2007; Tognon et al., 2008), influenced by chemical and physical structure towards other biomaterials employed in orthopedic surgery, the nano-structured form of non-stoichiometric Mg-HA may interfere with cell-substrate interaction. Indeed, particulate released from the biomaterial appears on the cell surface and cell morphology is characterized by a few short cytoplasmic bridges between the cell and substrate. These observations correlate positively with data obtained from viability and ELISA assays.

The results obtained in this study extend on the data reported earlier (Landi et al., 2006; Barrere et al., 2006). The HA derived biomaterials which were assayed did not induce cytotoxic effects while different cell behavior was elicited by its physical organization. The nano-structured form of non-stoichiometric Mg-HA is less favorable for cell adhesion and proliferation compared to non-stoichiometric Mg-HA and stoichiometric hydroxyapatite, in granular form. As shown by ELISA assay, biomaterials osteoconductivity may be related to FAK activation and thus, it can be inferred that biomaterials with low ability to induce FAK phosphorylation have low osteoconductivity capacity. In this study, the modulation of FAK activation by different biomaterial physical structures demonstrated that a non-structured powder is not the best biomaterial form for cell colonization and proliferation. However, both non-

stoichiometric Mg-HA and stoichiometric hydroxyapatite, in granular form, showed similar features in terms of FAK activation and influence on cell behavior and morphology. Moreover, the real effect of Mg^{2+} ions on cell adhesion and proliferation and the role of Mg^{2+} ions in osteoinductivity is as yet to be fully explained and clarified.

5 CONCLUSION

Employment of cellular model for biomaterial assay offer the possibility of better understand relationships between implant and host tissue at a cellular and molecular level. Moreover, in-vitro analysis of cell behavior during cultivation together with biomaterials may be useful to characterize cellular response to ECM stimuli. Indeed, it is known that extracellular environment contain physical and chemical cues capable of elicit specific cellular response that may induce bone progenitors cells differentiation. Although the easiest laboratory approach offered by the Saos-eGFP model in biomaterials characterization, hMSCs represent an important in-vitro tool to assay several biomaterials features such as osteo-inductivity and -conductivity, and cytotoxicity. The availability of a bone progenitor cell model, obtainable from different sources, allow a more insightful understanding of the earliest phase of cell-biomaterial interaction, resembling a more physiological-like environment. Moreover, hMSC represent an important tool for bone tissue engineering. In the future it is expected to obtain useful information by reverse engineering of gene networks during hMSC differentiation toward osteogenesis and analyzing its epigenetic control. This could lead to the discovery of molecular targets and designing of bioactive molecules capable to elicit a specific cellular response. This could allow the development of new soluble factors or new bio-functionalized materials that could be employed in tissue engineering strategies.

6 FIGURES LEGEND

Figure 1

pEGFP plasmid vector showing the eGFP insertion site and the Neo resistance gene.

Figure 2

The calibration curve was obtained by seeding Saos-eGFP cells at different densities and measuring the fluorescence emitted (excitation $\lambda=488$ nm, emission $\lambda=508$ nm). R value showed a positive correlation between cells number and fluorescence emitted.

Figure 3

Engipore® stoichiometric HA biomaterial. (A) Macrostructure, 3,000x magnification, and (B) microstructure, 20,000x magnification.

Figure 4

Sintlif® non-stoichiometric Mg-HA biomaterial. (A) Microgranules, 200x, and their surface (B), 3,000x. (C) Amorphous nanostructured paste, 20,000x magnification.

Figure 5

Adhesion index at 24 hours of incubation for controls and biomaterials, expressed as a ratio of living cells detected by fluorescence intensity measurement at 24 incubation hours, and cells seeded.

Figure 6

Proliferation index for control and biomaterials, expressed as a ratio of living cells detected by fluorescence intensity measured at 48 hours and 96 incubation hours respectively, and living cells detected at 24 incubation hours.

Figure 7

Direct observation by fluorescence microscope. At 48 h the Saos-eGFP cells appeared well attached to the control (A) as well as to the biomaterials. The assay revealed a homogeneous distribution of Saos-eGFP cells on the surfaces of the biomaterials, both the blocks (B) and granules (C).

Figure 8

SEM images showed that at 48 h, Saos-eGFP cells were homogeneously distributed on block biomaterials surface (A) and appear anchored to the granular scaffold by

cytoplasmic bridges also involved in cells interaction (B). Debris is also evident on the cell surface.

Figure 9

Histological evaluations indicate that tested biomaterial induces new bone formation, thus showing osteoconductive and osteoinductive properties. **Patient 1 (A)**. Biopsy consist of cortical bone (▲) associated with new bone formation and fibro-osseous tissue (★). Areas of cortical (▲) and spongius bone (■) are both index of bone ingrowth together with neoangiogenetic processes demonstrated by vascular channels (●). Magnification 10x. **Patient 2 (B)**. Black circles show exogenous material surrounded by mature cortical bone (▲) and fibro – osseous (★) tissue associated with macrophages and the infiltration of inflammatory cells (black arrows). Magnification 40x.

Figure 10

Surface antigenic profile of hMSCs obtained from bone marrow aspirates at the first culture passage. 50.61% of the first cell population passage expressed the Stro-1 antigen. In the analyzed samples, the level of hMSC purity was >95% towards the expression of CD105 and CD90, 89.61% towards the expression of CD73, 9.51% and 6.63% towards the expression of hematopoietic antigens CD235 and CD45, respectively. Samples also expressed CD29 (99.03%), CD44 (98.18%) and CD71 (52.08%)

Figure 11

The number of cumulative cell doublings during 5 cultivation passages of (n=3) donor samples were registered. As depicted in the graph, all the samples showed a similar growth kinetic associated to an increase in cell doublings over the culture passages.

Figure 12

The multipotent capacity of a mesenchymal stem cell. (A) Alizarin Red staining after 28 days of induction treatment. Calcium salt deposition is clearly visible. (B) Oil Red O staining showed adipogenic induction characterized by red colored cytosolic fat droplets after 28 days of induction. (C) Chondrogenic differentiation after 28 days of induction. The extra-cellular matrix sulfated proteoglycans appear pinkish and cartilage-bound lacunae are visible (white arrows). Size bar=10 μm .

Figure 13

hMSC metabolic activity measured by fluorescence intensity at t1=72 hours, t2=144 hours and t3=216 hours of cultivation on biomaterials. TCPS exhibited the highest value in cell viability while granular biomaterials exhibited the same metabolic activity. Statistically significant differences are evident between biomaterials in the granular and paste form and between TCPS and other samples ($p < 0.05$). SL=Sintlife®, EP=Engipore®.

Figure 14

Immuno-localization of activated pFAK (Tyr397) by polyclonal antibodies in hMSCs grown on TCPS. Nuclei were stained with DAPI. pFAK (Tyr397) is localized at cytosolic level as expected. 400x magnification.

Figure 15

Relative amount per sample of activated pFAK (Tyr397) quantified by ELISA assay (n=3). Values have been normalized versus total protein content measured by BCA assay. Statistically significant differences between granular apatite and paste form occurred ($p < 0.05$). TCPS exhibited the highest relative amount of pFAK (Tyr397) ($p < 0.01$). SL=Sintlif[®], EP=Engipore[®].

Figure 16

Cytoskeleton analysis by Phalloidin TRITC staining of hMSCs cultured on biomaterials. Actin filaments do not show alteration in the structural organization, confirming the compatibility of assayed biomaterials, (A) Engipore[®], (B) Sintlif[®] microgranules and (C) Sintlif[®] nano-structured paste. 400x magnification.

Figure 17

SEM morphology analysis of hMSCs cultured on different biomaterials. Cells appear to be well attached to the substrate with several pseudopodias and cytoplasmic extroflections when cultured on (A) Sintlif[®] micro-granules and (B) Engipore[®] Chips.

A large amount of debris is present on the surface of cells grown on (C) Sintlife® in paste form.

7 FIGURES

Figure 1

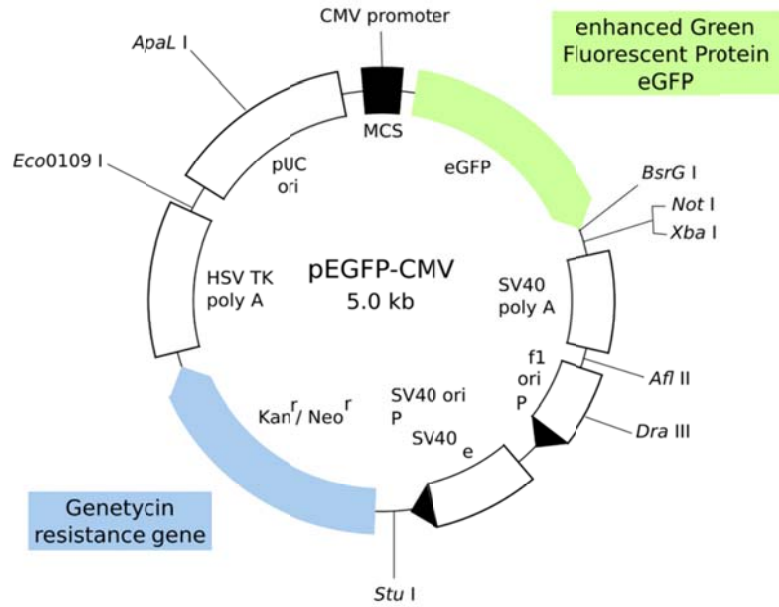


Figure 2

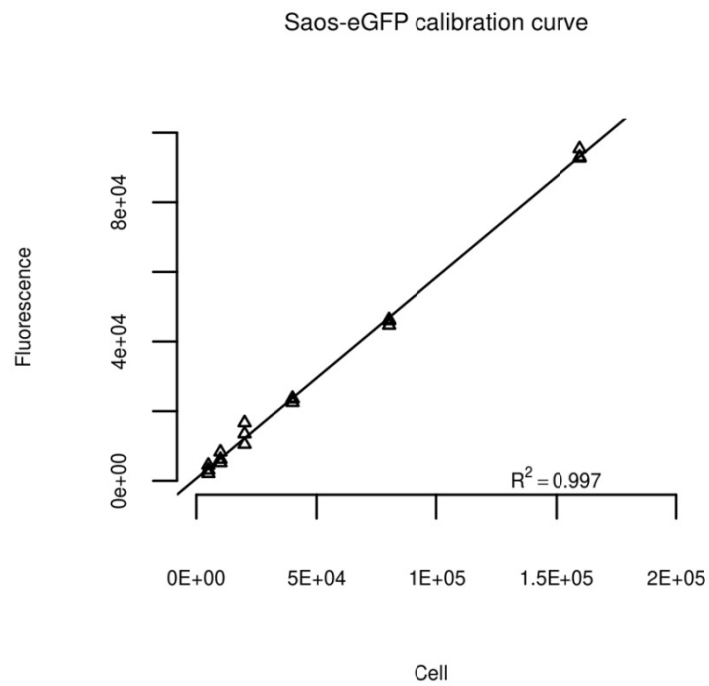


Figure 3

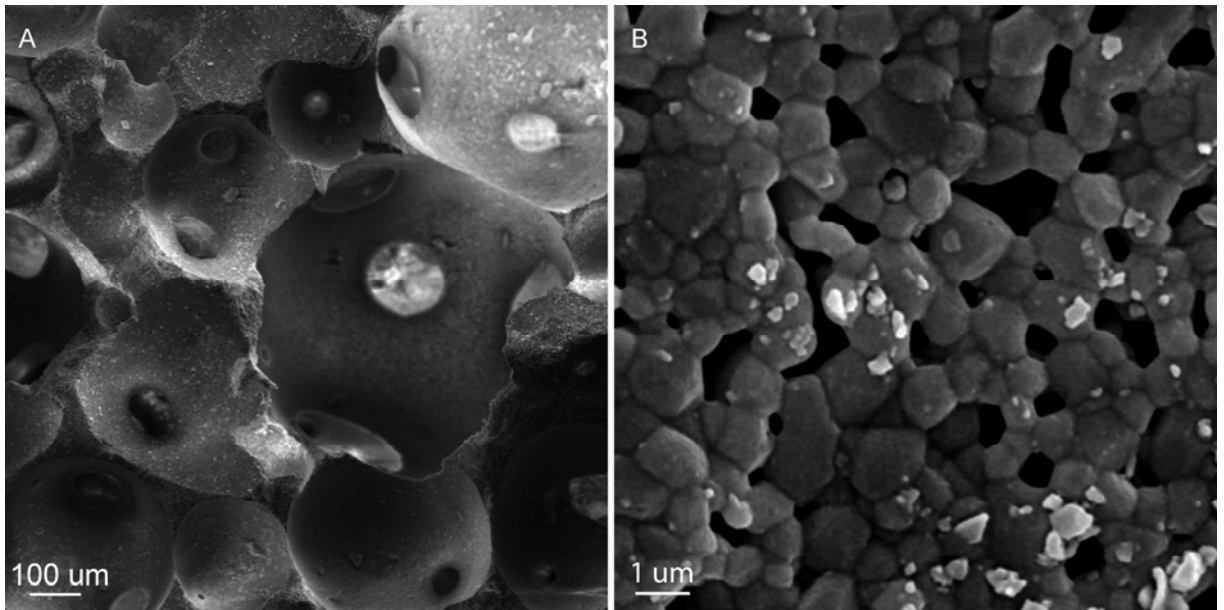


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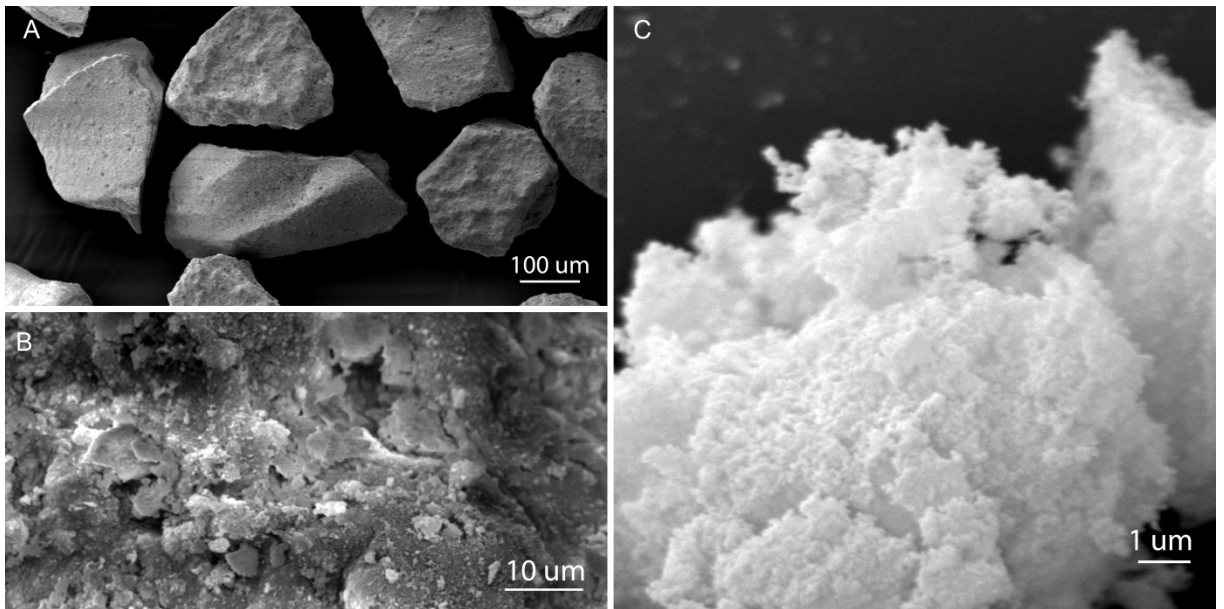


Figure 5

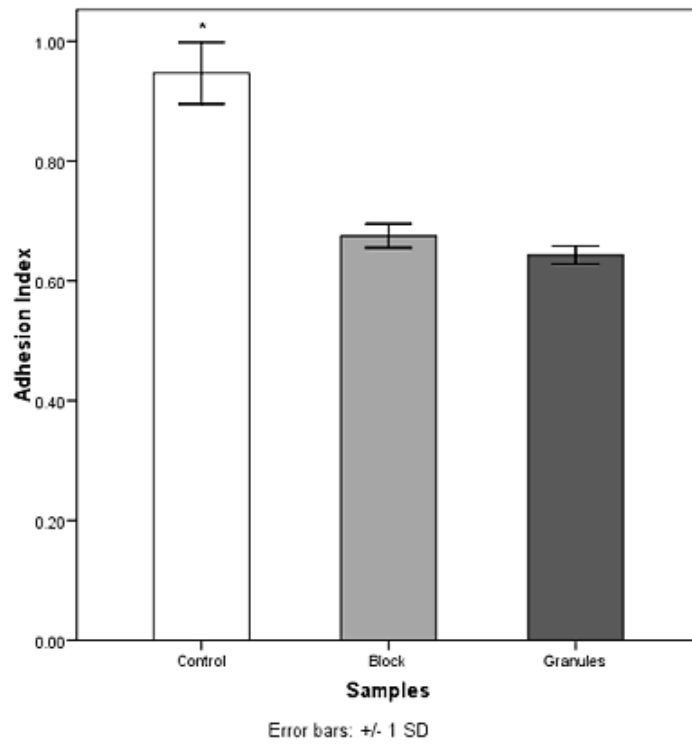


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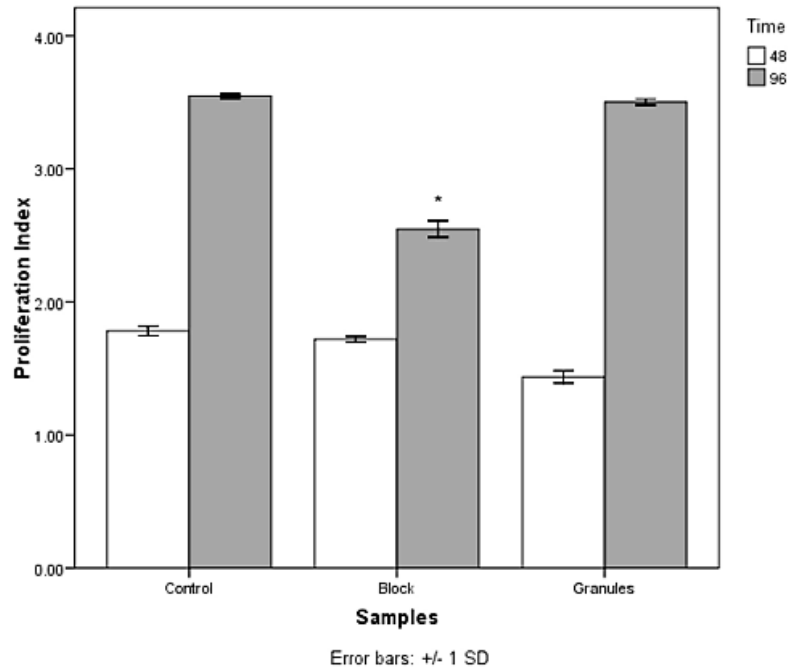


Figure 7

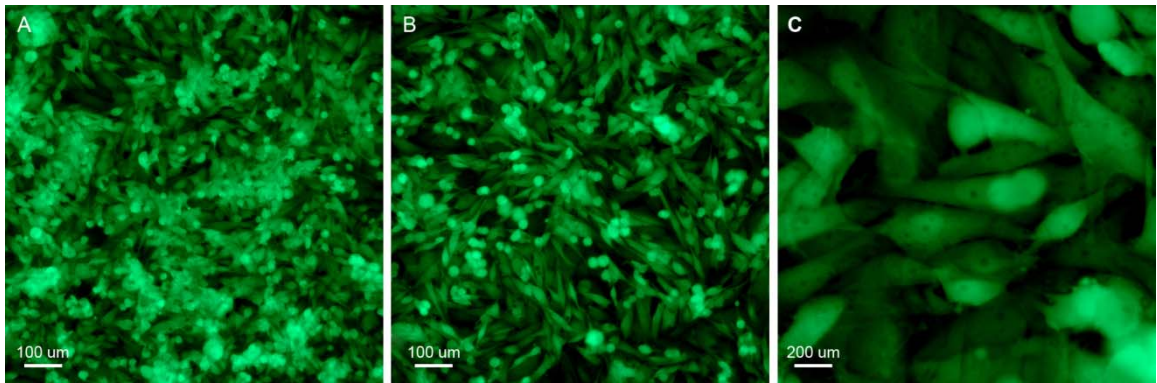


Figure 8

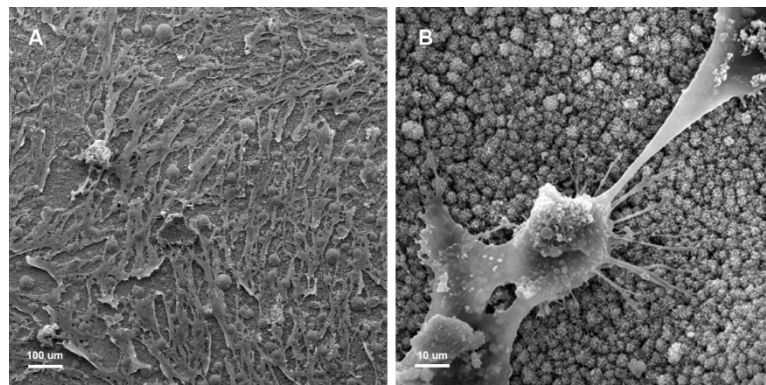


Figure 9

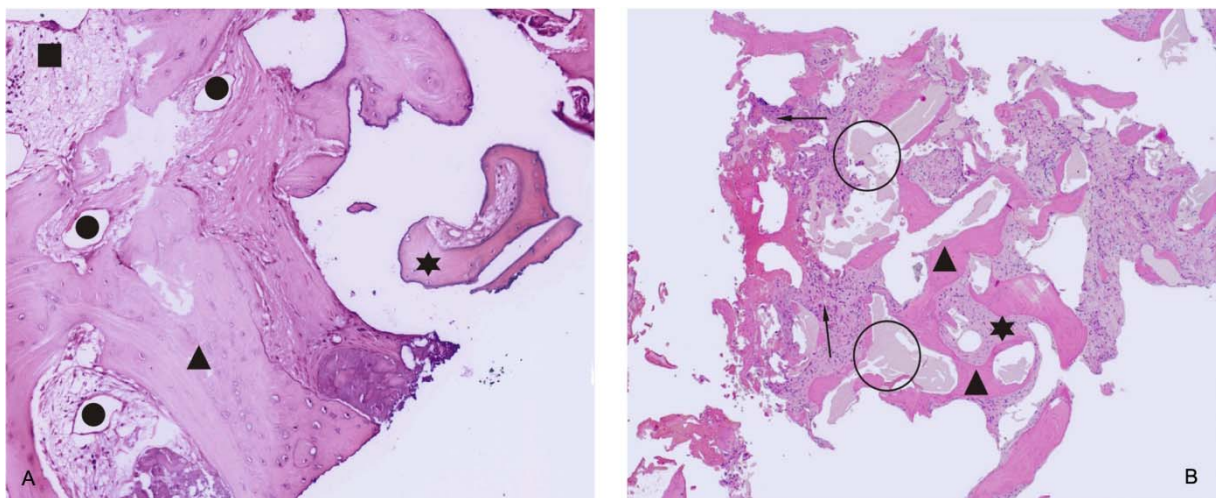


Figure 10

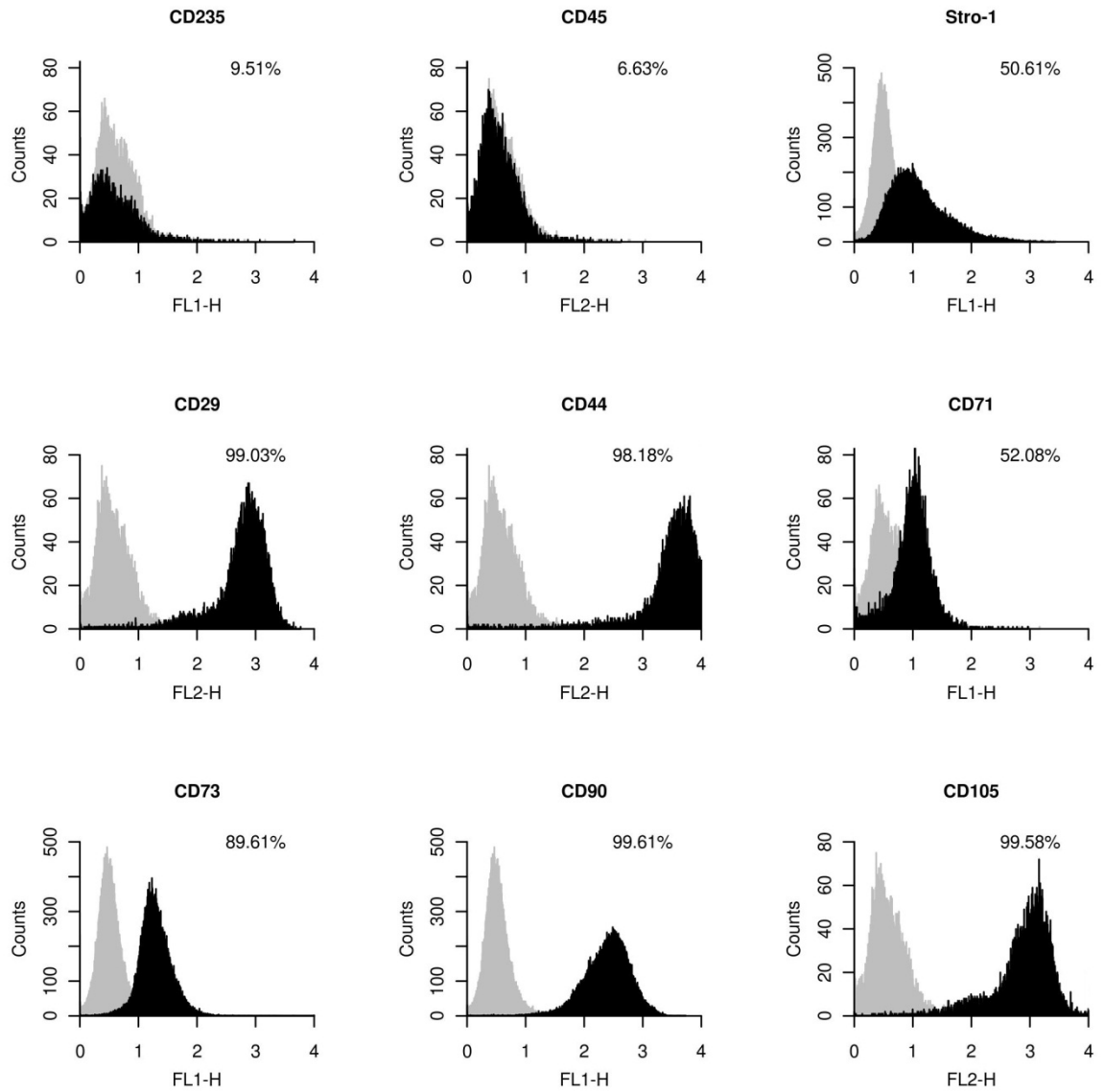


Figure 11

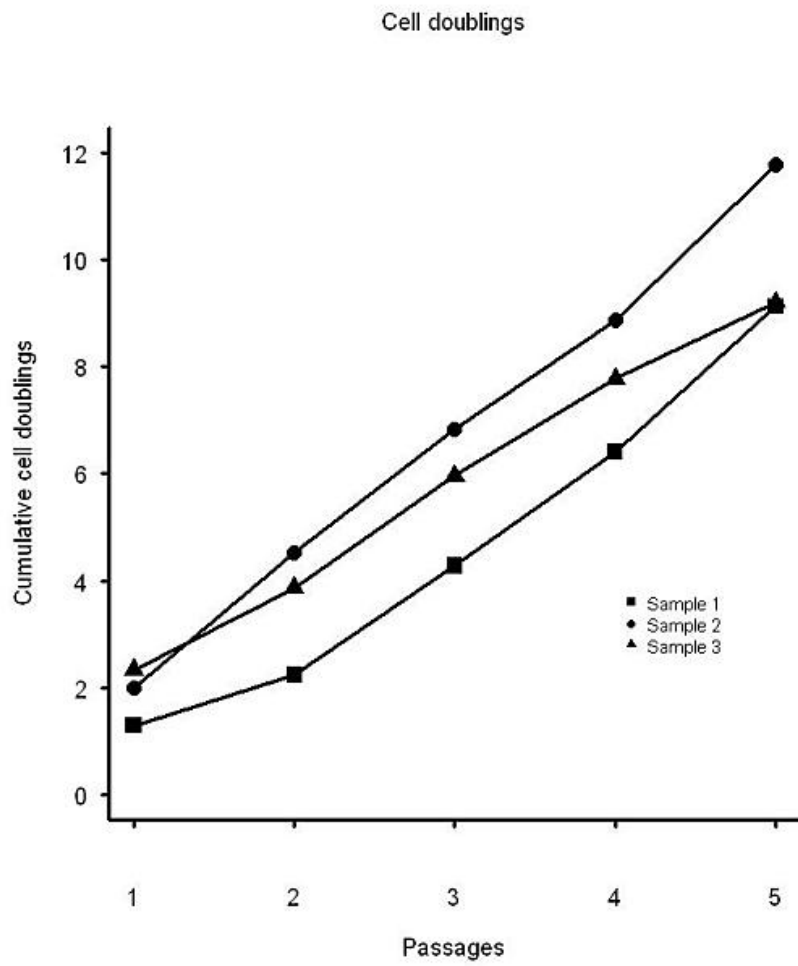


Figure 12

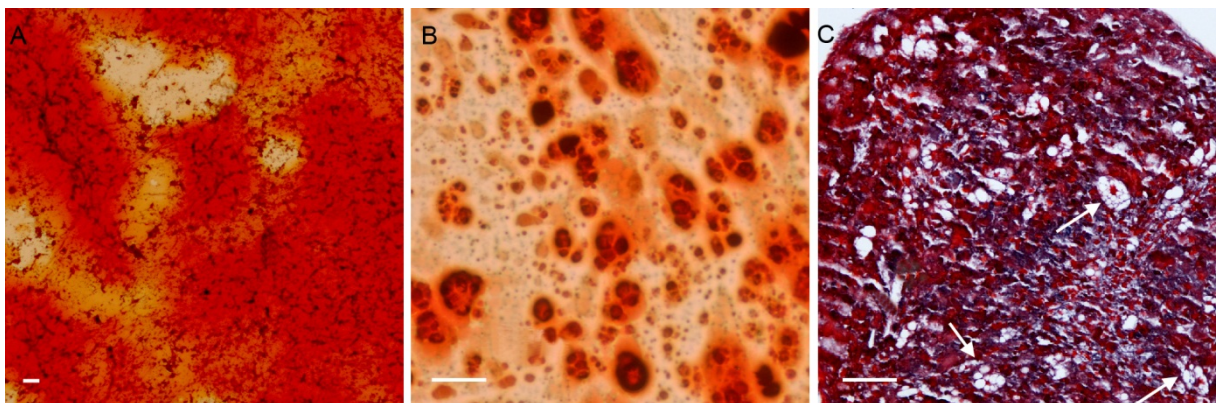


Figure 13

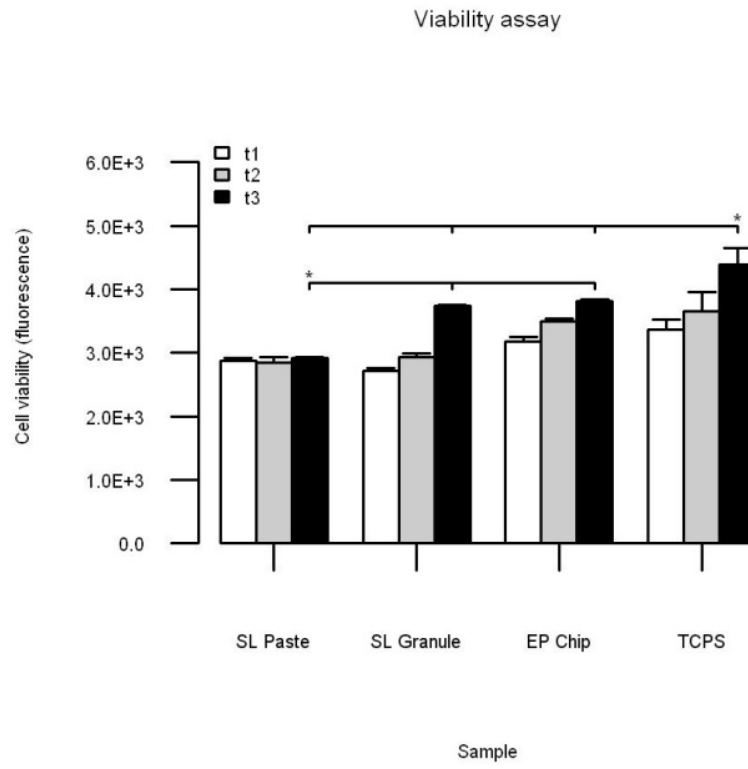


Figure 14

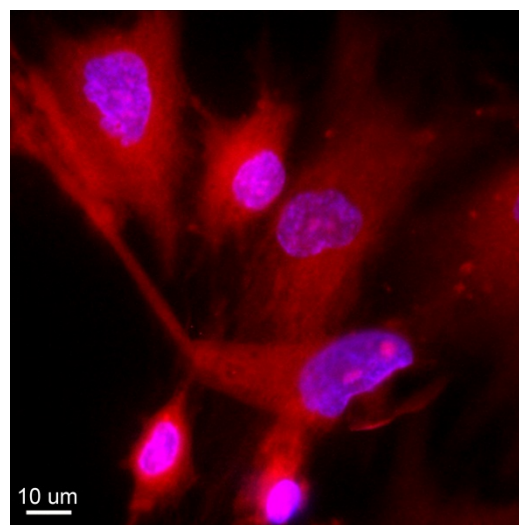


Figure 15

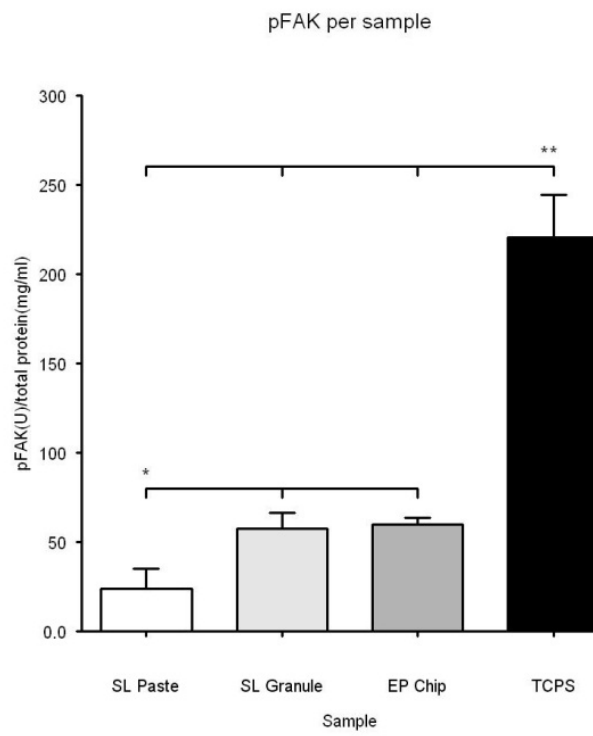


Figure 16

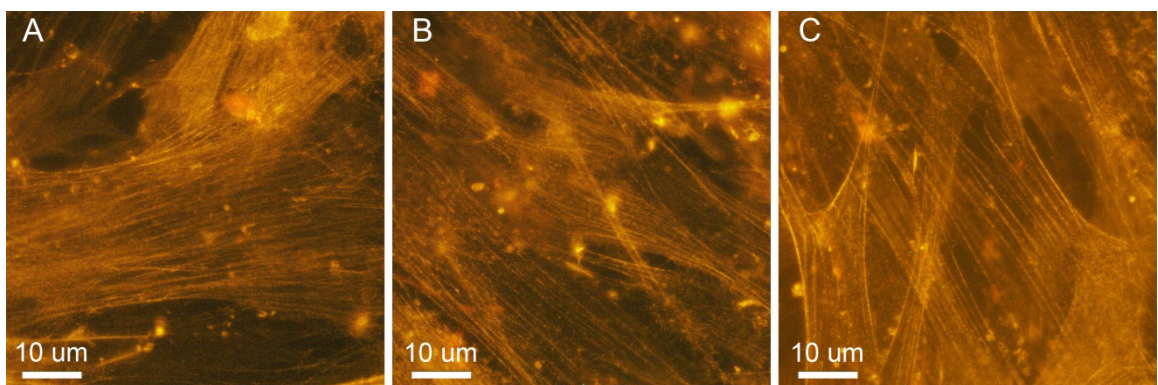
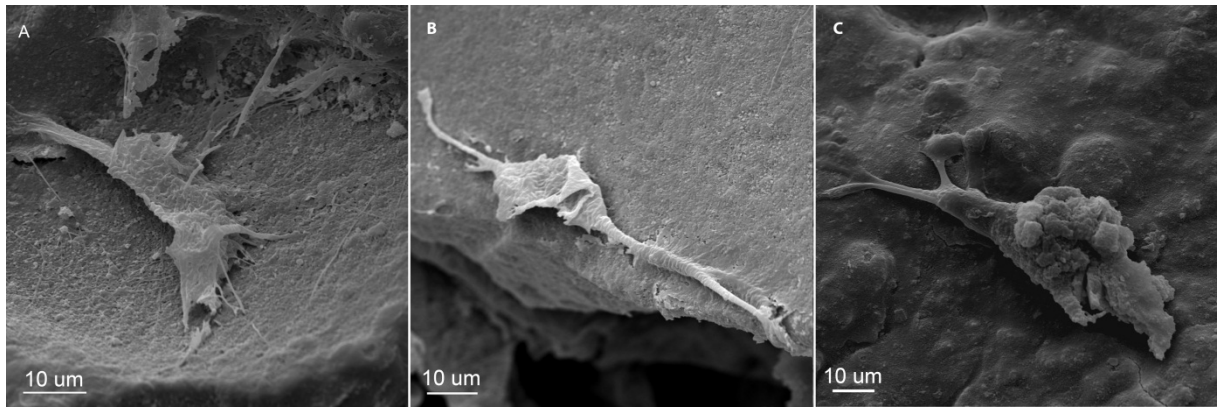


Figure 17



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ANNEX I

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ANNEX II

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